Telomerase activity in response to RBBP6-Cannabidiol co-treatment in cervical cancer

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Declaration

I, Goitsemang S. Morobe (Student number: 24539600), declare that the Dissertation entiled: ***"Telomerase activity in response to RBBP6 cannabidiol co-treatment in cervical cancer "*** is my own original and research work and has not been submitted to any other university. Also where citation from literature is made, the references have been adequately provided.

Signature of the student: _____ Date: November 2020

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"The only source of knowledge is experience"- Albert Einstein

Dedication

This Project is sincerely devoted to my late grandfather Dr Thebeetsile Mogorosi who has been my greatest inspiration and motivated me to study further and gain knowledge.

Research output

Poster presentation

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Abstract

Cervical cancer ranks as the 4th most cited reason for mortalities amongst women across the globe. Accordingly, in 2018, 570 000 new individuals were diagnosed of cervical cancer. Early screening, detection and treatment can reduce cervical cancer incidences by 50%, however, developing countries lack resources, trained personnel and education in the general population limits the implementation of these routine testing programs in developing countries of sub-Saharan Africa. Cheaper and alternative therapeutic options to the otherwise expensive current interventions of chemotherapy, radiation and surgical resections are urgently required.

Retinoblastoma binding protein 6, which interacts with tumour suppressor p53, is highly abundant and expressed in many cancers such as cervical cancer. Taken together, the enhanced expression of RBBP6 and association with p53, has opened up interesting research possibilities exploring the RBBP6 biomolecule as both as therapeutic agent and biomarker for cancer. Gene therapy is associated with toxic effects. Hence, the present study proposes combination therapy which relies on the use of natural anticancer agents such as cannabidiol (CBD) found in a medicinal plant, along with gene-targeted therapeutics such as RBBP6 as a novel and cost-effective strategy for eradication of advanced stages of cancer. The exact molecular mechanism by which CBD mediates anticancer effects remains to be fully elucidated. There is a speculation that CBD induces apoptosis in cervical cancer, hence, current study was commenced to elucidate whether the possible apoptotic mechanisms involved in the chemotherapeutic properties of CBD in combination with targeted gene therapy maybe resulting in decreased telomerase activity.

Co-treatment of cervical cancer cells with a silenced of RBBP6 and CBD was used to assess telomerase activity. Once telomeres lose their function, they become rearranged and unstable. This results in genetic instability and leads to an increase in telomerase activity. It is then possible that telomerase activity can be explored as a molecular biomarker for early screening prevention of cervical cancer. To explore this possibility, ME-180 cervical cancer cells were maintained and subjected to treatment with the anticancer agent, CBD. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) viability assay was conducted to evaluate CBD effects on the viability of ME-180 cells. Furthermore, apoptotic response of cervical cancer cells to siRBBP6 and co-

treatment with CBD was evaluated by DNA fragmentation and microscopy. Gene expression levels of RBBP6, MDM2 and p53 relative to GAPDH were evaluated by quantitative real-time PCR (qPCR). The effect of siRBBP6 and co-treatment was assessed using telomerase repeated amplification protocol (TRAP) assay.

It was found that CBD (0.5µg/ml) significantly reduces the viability of ME-180 cells, possibly by eliciting the apoptosis pathway. This was supported by observation of DNA fragmentation indicating both smearing that indicates necrosis in the combinational treatment. Microscopy revealed morphological features of apoptosis in cells treated with CBD alone and in-combination with siRBBP6. RBBP6 was successfully silenced and the combination therapy resulted in a down-regulation of RBBP6 and MDM2 expression levels and in contrast, p53 expression was found to be up-regulated. This study showed that RBBP6 promotes p53 degradation and that the p53 most likely central in telomerase activity. The likelihood that p53-dependent apoptosis is not related to telomerase activity in cervical cancer is being suggested based on the results obtained in the study. Overall, future endeavours should explore the cost effective and promising combination therapy as a powerful tool for halting cervical cancer

Keywords: Telomerase activity, cervical cancer, RBBP6, CBD, Apoptosis

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List of Abbreviations

ABS	Absorbance
AIDS	Acquired Immune Deficiency Syndrome
BAX-	Bcl-2-associated X protein
Bcl2	B-cell lymphoma 2
BP	Base Pairs
BSA	Bovine serum albumin
BID	BH3-Interacting Domain
Caspase	Cysteine aspartic-specific proteases
CBD	Cannabidiol
CCD	Charge-coupled device
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CFS	Chromosomal fragile sites
CO_2	Carbon dioxide
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DWNN	Domain with no name
FBS	Fetal bovine serum

FITC	Fluorescein isothiocyanate
Hdm2	Human double minute 2
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
hTERT	human Telomerase Reverse Transcriptase
IC50	Inhibitory concentration (half-maximal)
MTT	3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide
P21	Cyclin-dependent kinase inhibitor 1
P2P-R	Proliferation potential protein-related
P53	Phosphoprotein 53
PACT	P53-associated cellular protein testis-derived
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCD	Programmed cell death
PI	Propidium Iodide
PVD	Polyvinylidene difluoride
QPCR	quantitative Polymerase Chain Reaction
RBBP6	Retinoblastoma binding protein 6
RING	Really Interesting New Gene
RNA	Ribonucleic acid
RNAi	RNA interference

RT PCR	real-time polymerase chain reaction
shRNA	Short Ribonucleic acid
SiRBBP6	Retinoblastoma binding protein 6
TRAP	telomeric repeat amplification protocol

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Chapter 1 Introduction

In brief, cancer is defined by uncontrollable cell proliferation resulting from de-regulation of cell cycle. Cancer is often characterized by resistance to programmed cell death, high proliferation rates, activation of survival pathways. In addition, enhanced telomerase activity is strongly correlated to genomic instability which suggests that telomerase activity can be a biomarker of cancer (Reddy *et al.,* 2001). As a global epidemic, cancer kills more people than HIV/AIDS, TB and malaria (Iyoke and Ugwu, 2013). The exact cause of cancer is yet to be clearly defined, however, a series of genetic changes from nucleotide mutations to chromosomal rearrangements have been linked to carcinogenesis (Shalini *et al.,* 2011). As a consequence, cancer is found in almost all organs (brain, head and neck, mouth, lung, liver, breast, prostate, stomach, pancreas and kidney) of the body (Kourinou *et al.,* 2013; Young, 2013). The continued rise in the cases of the gynaecological breast and cervical cancers is an important concern, these have been predicted to worsen the economic impact of lower and middle income countries (Ifediora, 2019)

Cervical cancer is estimated to be fourth most leading cause of gynaecological malignancies and is linked to the majority of cancer-related mortalities in most women worldwide (Bray *et al.*, 2018; Arbyn*et al.,* 2020). In 2018, newly diagnosed incidences of cervical cancer were reportedly 570 000 and of these, 311 000 deaths occurred (Arbyn *et al.,* 2020). There has been increased prevalence of cervical cancer among women of low- and middle-income countries (LMICs) of the world (Arbynet al., 2020). The highest burden of the new cases cervical cancer was predominant in the eastern, western and southern parts of Africa (Arbyn *et al.,* 2020*)*. Epidemiological studies have linked risk factors such as sexual behaviours, HIV seropositive patients with aggressive course and poor treatment to cervical cancer development (Plummer *et al.,* 2012). It remains that roughly 99% of all cervical cancer related malignancies result from infection with high-risk human papillomaviruses (HPV) (WHO, 2018.)

When detected early, diagnosed and managed, cervical cancer is largely treatable. After successful implementation of cytology-based screening programs (WHO, 2002) and HPV vaccination (Pimple, 2016), high income and resource countries, reported marked reductions in cervical cancer-related incidences and deaths. In contrast, these reductions have not been seen in LMIC countries, where 290 000 (51%) of the 570 000 new

worldwide cases occurred in 2018 (Bray *et al.,* 2018). The highest occurrences of new cases and deaths occur in sub-Saharan Africa. Healthcare system challenges such as inadequate infrastructure, lack of availability and access to preventive HPV vaccines. Resource poor countries have also indicated that limitations in trained staff, insufficient screening and treatment options as some of the major impediment efforts for LMIC to curb disease progression (WHO, 2013; Sankaranarayanan, 2014). South Africa, being one of the LMICs, reported an estimated 7,735 new cases and 4,248 mortalities in women in 2017. Diagnosis was common made in women aged 15-44 years. Alternative therapies to available strategies are needed to accommodate the challenges experienced by LMIC countries. This study assessed the impact RBBP6 silencing and co-treating with a medicinal plant on telomerase activity. Medicinal plants have for centuries occupied an important place in the search for alternative therapies. Interestingly, extracts from *Cannabis sativa* have, in addition to other pharmacologically and biologically relevant activities, been shown to possess anti-cancer properties (Andre *et al.,* 2016)

1.1 Problem statement

Cervical cancer which causes more than 500 000 new cases and >250 000 deaths, annually continues to be a huge public health challenge. The most common reason for cervical cancer associated death is lack of effective treatments and late detection of the disease. Currently recommended therapies include chemotherapy, radiation treatment or medical procedure relying upon early detection. These treatment options are limited by the multiple liabilities such as being too expensive for LMIC countries and intense side effects resulting in patient abandonment have also been reported (Tervonen *et al.,* 2017). The pursue of medicinal plants for their therapeutic potential may alleviate the financial cost associated to cancer treatment, especially for LMICs.

The exact cause of cancers is poorly understood. Although, it has been adequately shown that deregulations in the activities of tumour suppressor genes such as p53 are critical for cancer development (Haupt *et al.,* 2016). For instance, HPV infection is strongly correlated with cervical cancer development. The E6 oncoprotein of HPV has been shown to degrade p53 and pRb proteins via the ubiquitin proteasome pathway. The degradation of these proteins subsequently leads to the development of tumorigenesis in the cervix (Hengstermann *et al.,* 2001). RBBP6 is also negatively regulated by p53 (*Li et al.,* 2007, Mbita *et al.,* 2012; Chen *et al.,* 2013; Moela *et al.,* 2014). Thus, targeting RBBP6 for

therapeutic development is a promising strategy. However, the potential toxicity associated with gene therapy is a huge risk for human health, necessitating this research approach which employs combination therapy in which RBBP6 in conjunction with a plant-derived compound exhibiting minimal toxicity to healthy human cells, is believed to eradicate cervical cancer cells. Together with finding the impacts of telomerase activity dependent on combinational treatment with a medicinal plant derivative known as CBD which possess an anticancer activity,

1.2 Research aim and objectives

1.2.1 Aim

The aim of the study is to assess the impact of telomerase activity on cervical cancer progression following co-treatment of siRBBP6 and CBD.

1.2.2 Objectives

- To silence RBBP6 gene in ME-180 adherent cervical cancer cell lines using RNAi technology
- To analyse the effect anti-proliferative effect of CBD ME-180 adherent cervical cancer cell lines using MTT assay
- To quantify the expression of p53 and MDM2 apoptotic genes in response to RBBP6 gene silencing and CBD co-treatment using quantitative PCR
- To analyse RBBP6 silencing and CBD co-treatment on apoptosis by confocal microscopy and DNA fragmentation
- To investigate RBBP6 silencing and CBD co-treatment on telomerase activity using TRAP assay

Chapter 2 Literature survey

2. 1 cervical cancer

2.1.1 Epidemiology

The global epidemic of cancer is an important public health concern (Ferlay, 2013: Zafra-Tanaka *et al.,* 2020). The World health Organization (WHO) provided data that indicated that in 2015 cancer killed millions of people aged 70 years in 91 in 172 countries making it is the second most important cause of death in the world. The incidences and mortality rates associated to cancer have been increasing dramatically. In 2018, WHO estimated that more than 18.1 million people across the world were diagnosed of cancer and of these 9.6 million died (WHO, 2018). The prevalence of cancer is higher in developed countries compared to developing, however, some cancers such as cervical are changing this epidemiological trend (Fidler *et al.,* 2017).

Many scientists have suggested that population growth, socioeconomic development, unhealthy eating and lack of exercise are some of the reasons for increasing trends of cancer (Omran, 1971; WHO, 2018). Risk factors such as smoking, HIV, exposure to harmful cancer causing chemicals have also been cited as contributing factor for increased prevalence of cancer (GLOBOCAN, 2018). In sub-Saharan Africa, breast and cervical cancers are the prominent cancers affecting women.

In Figure 4, an illustration of all the regions associated with poverty and infection related to cancer is depicted. Indeed, developed countries of Europe, North America, as well as high-income countries in Asia and Oceania still prevail in high burden of cancer. It is suggested that the so-called *westernization* of lifestyle is a major contributor to the rapid rise of cancer cases in the countries (Maulea and Merletti ,2012). The variance of cancer profiles in individual countries and between regions marked indicates that specific geographic diversity still exists. There is a persistence of local risk factors in populations of different social and economic perspectives (Bray, 2014). There are different types of cancers and in different organs of the body, some are found in the cervix, stomach, and liver. Cancer of the cervix is an important public health concern.

A decade ago, cervical cancer was rated to be third leading cause of cancer among women, globally. About 42 low-resource countries reported cervical malignancies as the most common cancer in their context (Arbyn *et al.,* 2011).

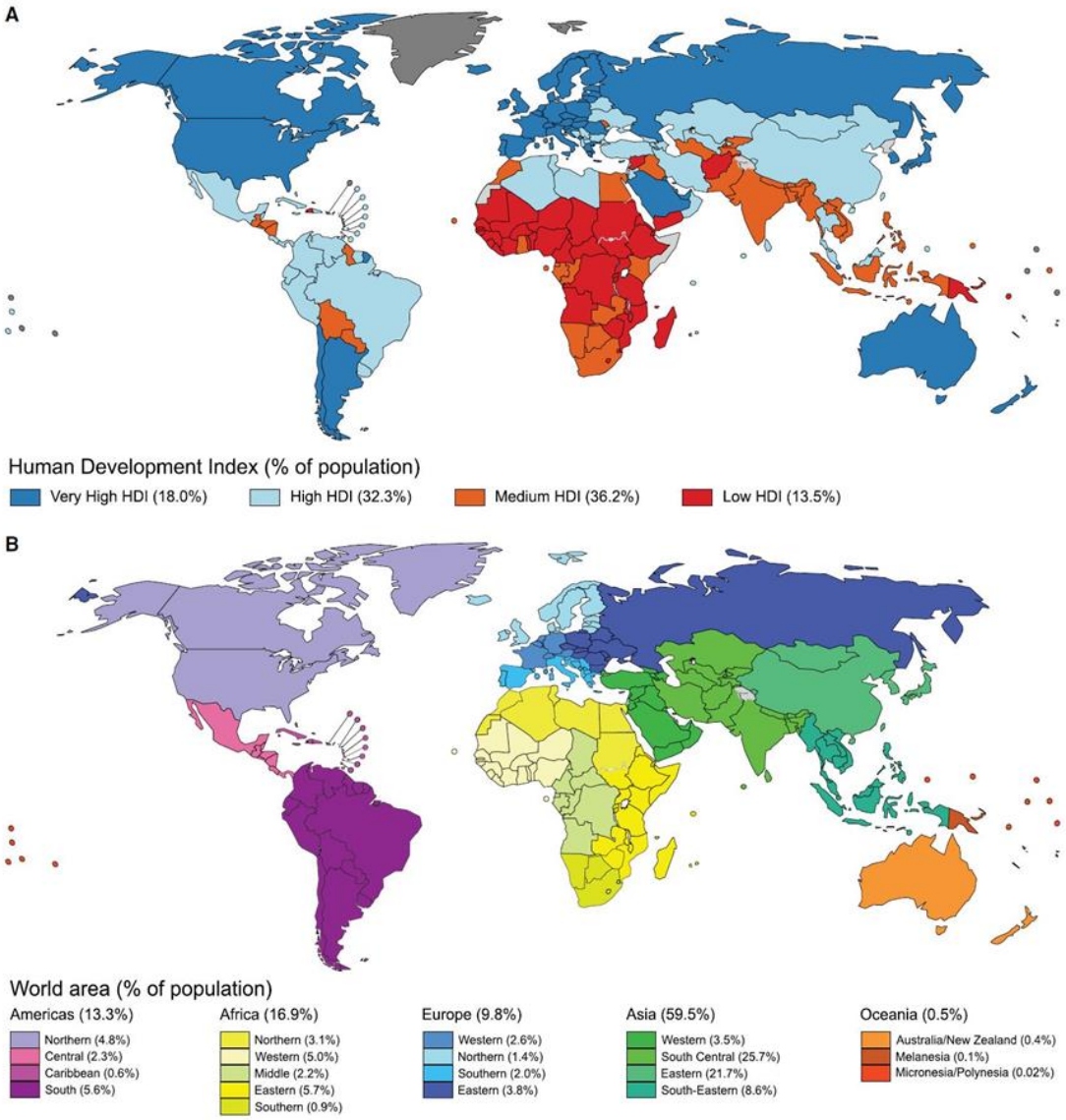


FIGURE 1. Global Maps representing countries affected by cervix, stomach, and liver. The 4-Tier human development index (HDI) is provided in A, and the bottom (B) represents areas of the world and their associated prevalence of cancer development. Source: United Nations Procurement Division.

It is generally accepted that HPV is the major causative agent of cervical cancer. This knowledge has opened avenues for understanding persistent infection of primary and secondary prevention of cervical cancer (IARC, 2007). One of the strategy being implemented for the prevention burden of cervical cancer is vaccination. High-risk HPV type 16 and 18 targeting vaccines have been licenced for worldwide use (Cutts, 2007). These vaccines contain antigens that have high efficacy and protection against infection and pre-cancerous cervical lesions associated with these types when individuals are not yet exposed (Arbyn *et al.,* 2018). Both high risk and low risk HPV cumulatively result in 70–75% of all cervical cancers and 40–60% of its precursors (de Sanjose, 2010; Bzhalava *et al.,* 2013).

2.1.2 Cervical cancer and HPV burden

The gynaecological malignancy of cervical cancer develops in the cervix, between the uterus and unction of the vagina (Saonere, 2010). There are two types of cervical cancer which are known to occur, squamous cell carcinoma which accounts for 75–90% and adenocarcinoma contributes to 10–25% of all cervical cancer cases (Vizcaino, 1998; Vizcaino, 2000; Seoud *et al.,* 2011). The aetiology of the invasive cervical cancer is strongly associated with the sexually transmitted infection known as HPV (Walboomers *et al.*, 1999; Ambior, 2000; Boulet *et al* 2007: De Vuyst *et al.,* 2009). HPV prevalence peaks during adolescent years especially in sexually active individuals and decreases with increasing age (Schiffman and Castle, 2005; Mbulawa *et al.,* 2018). HPV infection is the most common sexually transmitted disease with an estimated 75% of sexually active individuals being most vulnerable (Arybn *et al.,* 2011).

As small, non-enveloped double-stranded DNA viruses, HPVs belong to the *Papillomaviridae* family (Ambior, 2000; Boulet *et al.*, 2007) which comprise of 200 HPV. Of these, 40 types have been identified as genital tract colonizing (Chane *et al.,* 2019). Also, HPV infections are separated into two main types based on the severity of the infection and carcinogenic properties of the virus. High-risk types include genotypes as HPV 16, 18, 31, 33, 35. Others genotypes like HPV 50,66,70,73 and 82 are classified as potential high-risk. Although, HPV types 6 and 11 are considered as low-risk, there is a rising scientific evidence indicating that these types need to be re-classified into high-risk category (Munoz *et al.,* 2003; Halec *et al.,* 2013; Halec *et al.,* 2014).

HPV pathogenesis occurs when the virus gains access into the host cell. HPV targets the basal cells of the body, these are typically vulnerable via micro wounds. Once inside the host, HPV integrates into the host genome machinery and other accessory proteins by exploiting the biosynthetic cellular machinery for viral replication (Pellmans, 2003). From this, HPV virions penetrate by attaching to receptors such as alpha-6 integrin (Narisawasaito and Kiyono, 2007). Once HPV is incorporated into the host genome, then viral DNA replication occurs, yielding approximately 50–100 copies in each cell. DNA replication is followed by the expression of E1 and E2 proteins which are essential for the cell division and separation of the recently synthesized DNA. Furthermore, E1 and E2 ensure that the infected stem cells remain in their lesion state for a prolonged period of time (Graham, 2017; Wang *et al.,* 2018). Except for E1 helicase, more of the HPV proteins which function in DNA replication depend most profoundly on the host machinery to execute their function (Narisawasaito and Kiyono, 2007).

During replication, the E1 protein of HPV assumes a double hexamer conformation which assist in unwinding the strands of DNA (Côté-Martin *et al*., 2008; Münger *et al*., 2001). The sequence-dependent DNA binding protein, E2, which is a transcriptional activator recognizes a specific sequence motif on E1, thereby binding and recruiting it to the origin of replication (Côté-Martin *et al*., 2008). Overexpression of E2 protein has been implicated in suppression of E6/7 proteins expression. It is thought that E2 achieves this by acting as apoptosis promoting protein (Côté-Martin *et al*., 2008). However, it was also observed that E2 protein suppression induces overexpression of the modulatory proteins E6 and E7 of the HPV virus. Indeed, E7 repression activates the Rb pathway while E6 repression elicits the p53 pathway triggering both senescence and apoptosis (Côté-Martin *et al*., 2008). Ultimately, this activates deregulated proliferation and differentiation of cervical cancer cells (Côté-Martin *et al*., 2008; Lebelo et al., 2013). E6 protein has the ability to bind to p53 protein to form a complex resulting in p53 inactivation thus hindering the role of p53 protein in the tumour inhibition once the two bind it prevents phosphorylation. (Zur, 2002), resulting in a degradation of p53. The loss of p53 function prompts degradation of p21 resulting in a sequestration of cell cycle inhibitory function thus resulting in cell proliferation.

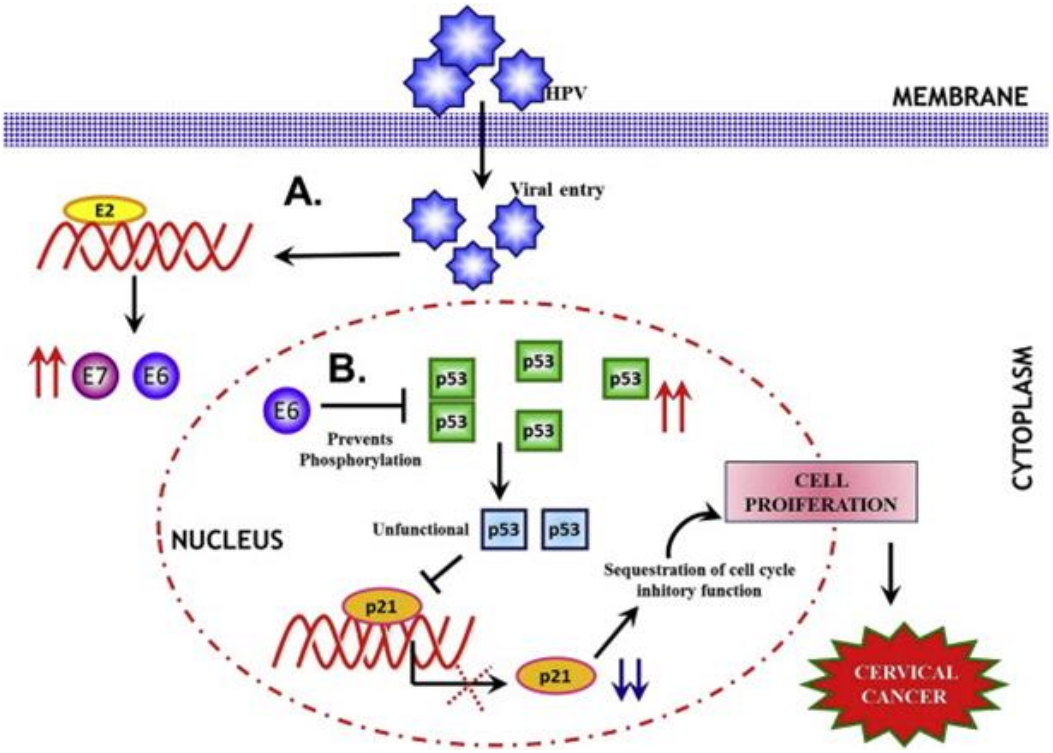


Figure 2; Illustration of viral infection by HPV following the six stages of a mode of transmission in response to the causation of cervical cancer. HPV binds to the receptor of the cell membrane injects its viral genome and disintegrate the host gene by replicating. Figure adopted from (Jawanjal *et al.*, 2015).

2.1.3 Other risk factors

Other factors such as age of first marriage, sex and oral contraceptives have been linked to the development of cervical cancer. In part, the preceding factors do not necessarily cause cancer, but they contribute to the escalation of the risk of developing malignant tumours. It has also been proposed that the age at which woman get married is also a determinant factor for the speedy spread of HPV infection in Sub-Saharan Africa (Kidanto *et al.,* 2002; Anorlu, 2008; Ogunbowale & Lawoyin, 2008). However, some investigators have also emphasized that it is in fact the age at which young women start to be sexually active and fall pregnant that determines onset of HPV infection (Rostad *et al.,* 2003; Louie *et al.,* 2009). In addition, a conclusion regarding an early pregnancy at an early age and number of lifetime births is related with this disease (Anorlu, 2008; Gichangi *et al.,* 2003; Rostad *et al.,* 2003). It is worthwhile noting that not all of the studies pointed to the positive association between high parity and cervical cancer. In fact, Ogunbowale and Lawoyin

(2008), in their study among Nigerian women in rural areas, found that low parity (0-3) is also correlated with the disease.

HIV/AIDS is also known as another casual and risk factor of cervical cancer. Indeed, a positive correlation between HIV and acceleration in the development of precancerous lesions results in higher risks of cervical cancer (Clifford, 2006; Massad, 2001). Infection associated with HIV have a wide extensive lesions of the cervix, about 3-5 times lesions are common in women living with HIV/AIDS (WLHIV) than in those with no history of HIV (Horo, 2012). It has also been reported that WLHIV die at a rate 2-fold higher than HIV-negative women (Peterson, 2016).

Oral contraceptives are another important risk factors for cervical cancer development. It was found that the longer (more than 5 years) a woman is on most oral contraceptives the more likely it is that she will develop cervical cancer (Holmes *et al.*, 2009). In another study, women on oral contraceptive for under 5 years were reported to be at 60% risk while those on the pill for 5 to 9 years use of the pills were considered to even more highly at risk (Smith, 2003). As expected with increasing years, as women move past adolescence and birth-giving age, the risk to cervical cancer development declines sharply (Appleby, 2007; Roural, 2016; Iversen, 2017). Oral contraceptive contains synthetic versions of female hormones such as oestrogen and progesterone which stimulates the development and growth of some cancer which has the potential to increase the risk of spread and metastasis of cancer (Murphy, 2017). In addition, oral contraceptives might increase the susceptibility of cervical cancer cells to persistent infection with HPV (Murphy, 2017).

2.1.4 Prevention, screening and treatment of cervical cancer

The primary prevention of cervical cancer mainly relies on conventional strategies such as abstinence, delaying sexual intercourse and practicing safe sex by using condoms (Sawaya and Smith-McCune, 2016; Mariani *et al.*, 2015). It is also important women of all age groups to constantly screen cervical cancer. Screening programmes have been implemented in South Africa in order to reduce the burden of cervical cancer (Dreyer *et al.,* 2019). Routine screening allows for an early detection and treatment of pathological changes brought by HPV. (Arbyn, 2007; Saslow *et al.,* 2012; Torres-Poveda *et al.,* 2019). There are two types of screening cervical cancer namely, HPV detection and

Papanicolaou (PAP) smear test. In 2014, a new test known as cobas14800 HPV test was introduced and approved by the U.S. Food and Drug Administration (FDA) for primary screening in cervical cancer (Dreyer *et al .,*2019). This HPV screening test can detect as high-risk sub-types of HPV in a single run and additionally, genotype HPV 16 and 18 (Micalessi *et al.,* 2012).

It is recommended that women over the age of thirty should go for Pap smear test every second year (Kim, 2011). The most important thing about Pap smear test is their ability detect both cervical cancerous and precancerous cells. The Pap smear detects abnormal cells in the cervix (Azamimi *et al.,* 2019). In South African after receiving positive results of large loop excision of transformation zone, (LLETZ) treatment is recommended for treating abnormal cancerous cells (cancer screening, 2015). These current techniques such as Pap smear technique and visual inspection with acetic acid/ Lugol's iodine are very challenging to implement in many sub-Saharan African countries (Denny *et al.,* 2006). Because of the lack of sufficient trained personnel, especially in poorer countries, these screening programmes are barely rolled out on public health scale. However, a new strategy involving simultaneous detection and vaccination of HPV is receiving scientific research consideration and is possibly an emerging as a complementary tools for Low and Middle Income Countries (LMICs) (Qiao *et al.,*2008)

HPV vaccines remain a powerful tool used for primary prevention of cervical cancer and other HPV-associated diseases (Bloem, 2017). The HPV vaccine was first developed in 2006, by Ian Frazer (Australia). Since then, HPV vaccines have been rolled out to 71 countries and forms part of their national immunisation programmes for young women of pre-adolescent and school going age (WHO, 2017). Eleven countries have also implemented the HPV for their male adolescents (WHO, 2017). By 2017, globally. The introduction of HPV vaccines in the developing world is stalled by the financial challenges (Botwright, 2017; Black, 2018; Gallagher, 2018). However, in more recent years, maintenance from organizations such as global alliance for vaccines and immunization (Gavi) and the Vaccine Alliance and the Pan American Health Organization (PAHO) has permitted Latin American and Caribbean countries to introduce HPV vaccination in their national immunizations programmes (Botwright, 2017; Black, 2018; Gallagher, 2018).

The name of this vaccine is Gardasil 9 developed to protect against nine HPV types (6, 11,16,18,31,33,45,52 and 56) (Richwine, 2009). Gardasil 9 became available in 2006 in

the United State designed for females 9-26 years of age to protect against cervical cancer caused by HPV (Siddiqui and Perry,2006; Reissner,2015) and in April 2014, a Gardasil vaccination program was launched in South Africa, targeting about 550 000 young girls in grade 4 over the age of 9 years (Palmer *et al.*, 2014). These grade 4 girls were given 2 dose (6 months apart) as requested by the national department of health. When the programme was implemented over 350 000 grade 4 females were vaccinated in more than 16 000 public schools across South Africa (Delany-Moretlwe *et al.,* 2018).

2.2 P53

2.2.1 History of p53 and function of p53

P53**,** is also known as TP53, was discovered in 1979 and following exhaustively studies (Kress et al., 1979; Rotter, 1983; Strachan and Read, 1999; Jiang et al, 2013; Toufekchan and Toledo, 2018) became dubbed as a tumour suppressor. *P53* is found on chromosome 17 of the human genome (Sager, 1992) and is fondly called guardian of the genome based on its ability to conserve stability of cells by preventing genome mutation or DNA damage (Lane, 1992; Strachan and Read, 1999; Toufekchan and Toledo, 2018). This 53kDa phosphoprotein is made up of 392 amino acids comprising of the following discrete domains: transcriptional activation, DNA binding, and oligomerization (Bell *et al.,* 2002) Indeed, the N-terminal transactivation domain of p53 identifies specific DNA sequences, domain that is responsible for tetramerization of protein and non-specific DNA binding domain that binds to damaged DNA (Shangary and Wang, 2008). This region plays a role in suppressing central domain from DNA binding (Shangary and Wang, 2008). The N-terminal transactivation domain of P53 comprises of highly conserved domain I (HCD I) region and an MDM2 binding site.

One of the best-characterized and studied property of the p53 is its ability to activate gene transcription. As such, atleast 350 genes which are involved in cell cycle arrest/senescence, programmed cell death, DNA repair, controlling metabolism and translation regulation have been found to be activated by p53 (Adimoola and Ford, 2003; Meek, 2004; Fischer, 2017). Previous studies revealed that p53 is down-regulated in normal cells (Hinds and Weinberg, 1994). However, exposure to some cellular stressors such as damaged DNA and hypoxia causes enhanced production of the p53 protein. Once abundant amounts of p53 are expressed in the cell, essential process such as cell

cycle arrest. DNA repair or apoptosis become triggered and activated (Freeman *et al.,* 1999; Meek, 2004). The levels P53 expressed in the cells are facilitated by protein stabilization via a presence of MDM2 is a negative feedback loop. Post-translational modifications (PTMs) processes including methylation, acetylation, ubiquitination and phosphorylation have also been shown to enhance the amount of p53 expressed (Shangary *et al.,* 2008; Shangary and Wang, 2008; Meek and Anderson, 2009). Activation of p53 results in the production of important genes that involved in various tumour suppressor mechanisms such as Bax, PUMA, and Noxa which are important for the apoptosis pathway. Other genes such as TSP1 and maspin serve as inhibitors of angiogenesis by stimulating endothelial cell apoptosis. Also, activation of p53 cause the expression of the cyclin-dependent kinase p21 which promotes cell cycle inhibition and effect anti-proliferation in normal cells (Vousden, 2000; Vousden, 2002). DNA damage causes enhanced protein expression of p53 which then activates induction of MDM2 expression in the cells resulting in the blockage of p53 activities. Thus, MDM2 is confirmed as negative regulator of p53 which mediates its degradation (Freeman *et al.,* 1999). And conversely, p53 positively regulates MDM2 (Freeman, Wu, and Levine., 1999).

2.2.2 Role of p53 in cervical cancer

P53 serves primarily as a tumour suppressor repairing damaged DNA during the process of cell division (Hinds and Weinberg, 1994; Adimoolam and Ford, 2003; Chen, 2016). However, the loss of its function leads to development of tumour malignancies. Tumorigenesis is influenced by the occurrence of mutations, activation of proto-oncogenic genes, and abnormal over expression of p53 (Sager, 1992). A mutated p53 allow aberrant cells to proliferate therefore resulting in cancer (Hinds and Weinberg, 1994; Chen *et al.,* 2013). In the HPV associated cervical cancer, It is believed that HPV E6 protein degrades p53 (Hietanen *et al.,* 2000). In this regard, stabilization of p53 has been achieved by short interfering RNA (siRNA) molecules targeting E6 mRNA (Kiovusalo, 2006). High-risk types of HPV are most critical in the pathogenesis and development of cervical cancer (Sasagawa, 2003; Hirte *et al.,* 2008). E6 and E7 are overexpressed after the infection of genital mucosa and entry of virus into the host genome, which then immortalizes the host cells by disrupting the functions of p53 and pRb tumour suppressor genes (Munger, 2002; Artaza-Irigaray *et al.,*2019). Ubiquitin-mediated degradation of p53

is mediated upon the binding of HPV E6 protein, while E7 on the hand inactivates another tumour suppressor gene pRb (Munger, 2002; Yi *et al.,* 2013). P53 initiates cellular responses to various stresses, e.g. DNA damage. This is because once p53 has been activated, damage repairing processes such as programmed cell death and cell cycle arrest become triggered and activated (Vousden, 2002: Altin and Schulze, 2011; Wang *et al.,*2018). Interestingly, RBBP6 works in conjunction with MDM2 to interact with two major tumour suppressor proteins p53 and pRb. Thus, the chemotherapeutic potential of RBBP6 is an interesting area of exploration in cancer research

2.3 Retinoblastoma binding protein 6 (RBBP6)

2.3.1 RBBP6, its domain and isoforms

The retinoblastoma binding protein 6 (RBBP6), is also known as p53-associated cellular protein-testes (PACT), proliferation protein-related protein (P2P-R/PP-RP) and retinoblastoma binding Q protein-1(RBQ-1), is eukaryotic, nuclear-associated protein, which has been shown to associate with p53 and pRb in humans and mice. In humans, RBBP6 is situated on chromosome 16p12.1 is transcribed into three proven splice variants encoding for 3 protein isoforms (Sakai, 1995: Gao, 2002). However, the existence of a fourth variant possibly due to alternative splicing has been reported, although it is yet to be experimentally validated (Ntwasa *et al.,* 2018). RBBP6, has been implicated in biologically important processes such as tumour development (Chibi *et al.,* 2008; Li *et al.,* 2007; Motadi *et al.,*2010), cell cycle regulation (Jones et al., 2006) and mRNA processing (Vo *et al.,.* 2001; Di Giammartino *et al.,* 2014). RBBP6 negatively regulates p53 by activating the poly-ubiquitination activity of MDM2 (Li *et al.,* 2007)

In order to facilitate these cellular functions, RBBP6 variant 1 proteins contain a number of features such as the N-terminal domain with no name, zinc knuckle and E3 ligase activity RING finger domains. RBBP6 has been shown to use the RING finger domain for p53 degradation and MDM2-mediated ubiquitination (Li *et al.,* 2007) box binding Protein 1 (YB-1) and the pro-proliferation transcription factor (Chibiet *et al.,*2008). The knockdown of RBBP6 led to reduced replication fork movement (Miotto *et al*., 2014). This is because owning its E3 ligase activity RBBP6 ubiquitinates a transcription repressor, zinc finger and BTB domain containing 38 (ZBTB38), that controls the levels of replication factor mini-chromosome maintenance 10 (MCM10) (Miotto *et al*., 2014)

In addition to classical domain configuration, the mammalian RBBP6 comprise of the retinoblastoma (Rb) binding, p53-binding, proline-rich and serine rich regions. In fact, RBBP6 was first identified in mouse as Rb (Saijo *et al.*, 1995; Sakai *et al.*, 1995) and p53 binding protein (Simons *et al.,*1997) from which it derived names such as p53-associated cellular protein testes derived (PACT) and proliferation potential-related protein (P2P-R or PP-RP) (Witt and Scott 1997). RBBP6 has been demonstrated to be essential for cell viability, as its absence leads to early embryonic lethality in mouse (Li *et al.,*2007), flies (Mather *et al.,* 2005) and worms (Huang *et al.,* 2013). Knock-down of truncated derivative of RBBP6 in mice was shown to significantly decrease the p53-Hdm2 connection, reducing p53 poly-ubiquitination and degradation, and enhances p53 accumulation (Li *et al.,* 2007). The accumulation of p53 results in both apoptosis and cell growth retardation. Overexpression of RBBP6 in mice results in apoptosis (Gao and Scott, 2002; Scott *et al.*, 2003) and equally, silencing of RBBP6 variant 1 by RNA interefence technology in mouse 3T3 cells led to resistance towards campotothecin-induced apoptosis (Pretorius *et al.,* 2014). Moela, 2016 demonstrated that RBBP6 knockdown is a results of growing cell populations in G0/G1 phase and as well as in the S-phase in response to RBBP6 overexpression. Furthermore, the study suggested that RBBP6 plays a significant role in prompting cell cycle arrest (Moela and Motadi, 2016). RBBP6 is an important regulator of the cell cycle and cell proliferation.

In human beings, a single copy of RBBP6 gene is located on chromosome 16p12.2 encoding four protein isoforms which are generated through the process of alternative splicing and poly-adenylation. Human RBBP6 is a promising preventative therapeutic agent against cancer, its upregulation was strongly correlated with lung (Motadi *et al.,*2011), stomach (Chen *et al.,*2013; Morisake *et al.,* 2014) and cervical (Moela and Motadi 2016) cancer progression. Indeed, enhanced levels of RBBP6 relates with poor clinical progression and has been associated to poor outcomes and minimal survival rates in oesophageal cancers (Yoshitake *et al.,* 2004). Furthermore, cytotoxic T-cells specific for RBBP6 derived peptides were able to lyse cultured oesophageal cancer cells and in mouse xenograft models restore oesophageal tumours to their normal phenotype. Furthermore, RBBP6 is a mammalian nuclear protein that plays a vital role in cell cycle and apoptosis regulation through its interaction with p53 and RB1 tumour suppressor genes (Pugh *et al.,* 2006)

The interaction of RBBP6 with the tumour suppressor genes is mediated via E3 ubiquitin ligase owing to the presence of RING finger domain (Pugh *et al*., 2006). RBBP6 promotes the deregulation of p53 whereby increased cell proliferation occurs. Isoform 3 is encoded by a 1.1 kb transcript is also called DWNN (Pugh *et al.,* 2006).

DWNN

The domain with no name (DWNN) being the third spliced variant of RBBP6 is situated at the N-terminus of the protein (Figure 3). The three-dimensional structure of the DWNN was resolved by Pugh and colleagues (2006), and was revealed to ubiquitin-like in configuration (Pugh *et al.,* 2006). Little is known about the function DWNN, an independent module referred to as RBBP6 variant 3. Although RBBP6 variant 3 comprise of the DWNN (1-76) and an unstructured C-terminal, only the DWNN which adopts an ubiquitin-like fold (Pugh *et al.,* 2006). The DWNN domain has been shown to mediate an interaction to Cst64, in which RBBP6 and RBBP6 variant 3 compete for binding (Di Giammartino and Manley 2014). Overexpression of variant 3 stops cleavage raising intriguing possibilities of modulation of 3' processing by reasonable tuning the levels of the two RBBP6 isoforms. Furthermore, RBBP6 variant 3 seems to be involved in both cell cycle regulation and camptothecin (CPT)-induced apoptosis. Also, differential expression levels of variant 3 were seen in colorectal, breast, cervical, ovarian, and prostate cancer when compared to normal samples (Mbita *et al.,*2012).

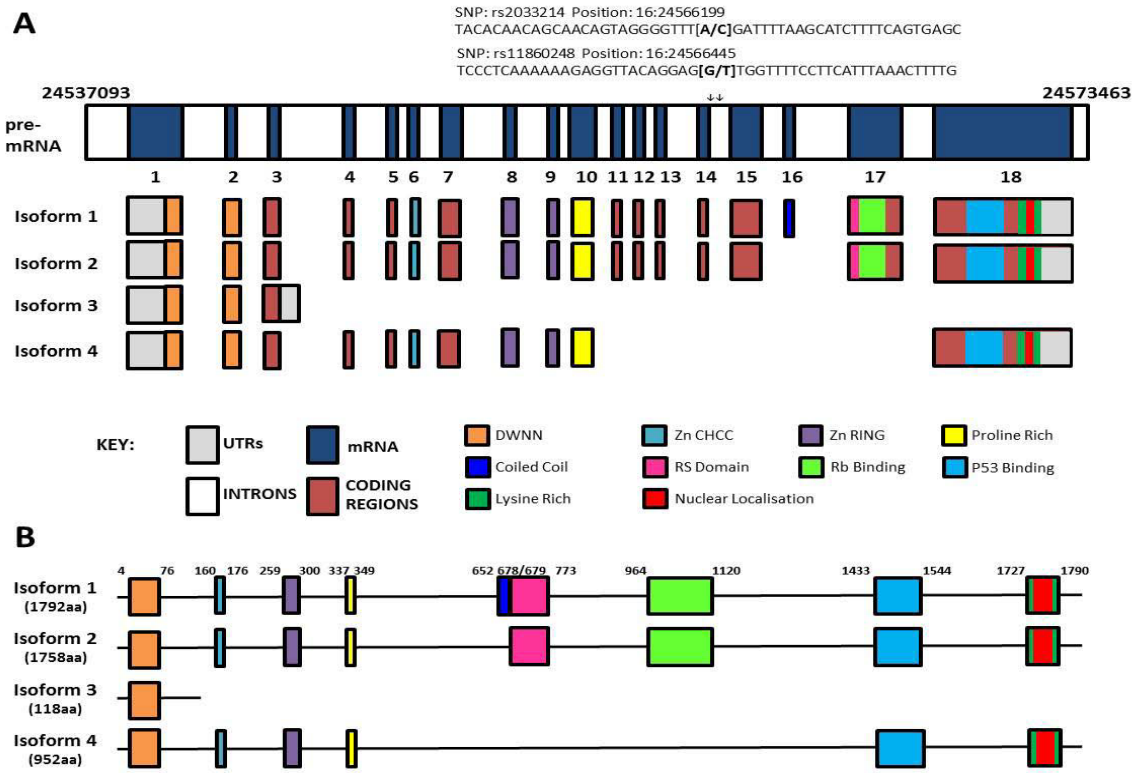


Figure 3: Organization of the *RBBP6* gene and domain organization of its protein product. A. Gene structure and configuration of exons in the spliced transcripts. The locations of SNPs in intron 14 associated with glioma are indicated. Pre-mRNA sequences that are landmark for splicing reactions with their associated binding proteins are shown. Location of exons are shown in the different RBBP6 isoforms B. *RBBP6 i*soforms and domains. Arrangement of several domains in RBPP6. Domains contained in each isoform are shown. (Ntwasa *et al.,* 2018)

Ring finger

Ring finger domain is best portrayed as a protein structure domain of zinc finger-like which contains C3HC4 amino acids motif and can bind two zinc cations (seven cysteines and one histidine orchestrated non-sequentially) (Borden, 1996; Hanson, 1991; Freemont, 1991). Zinc cations bind with ring finger domain they form a fold that leads to a secondary structure made up of antiparallel β-sheets and a α-helix linked together by long peptide loops (Pugh *et al.,* 2006). The Independent folding of the RING finger motif is made possible by the interaction with two Zn^{2+} ions via cysteine and histidine residues, forming a stabilized zinc-finger structure (Liew *et al.,* 2000; Laity *et al.,* 2001). This protein domain has about 40 to 60 amino acids. Most proteins with a presence of a ring finger domain play a vital role in the ubiquitination pathway.

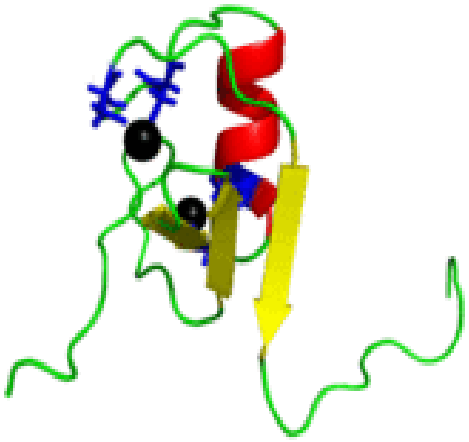


Figure 4: Structure of the C3H4 domain. Black spheres represent Zinc ion, synchronized by cysteines residues (blue) (Barlow, 1994)

Zinc finger

A zinc finger domain is best portrayed as a small protein structure motif described by the organization of at least one zinc ions (Zn2+) to balance out the overlap (Klug, 1987). They are a diverse family of proteins that serve a wide-range of biological functions. It is hard to bring up a best a definition of what connects all ZnF proteins because of its decent variety; in any case, the most ideal way is to characterize them as small functional domains that needs coordination by at least one zinc ion (Laity *et al.*, 2001).

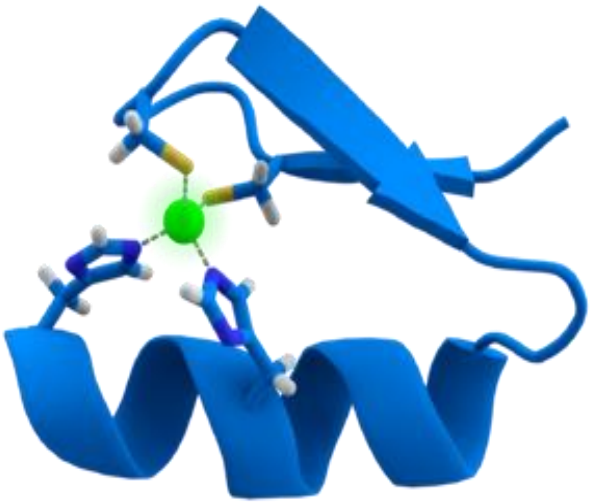


Figure 5: Illustration of the structure of the Cys2-His2 zinc finger motif. This structure consists of α helix and an antiparallel β sheet. The zinc ion is indicated by the colour green and forms interactions with histidine and cysteine residues. (Thomas Splettstoesser, 2007)

2.3.2 RBBP6 role in cancer

RBBP6 is overexpressed in various cancers; and it was specifically shown to be upregulated in cervical cancer (Moela and Motadi, 2016). In a study wherein cervical squamous carcinoma tissues (T) and adjacent non-cancerous tissues (N) were obtained from clinical specimens. A determination of RBBP6 gene and protein expression levels, revealed a significantly enhanced expression level in cancerous tissues versus to non-cancerous tissues (Teng *et al.,* 2018). Another study found elevated levels of RBBP6 in testis and oocytes (Simons *et al.*, 1997; Yoshitake *et al.,* 2004; Mather, 2005; Huang *et al.,* 2013) also in the intestine (Huang *et al.,* 2013). The observation that higher levels of RBBP6 are expressed in proliferating tissues compared to the low level produced in differentiated cells raises important possibilities of exploring it as a regulatory biomolecule in the development of cancer (Witte & Scott, 1997; Jones *et al.,* 2006). RBBP6 has a vital function in the transcription-related activities as RBBP6 forms part of the oestrogens-transcription complex (Peidis *et al.,* 2010) and localizes to nuclear speckles (Gao & Scott kl;2002; Scott *et al.,* 2003; Huang *et al.,* 2013).

2.3.3 The relationship of RBBP6 and MDM2 in degrading p53

RBBP6 and MDM2 most certainly share important similarities, the most prominent of which, being their ability of interact with p53(Mather *et al.,* 2005; Simons *et al.,* 1997). Structurally, both RBBP6 and MDM2 exhibit E3 ligase activity and they form a complex with p53 then catalyse it via ubiquitination (Chibi *et al.,* 2008). Interestingly, null mutants of RBBP6 and MDM2 die during embryogenesis suggesting that they're is important for cell development (L i *et al.,* 2007). The effect brought by RBBP6 and MDM2 null mutants can be reversed p53 deletion.

RBBP6 degrades p53 via its RING finger domain which facilitates its interactions with both p53 and pRB proteins (Kappo *et al*., 2012). The activation of p53 in cell occurs as a response to cellular stresses such as DNA damage, hypoxia, exposure to UV and radiation light. This leads to a reduction in MDM2 expression in the cell (de Bruin and Medema, 2008). Mutation of p53 is associated with many human malignancies. RBBP6 and MDM2 are both negative regulators of p53, overexpression of both RBBP6 and MDM2 results in a degradation of p53.

RBBP6 is receiving a lot research attention investigating its gene therapy potential for various cancers. In a study conducted by Moela and Motadi (2016), relationship between the expression of mRNA and protein levels of MDM2, RBBP6 and p53 was shown in cervical cancer cells. In MDM2 and RBBP6 knockdown cells and co-treatment with staurosporine, increased protein expression of bax and constant/reduced expression of Bcl-2 was seen (Moela and Motadi, 2016). Another study suggested that RBBP6 has Mdm2-independent activities that are crucial for development (Ntwasa *et al.,* 2018). The functional cooperation of RBBP6 and MDM2 is an interesting subject of investigation in the field of cancer therapy. This dissertation focuses on silencing RBBP6 and co-treatment with CBD

2.4 Cell cycle

Cell cycle is the process whereby cells divide and progress. In normal cells, cell cycle is controlled by signalling pathway whereby cells divide and replicate DNA. As shown in Figure 7, the cell cycle is controlled by four sequential phases (Wang *et al.,* 2009). S phase whereby DNA replication occurs, G2 phase also known as second gap phase whereby cell prepares to divide for an entry into the M phase. M phase is a process whereby cell division occurs. (Murray and Hunt, 1993; Vermeulen *et al.,* 2003). G0 represent the stage when cells can withdraw from cell division in response to cell density or mitogen deprivation (Zetterberg, 1985). This organized and highly regulated process of cell cycle ensures that normal cells divide in the S-phase, followed by an equal distribution of identical chromosomes into daughter cells and that all the errors are corrected during cell division if not the undergo cell death (DiPaola, 2002)

During the process of cancer development in the cell cycle, cells have a way of progressing through the cell cycle without being checked due to deregulated cell cycle checkpoints. Oncogenic proteins such as RBBP6 plays a fundamental role during cell division by stabilizing chromosomal fragile sites (CFSs) in order to accelerate cell cycle progression (Miotto *et al.*, 2014). Therefore, it is suspected that an overexpression of RBBP6 in tumorigenesis might be responsible for the uncontrolled cell proliferation. This makes functional studies of RBBP6 in relation to the cell cycle necessary (Miotto *et al.*, 2014). Previous studies have also shown that p53 is known to be a negative regulator of RBBP6, an overexpression of RBBP6 during cell cycle makes p53 to lose its function thus cells are able to move to the next stage without being repaired (Freeman *et al.,* 1999).

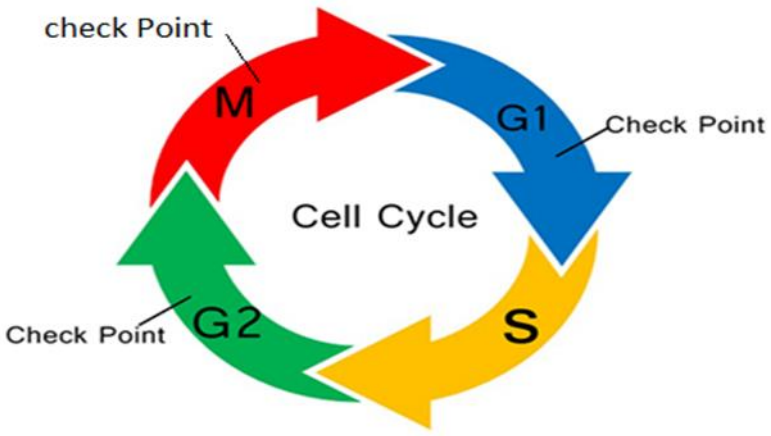


Figure 6: Cell cycle stages and checkpoint points.

2.5 Telomere and telomerase

2.5.1 Location and structure of telomeres

Telomeres are typically found at the end of every chromosome of all living organisms. They are responsible for protecting chromosomes from deterioration, fusion at the end of chromosomes. With each cell cycle, telomeres shorten by 50–200 nt for each cycle, bringing about cell senescence and apoptosis and in this way repairing the chromosomes during the process of cell mitosis (Shay, 2002). Telomere control genomic integrity in most cells and their shortening process initiates chromosomal instability. Telomere length of all normal cells are maintained by telomerase (Shay, 2002: Blackburn, 2006: Rudolph, 2006). This is achieved by continuous addit ion of telomeric DNA (TTAGGG) sequences at the end of the telomeres to by the telomerase, thereby avoiding cell death. Thus, telomere length and telomerase activity are an important biomarker, as these play a critical role in formation and continuous proliferation of cancer (Xiao *et al.*, 2004).

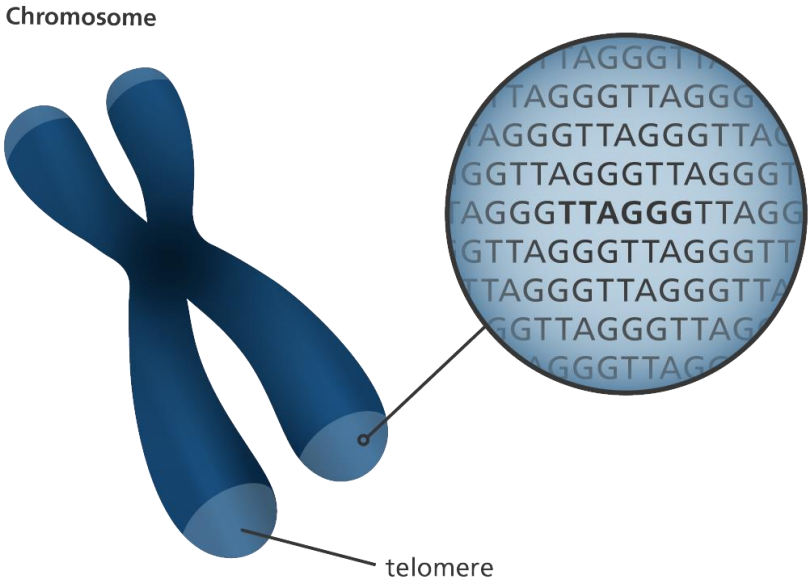


Figure 7: Illustration demonstrating the position of telomeres toward the end of the chromosome. (Image credit: Genome Research Limited)

2.5.2 Telomerase in cancer

Cancer is believed to be an age-related genetic disease. One of the hallmarks of cancer is that normal cells aggregate resulting from genomic instability of an undefined period of time and as a consequence lose their replicative immortality (Akincilar, 2016). Cancer cells tends to have a high telomerase activity in contrast to normal cells (Kim, 1994; Shay, 1997). In the process of cell mitosis an overexpression or upregulation of telomerase prevents telomeres from shortening, making cancer cells in mitosis indefinitely (Kim, 2015). The relationship between telomerase activity and cancer induction raises interesting potentials of exploring this activity as a valuable biomarker for an early detection and possible therapy for cancer. The TRAP assay which is polymerase chain reaction via a gel based is one of the most common and widely used technique for determining telomerase activity. The advantage of TRAP remains that it is a fast, sensitive and contamination free (Hou, *et al.,* 2001; Wright *et al.,* 1995).

2.5.3 Telomerase in cell cycle

Telomerases are crucial for maintaining telomere length. Once telomeres are damaged, cell division becomes disturbed whereby damaged DNA is transformed leading to instability of the cells. Hence, increased telomerase activity is considered to be an indicator of proliferating cells. The human telomerase RNA gene (hTERC) is able to bind and inhibit protein kinase ATR, which plays an important role in targeting p53 and CHK1 protein kinase for cell cycle checkpoint control. P53 and CHK1 protein kinase of the system of signal transmission from the DNA that is damaged (Zhang, 2013). P53 loses its function of repairing genes in the G1 phase when subunits such as hTERT become upregulated resulting in high telomerase activity (Zhang, 2003). A decrease in the hTERC expression level leads to a halt in the cell cycle at G1 and G2 stages as a result of the activation of the p53 protein and CHK1 protein kinase (Kedde *et al.,* 2006).

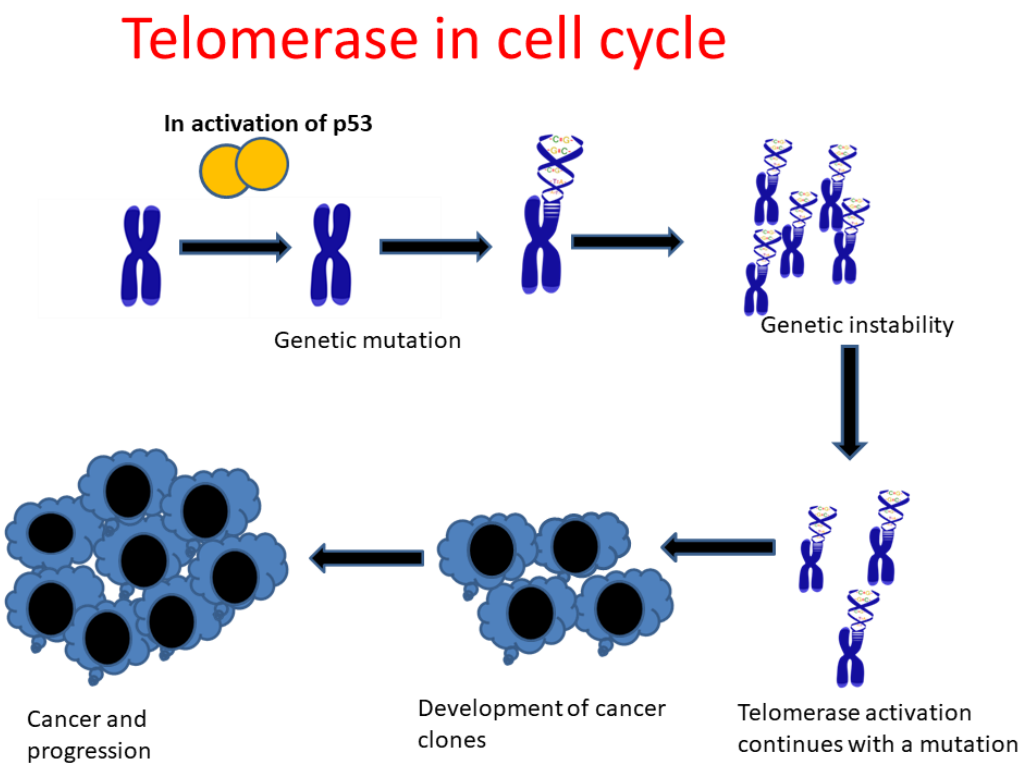


Figure 8: Telomerase in the cell cycle. During successive cell division when telomeres are damaged, DNA mutation occurs followed by replication (Sharpless and DePinho, 2007). Which may result in genetic instability. This transformed DNA continues with the mutation and disables p53 to perform its function of repairing the gene leading into cancer. In the G1 phase if p53 is unable to repair the DNA. It negatively regulates RBBP6 to become upregulated. Telomerase is activated when there is a genomic instability adding up DNA repeats to telomeres increasing

telomere length, which automatically becomes unstable and leads to the development of cancer clones.

2.6 Apoptosis and cervical cancer

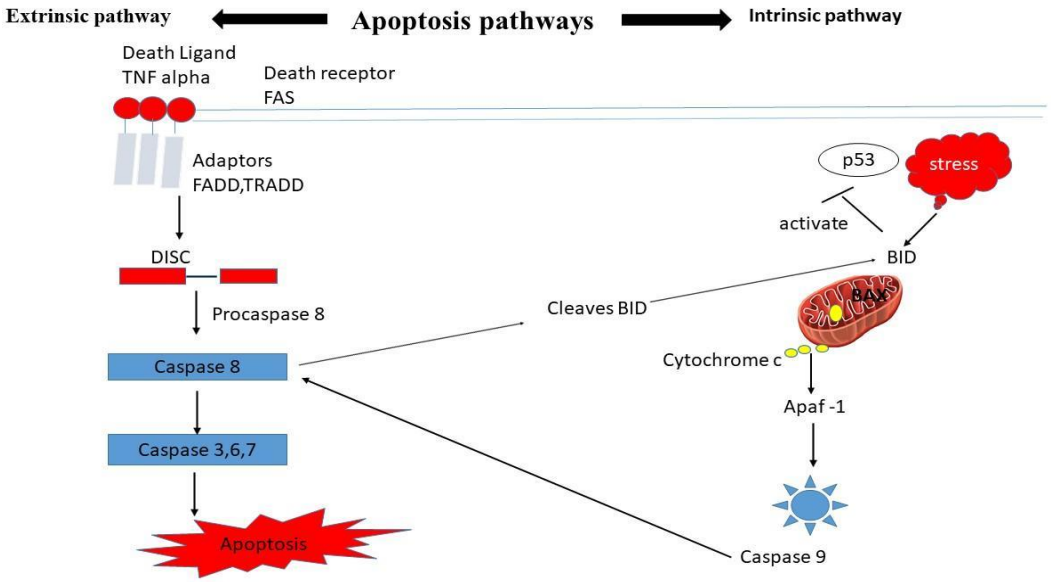
Carcinogenesis was previously regarded as a result of mitotic disorders. However, in more recent years there has been a revision to this thought. Failure of apoptosis, possibly caused by deregulation or mutation of regulatory genes and their protein products is increasing (Hassan *et al.,* 2014) becoming the apparent cause of carcinogenesis (Mokbel and Mokbel, 2018). Apoptosis is a form of programmed cell death with defining features such as distinctive cell morphology and energy-dependent biochemical mechanisms (Kerr *et al*., 1972; Paweletz, 2001; Kerr, 2002). Apoptosis is characterised by series of morphological changes of features which include cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay in the cells or body (Karam, 2009). Apoptosis plays a vital role in human development as well as homeostasis (Hassan, 2014). It is believed to be one of the most highly effective processes as it serves in eliminating any unnecessary or unwanted cells (King and Cidlowski, 1995; Pucci *et al.,*2000; Yeung, 2002). There are numerous conditions which can lead to stimulation of the apoptotic pathways including DNA damage or uncontrolled proliferation of cells. Apoptosis mechanism is activated by both extracellular and intracellular signals (Lopez and Tait, 2015). This signals are suspected to be external stimuli such ultraviolet light, hypoxia, DNA damage, activation of death receptors, and chemotherapeutic agents that exposes the cells and induce apoptosis (Igney and Krammer, 2002; Zangemeister-Wittke and Simon, 2001). Aberrant apoptosis has cited as the main cause for many disorders such as cancer, diabetes, various neurological and autoimmune disease (Magal *et al*., 2005). Most often referred to as mitochondrial and death receptor pathways respectively (Zaman *et, al.,* 1992).

Intracellular signals are associated with DNA damage growth factor deficiency and cytokines deficiency whereas extracellular signals are most commonly associated with death-inducing signals produced by cytotoxic T cells from the immune system in response to damaged or infected cells (Zaman *et, al.,* 1992). Apoptosis pathways are carried out by executioner caspases (Salvesen, 1997) Caspase activation occurs when there is a signal sent within the cell. In case of an intrinsic pathway largely caused by stresses that result in the mitochondrial membrane potential change. For example, exposure to reactive

oxygen species (ROS) and oncogene activation may trigger the expression of protein folders and pro-apoptotic members of the Bcl2 family sensors. Upon disruption of the outer mitochondrial membrane, a set of proteins, including cytochrome c begin moving through the permeable membrane of the mitochondria forming pores. Cytochrome c forms a complex on the membrane space of the mitochondrion with Apaf 1 an apoptosis-protease-activating factor 1(Chipuk *et al*., 2004; Fulda and Debatin, 2006; Li-weber, 2013). This complex binds to dATP resulting in an apoptosome complex which induces the production and activation of caspases-9 (Wong, 2011; Cárdenas-García and González-Pérez, 2013). Once caspase 9 has been activated, the resulting consequence are a cleavage of caspase 3 and induction of caspase 8 activity via a protein cascade which leads to apoptosis (Gewies, 2003; Magal *et al*., 2005; Spierings *et al.,* 2005).

Following the extrinsic pathway, there is an external death signalling directed to the death receptors located on the surface of the cells. The death ligand TNF alpha binds to death receptors (FADD) present on the cell (Walczak, 2013; Sedger, 2014). The binding of the death ligands to the receptors targets cell triggers receptor clustering on the cell surface (Elmore, 2007). This aggregation recruits the adaptor proteins on the cytoplasmic site receptors to form death-inducing signalling complex (DISC). Fas receptor employs the use of Fas-associated death domain (FADD) adaptor protein to facilitate the binding of procaspase-8 (Wong,2011) by bringing procaspase molecules close to one another, by facilitating their autolytic activation and release into the cytoplasm where they activate caspase cascade (Kischkel *et al.*, 1995).

Active caspase 8 mediates cleavage of BID, a proapoptotic protein (Kataoka *et al.*, 1998; Scaffidi, 1999).BID releases mitochondrial proapoptotic factor linking the two pathways (Kantari, 2011). Caspase 8 activates other caspases such as 3.6,9 which results in apoptosis and remove dead or unwanted cells. The following schematic diagram represents an apoptosis mechanism via an intrinsic and extrinsic pathway (Hussein,



2005).

Figure 9: Apoptosis pathway, intrinsic and extrinsic pathways

2.7.1 *Cannabis sativa*

Despite current chemotherapeutic intervention, cancer incidences keep rising at alarming rates, annually. An important concern is the recent high prevalence of cervical cancer in LMICs countries of sub-Saharan Africa. Therefore, new strategies investigating new mechanism to prevent and manage cancer are highly imperative and urgently needed. Ideally, these strategies should be capable of inhibiting, reversing and preventing aggressive cancer without causing adverse effects to humans. Sub-Saharan Africa is rich with natural fauna and flora, and since medicinal plants extracts which have long been used worldwide to treat numerous ailments including cancer, nausea, microbial and fungal infections should be explored (Desai *et al.,* 2008; Ghali *et al.,* 2014).

Cannabis sativa (marijuana or weed) is a dioecious plant of Cannabaceae family. It originates from Central and Eastern Asia (Turner *et al*., 1975; Flemming *et al*., 2007). *C. sativa* is widely distributed and grown mainly in Morocco, South Africa, United States of America, Brazil, India, and parts of Europe (Turner *et al*., 1975; Happyana *et al*., 2013). The main setback of using *C. sativa* for recreational and medicinal use is that it has not been legalized for private use in many countries. This is partly due to the fact thatof *C. sativa* is addictive and often leads to unproductive and criminal behaviour (Ayenigbara, 2012). When smoked at high concentrations, it releases tetrahydrocannabidiol (THC) that is absorbed in blood stream through the lungs, result in respiratory problems (Hunault *et*

al., 2008). However, despite these controversial functions associated to it, *C. sativa* is considered as one of the most useful drug in the medical research. This plant has an anticancer proliferative activity and the ability to treat other health related issues such as anorexia, chronic pain such as fibromyalgia, rheumatoid arthritis and sclerosis and brain disease. (Ayenigbara, 2012).

A total of 554 bioactive secondary metabolites have been identified in *C Sativa,* of these the highest constituents of 113 cannabidinoids are known to be present in the plant (Calvi *et al.,* 2017). Phytochemicals such as flavonoids, steroids and terpenoids have also been found in *C. sativa* (Elsohly and Slade, 2005). This study uses CBD- a phytochemical constituent of *C. sativa* compound to elucidate the anti-cervical cancer properties and efficiency of combination therapy.

2.7.2 Cannabidiol

Plant derived cannibidiol (CBD) which constitute 40% of the *C. sativa* extracts is a potent anti-cancer agent. CBD inhibits prostate (Rock *et al.,* 2011; Pisanti et al., 2017), Lung (Ramer *et al.,* 2011; Sharma *et al.,* 2014) and cervical cancers (Lukhele and Motadi 2016). In breast cancer cell lines, CBD has been shown to inhibit cell proliferation and induces apoptosis (Shrivatastavas, 2011) while for prostate cancer cells, CBD seems to induce apoptosis via a down regulation of AKT protein (Sharm M, *et al* 2014). Lukhele and Motadi (2016) provided a correlation of results found by Romano *et al* (2014) that showed CBD was able to reduce cell proliferation in two colorectal cancer cell lines and also induce cell death in prostate cancer. Although it has been shown that CBD inhibit the growth of cells and induce apoptosis in cervical cancer cells (Lukhele and Motadi, 2016), the effect of this on telomerase activity remains to be established. Interestingly, in contrast to the whole plant extract of *C. sativa*, CBD has been described for its non-psychoactive effects making it a safe and most recommended anti-cancer drug in development (Bergaschi, 2011; Ramer *et al.,* 2012)

The molecular mechanism by which CBD destroys tumour cells is not thoroughly understood. However, several observations have been made, for instance CBD is cytotoxic to gliomas and also possesses the ability to inhibit tumour cell migration in vitro (Vaccani *et al.,* 2005). Also, it is reported that CBD has the ability to induce apoptosis in human leukaemia cell lines by activating standard caspase pathways, improving NOX4,

and p22 function. A recent study revealed that CBD has the potential to inhibit cancer growth by downregulate ID1, a regulator of metastasis in breast cancer cell lines (Ligresti, 2006; McAllister, 2007). Furthermore, a conjugation of CBD and THC induces apoptosis in glioma cells (Marcu, 2010). Despite these extensive studies, the precise molecular mechanism by which CBD uses for its anti-cancer properties remains highly debated. More investigations into signalling cascades that could lead understanding the potentiality of CBD as anti-cancer agent are warranted.

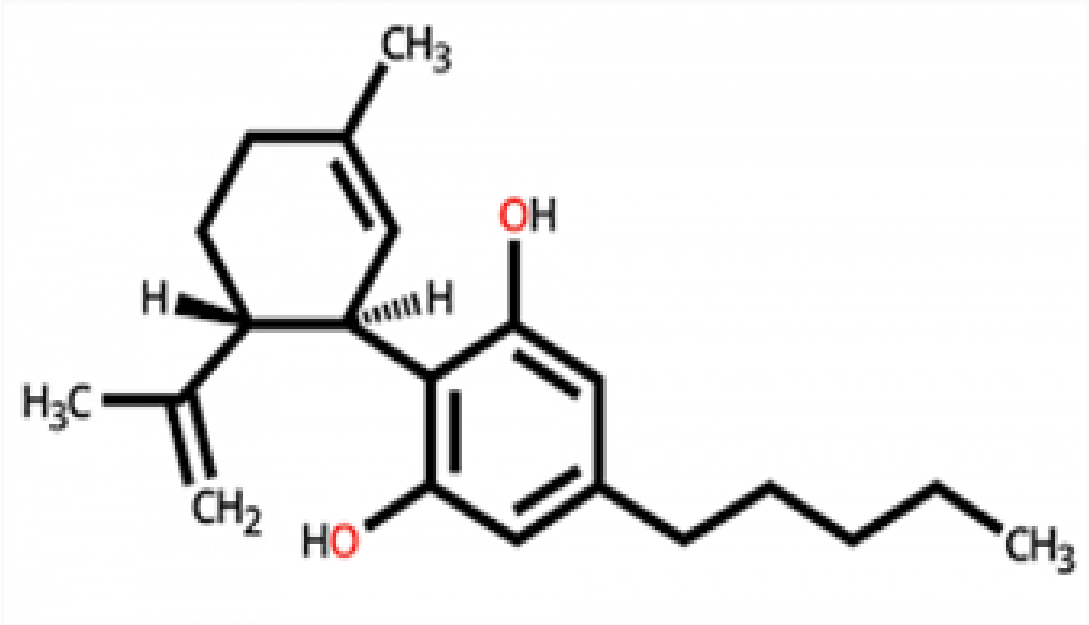


Figure 10: Cannabidiol structure. (Russo *et al.,* 2018)

2.8 Combinational therapy

Combinational therapy is a new and reliable source of treatment for cancer. This therapy benefits cancer treatment by supressing one pathway and therefore synergistically eradicating several clones that appear in a tumour (Lord *et al,* 2015). Evidence for the effectiveness of combination therapy has also been shown in the laboratory setting with cell culture studies and in some cases on clinical trials (Brechbiel *et al.,* 2014; Ma *et al.,* 2014; Andersen *et al.,* 2015; Li *et al.,* 2016). Considerably, some of the most common therapies used in the treatment of human cervical cancers are radiotherapy and chemotherapy, however, these therapies are toxic and expensive. Furthermore, these therapies cause DNA damage in normal cells which then limits their clinical efficacy and desirability (Moding *et al.,* 2013). Therefore, research on combinational therapy has intensified, it is believed that this promising strategy can be exploited for treating various

cancer. It seems through combination therapy non-cancerous cells can maintain their normality while cancer cells are being inhibited. The benefits of combination therapy are increasingly being realized.In a study, Moela and Motadi (2014) co-treated cervical cancer with silenced RBBP6 and anticancer agents staurosporine resulting in inhibition of cell proliferation and apoptosis furthermore this study is intended to check how telomerase activity responds to combinational therapy by knocking down RBBP6 and co treating with cannabidiol and also observe apoptosis.

Chapter 3: Methodology

3.1 Cell culture

The metastatic cervical cancer cell lines, ME-180, were purchased from ATCC and used as a source of mRNA for studying the growth and morphological changes after co-treatment with siRBBP6 and CBD. In brief, cell culture media was prepared using Dulbecco's modified eagle's media (DMEM) (Highveld biological) supplemented with 10% fetal bovine serum (FBS) (Highveld biological) and 1% penicillin and streptomycin antibiotic (pen/strep) (Sigma) and incubated at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2). To reduce contamination, a vial containing frozen ME-180 cervical cancer was wiped with 70% ethanol and thawed in a lamina flow hood for approximately 45 seconds. And for growth, ME-180 cells 50% of supplemented DMEM was mixed with 50% high glucose nutrient media (Thermofisher, USA) were incubated until they were 70% confluent. The cells were fed with new nutrient growth media every second day of the week, following this, cells were washed twice with 2 ml with PBS. The cells were sub-cultured to confluence by trypsinization twice per week. sub-cultures (excess) were then stored -80°C in DMEM/High Glucose growth medium containing 10% FBS and 20% DMSO (dimethyl sulfoxide). The cells were counted using cell counter machine and used for MTT assays and subsequent analyses.

3.2. MTT assay

MTT assay is a colorimetric method used to assess cell proliferation, viability, cytotoxicity following treatment of cells with CBD. The MTT assay measure cytotoxicity and cell proliferation by employing a succinate dehydrogenase enzyme which reduces a yellow 3-(4, 5-dimethythiazol-2-yl)-2-5 diphenyl tetrazolium bromide to an insoluble formazan (Berridge, 2005). Upon entering the cell, MTT goes through the mitochondria, where it is metabolized to an insoluble, shaded (dark purple) formazan product. The cells are then solubilized with a natural solvent known as isopropanol and released. The solubilised formazan reagent which can be measured spectrophotometrically (Kerr, 2011). The decrease of the MTT occurs metabolically inactive cells that have a level of activity as a measure of cell viability. This study used the MTT assay to evaluate the cytotoxic effect of CBD on ME-180 cells and also account for IC_{50} which represents the half maximal concentration that induces 50% cell death.

Approximately 80µL of ME-180 cells with a density of five thousand were seeded into a 96 well plate incubated overnight at 37°C under a humidified atmosphere of 5% CO_2 for attachment and 80µL of growth medium only were added in some wells to serve as a blank. Following attachment, cells were treated with various concentrations of CBD (0.5 -3µg/ml) and incubated for an additional 24h. Camptothecin with a concentration of 0.3 µM and 0.1% DMSO were used as a positive and vehicle control respectively. Subsequent to this, 20µL MTT solution (5mg/ml) was added to the wells except for the blank for a 4h incubation. After incubation DMSO was added into wells for another 4h to dissolve the formazan crystals and absorbance was measured using a spectrophotometer plate reader (Bio-rad, USA) at a wavelength of 570nm.

$$\%\ \boldsymbol{Cell\ viability} = \frac{\boldsymbol{Abs\ treated\ -\ Abs\ blank}}{\boldsymbol{s\ untreated\ -\ Abs\ blank})}\ \boldsymbol{X100} \qquad \text{eq…1}$$

% cell death = 100-cell viability eq…2

The formulae used to determine IC_{50} and **Abs** represents the absorbance readings.

3.3. RNAi transfection.

RNA interference is also known as post –transcriptional gene silencing, a eukaryotic regulatory process by which the expression of a particular gene is silenced at the stage of translation (Hanmon, 2002). In this process a short interference RNA is introduced in the cells and it binds with the complementary nucleic acid pairs of the target mRNA. The binding of the shRNA to the target gene functionally inactivates the target gene, or in some cases, renders the degradation of the target mRNA (Aagaard and Rossi, 2007). This study achieved RBBP6 silencing using RNAi technique. Briefly, once 70-80% confluent, ME-180 cells were transfected with siPORT™NeoFX transfection reagent (Thermofisher, USA) which contained 30nM siRNAs specific to RBBP6. Once inside the cells, siRBBP6 forms complementary base pairing thereby inactivates the expression of RBBP6. Gene silencing was induced over 24 hours, following which, adherent cells were trypsinized and re-suspended in free serum media without antibiotics. The suspended cells were then mixed with siRNA/transfection agent from complex in T25 flasks and incubated at 37°C for a period of 24 hours. Post-transfection cells were exposed to

0.5µg/ml of CBD incubated for an additional 24 hours. Transfected cells were used for subsequent experimental analysis.

3.4 Apoptosis detection

Apoptosis detection was conducted by microscopy and DNA fragmentation. Microscopy is one of the most reliable and powerful techniques in the biological studies (Thorn, 2016), it uses fluorescence and phosphorescence to study the properties of an organic and inorganic specimens. The fluorophores are absorbed at a specific wavelength and emits light at a specific wavelength. In a sample a specific dye is added which binds to the cells and light is emitted and absorbed at specific wavelengths. This fluorescence microscope shows a high resolution picture with a detailed shape and colour unlike a conventional light microscope (Bootman, 2014).

Approximately, 10 thousand cells were transfected with siRBBP6 seeded onto a 6-well plate with coverslips and incubated overnight to allow attachment. Next, the media was discarded; cells were washed with 2ml PBS. Transfected cells were then treated with 0.5µg/ml CBD for an additional 24h, to confirm the induction of apoptosis and the morphological changes that may have occurred after treatment. After 24hrs, old media was discarded, cells were washed with 1x binding buffer. 5 µL of Annexin- V and PI respectively were added to the cells then incubated for 15 minutes in a dark room. Cells were fixed with two per cent (2%) formaldehyde and incubated for another 15 minutes at room temperature. A fluorescent microscope was used to observe a morphological change.

DNA Fragmentation

The DNA fragmentation is one of morphological changes that were identified (Basnakian and James, 1994). For fragmentation to occur there is specific caspase responsible for the cleavage of the cells that undergoes apoptosis known as caspase 3 (Nagata *et al.,* 1998). Caspase 3 is responsible for cleaving ICAD to dissociate the CAD: ICAD complex, allowing CAD to cleave chromosomal DNA. Cells that have the absence of ICAD or that express caspase-resistant mutant ICAD are unable to show DNA fragmentation during apoptosis, although they do display some other features of apoptosis (Shakari, 1998). The fragmentation of DNA is shown in an agarose gel to confirm apoptosis. Fragmentation is observed in 180 bp size when the enzyme has cleaved the ICAD

Cells that were 70-80%confluent were transfected with siRNA and siRBBP 6 for 24 hours followed by additional 24-hour treatment with CBD. After treatment cells were washed twice with 2 ml 1X PBS and trypsinized to allow detachment. Cells were then collected by lysis buffer, centrifuged for 10 minutes at 10 °C at 12000 rpm. DNA was extracted following the Nucleospin® user manual. The concentration of DNA was read using the Nanodrop at a ratio of 260/280. DNA was run in 1% agarose gel. 1kb marker from NEW ENGLAND BioLabs #N3232L was used as reference. Bio-RAD GEL Doc

3.5 RNA Extraction

RNA is used in real-time PCR whereby it is reverse transcribed into cDNA. Firstly, it should be isolated from cells or tissues. To ensure a purified RNA the following conditions should be met. It should be free from contaminants; a ratio of A260/280. High quality total RNA is used for the performance of molecular techniques (Huggett *et al.*, 2013; Tan and Yiap, 2009). This study used a nucleospin kit which contains RNAse inhibitory agents such as chaotropic ions (e.g. guanidine salt), sodium dodecylsulfate (SDS) denaturant or phenol-based compounds to ensure that highly pure RNA has been extracted. In this study, total RNA was extracted from cells transfected with siRBBP6 and co-treated with CBD as described in section **3.2**

Transfected cells were lysed with150 µL of RA1 buffer and 1.5 µL of β—mercaptoethanol mixed vigorously with a vortex for 5 seconds to lyse the cells. The lysate was transferred onto a new Nucleospin RNA filter in a collection tube and centrifuged for 30 seconds at 11000 x g. The Nucleospin RNA filter was discarded, the sample was added to a new tube and 70% ethanol was added to the lysate to homogenous the lysate in order to adjust the RNA binding. The new Nucleospin RNA was placed in the new collection tube the lysate was loaded into the tube and centrifuged at 11000 x g for 30 seconds. The lysate was then transferred through the RNA binding silica membrane. 100 µL of MBD buffer was applied onto the tube centrifuged for 30 seconds to desalt the silica membrane. 25 µL rDNase reaction mixture was added to the lysate for 15 minutes for incubation at room temperature. After incubation cell lysate was washed 3 times at 11000 xg 1 minute after wash the RNA Nucleospin tube was air dried and 15 µL of RNase free water was added to the membrane to elute the RNA centrifuged for 30 seconds at 11000xg.

The concentration of the total RNA extracted was done using the nanodrop spectrophotometer (Thermofischer, USA) and the purity of the RNA was confirmed by ensuring that A260/A280 ratio was >1.7 as indicated on **Appendix C.** After extraction of RNA, it was reverse transcribed to cDNA using (SuperScript ™ VILO™ cDNA synthesis) (Roche, Switzerland), following the manufacture s protocol, the synthesis of cDNA was briefly carried out as follows. Cocktail mixture containing the following 4 µLof 5X VILO™Reaction Mix, 2 µL of 10X SuperScript ™Enzyme Mix and 1 µL of RNA (up to 2.5 µg) was prepared. The volume of the reaction was brought to 25 µL using 13 µL of DEPC-treated water for a single reaction and this was further added onto the tube on ice.

The reaction was run on a BioRad CFX-real-time thermocycler, which was programmed to run the reaction as follows:

Table 1. The reverse transcription PCR protocol

Temperature (°C)	**Time (mins)**
25 °C (denaturation)	10 minutes
42 °C (annealing)	60 minutes
80 °C (extension)	5 minutes

Table 2. Master Mix for Optimization of primers

Master Mix for optimization of all the genes was as follows in a 50 µL:

Components	**Quantity**
PCR Master Mix 2X	25 µL
Forward	0.1-1.0 µM
Reverse	0.1-1.0 µM
Template (RNA)	10pg-1 µg
Nuclease free water	Up to 50 µL

3.6 Real-Time Quantitative –PCR

Real time quantitative PCR (RT-qPCR) was performed to ensure that transfection of cells was successful and to also study gene expression changes following co-treatment of cells (Huggett *et al.,* 2013; Tan and Yiap, 2009). The main purpose of RT- qPCR is to monitor the amplification of the targeted DNA molecule in real time. In this case, the total isolated mRNA from the co-treated cells was synthesized to a complementary DNA

In real-time PCR to measure the amount of DNA at each cycle, a fluorescent dye was used which has the capability of intercalating each newly synthesized dsDNA to produce a signal that's directly proportional to the amount of PCR produced. The fluorescent dye used was SYBR Green which is inexpensive, easy to use and highly sensitive. (Valasek and Repa, 2005; Ginzinger, 2002).

Table 3. PCR protocol for primers

Steps	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	95	1-3min	1
Denaturation	95	The 30s	34
Annealing	Tm-5	30s	34
Extension	72	1min/kb	34
Final extension	72	5-15 min	1

ME -180 cells were exposed to a 48-hour combinational treatment (transfection with RNAi and siRBBP6 and co-treated with 0.5ug/ml CBD). Total RNA was isolated from treated cells, cDNA was synthesised using SuperScript™ VILO ™ cDNA kit from the isolate and quantified by SYBR® Green JumpStart Taq Ready Mix (SIGMA, USA) dye. The qPCR reaction was performed in a 10µl mixture of 2122ng/ µL cDNA, SYBR Green, forward and reverse primers under optimized conditions: denaturation at 94°C for 35s, followed by

annealing of the primers at 59°C for 45s, and extension 72°C for 45s, the reaction occurred for 36 cycles

3.7 TRAP Assay

The ribonucleoprotein, telomerase is an enzyme that is responsible for the maintaining telomere length in immortal and cancer cells (Capkova, 2011). Therefore, for early detection of cancer and the fast screening of compounds, new cancer drugs discoveries should be designed based on the detection of telomerase. The most widely used method for determination of telomerase activity has been a PCR- based technique known as the TRAP assay. This method was developed for fast and efficient detection of telomerase activity. In the vast majority primary human tumour examined by researcher all over the world, TRAP assay has proven to be an adequate technique for measuring telomerase activity (Vicentini *et al.,* 2004).

For the TRAP assay, using 200 µL ice-cold 1x Chapsis lysis buffer (10mM Tris-HCl, pH 7.5,1 Mm $MgCl_2$,1 mM EDTA, 0.1mM Benzamidine, 5mM β--mercaptoethanol, 0.5% CHAPS,10% Glycerol) transfected and co-treated ME-180 cervical cancer cells were lysed. The process of cell lysis was allowed to occur on ice for 30 minutes. The lysate was then centrifuged at 12000xg for 20 minutes at 4˚C. The supernatant was transferred to a new tube and the total protein was measured using a UV/Vis spectrophotometer. For experimental use telomerase samples were divided into two groups of all the four treatments (untreated, siRBBP6, siRBBP6+CBD and CBD) for heat of 80˚C and no heat. Positive cells were used as control and 1X CHAPS buffer. For PCR reactions, 2 µL of all samples were added into the pcr tubes. The master mix containing 5.0 µL of 10X TRAP Reaction Buffer, 1.0 µL 50X dNTP Mix, 1.0 µL TS Primer, 1.0 µL TRAP Primer Mix and 0.4 µL (2 Units) Taq Polymerase (5 units/µL) was prepared . The volume was adjusted to 48.0 µL with dH2O and 2 µL of protein cells was added to the mixture and mixed vigorously. About 2 µL of all the samples were transferred to 1.5 µL PCR tubes and placed in the thermocycler following the PCR amplification conditions: Cycle 1 of 30°C for 30 mins, Cycle 2 of 95°C for 2.mins,Cycle 3 of 94°C for15 sec, 59°C for 30 sec, 30-34 cycles of 72ºC for 1.0 min and 1 cycle of 4°C for ∞ The PCR products were run on a 12% native page for 4 hours and the GEL-Doc machine from Bio-RAD was used for imaging

Chapter 4: Results and discussion

4.1 Introduction

In this chapter, the results and discussion of the study aim and objectives are detailed. The first objective was to measure the cell viability of ME-180 cervical cancer cells after treatment with CBD. This was followed by apoptosis detection using DNA fragmentation and microscopy. The cells were then co-treated with siRBBP6 and CBD and the relative expression of RBBP6, MDM2 and P53 were measure quantitatively. Finally, Trap assay was conducted to assess telomerase activity.

4.2 Results

4.2 .1 MTT assay

MTT assay was conducted to evaluate the cytotoxic and cell proliferation effect of CBD treated ME-180 adherent cervical cancer cells. The experiments were conducted in duplicates and cells were treated with increasing concentrations of 0.5µg/ml, 1.0 µg/ml, 1.5 µg/ml, 2.0 µg/ml and 3.0 µg/ml CBD. Untreated cells were used as reference for evaluating the efficacy of CBD, while DMSO and camptothecin were used as vehicle and positive control, respectively.

As can be seen in Figure 13, DMSO, which has been for years been used to preserve the integrity of viable cells because at lower concentrations it is not toxic did not represent a significant change on ME-180 cervical cancer cells, maintaining their viability of 89% of the cells. The data obtained for DMSO was found to be insignificant, however, with p-values of 0.05 and lower, campothecin inhibited more cell proliferation compared to CBD. The positive control, camptothecin showed a more significant cell death effect (80% dead cells) compared to CBD which also exhibited a significant inhibition effect almost 70% of the cells in all the treatments of the cells (Figure 11), indicating that CBD sensitized ME-180 cells.

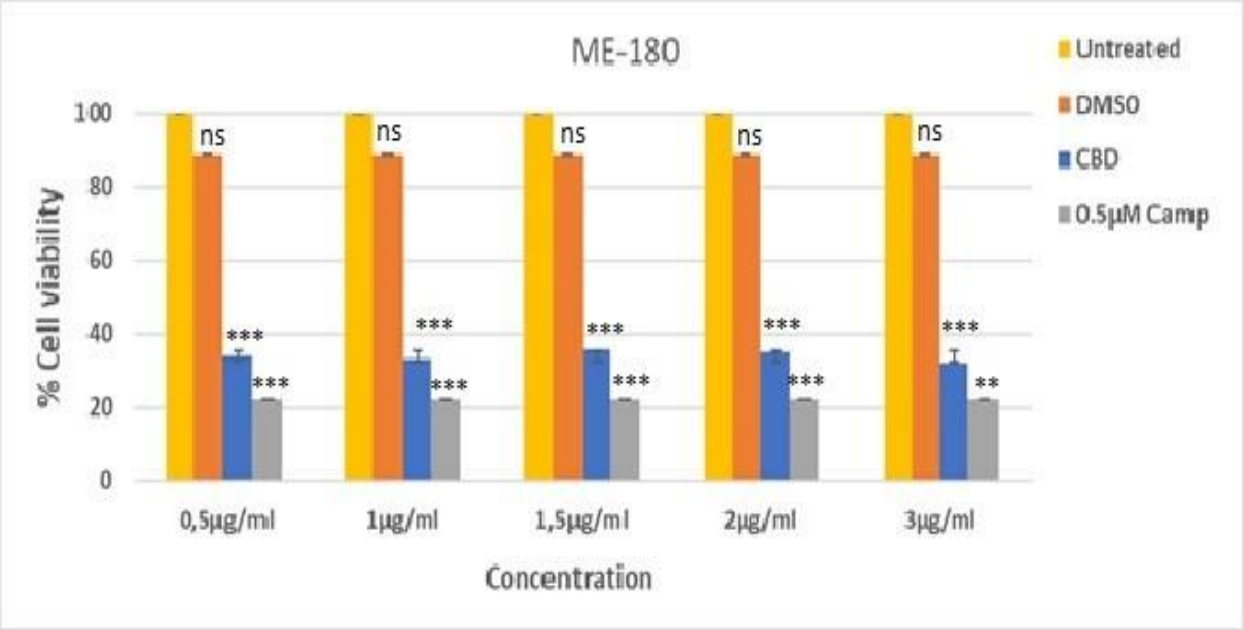


Figure 11: Cytotoxic effects of CBD on the cervical cancer cell line. ME-180 cells when treated with various concentrations of CBD (0.5, 1, 1.5, 2 and 3 µg/ml) for a period of 24 hrs. Data are expressed as the mean ± SD and ***p<0.001, **p<0.01,

4.2.4 Apoptosis detection

DNA fragmentation

The morphological changes in nuclear DNA can be used to differentiate between cells undergoing apoptotic cell death from viable cells. This is because apoptosis is often accompanied by chromatin condensation. And because of the catalytic cleavage that is often facilitated by caspases resulting in nuclear DNA fragmentation, this morphological change is also used as another indicator of apoptosis (Kijima and Mizuta, 2019). In the experiment, DNA fragmentation was performed for evaluating the approach of cell death induced by CBD and the co-treatments

In principle, DNA fragmentation uses an agarose gel electrophoresis to visualise the apoptotic bodies by detecting DNA laddering or smearing. Thus, in order to examine whether the isolated genomic DNA of treated or untreated DNA detection and ladders were visualised by ethidium bromide. The results obtained indicate that little to no smearing but a faint DNA band untreated cells, confirming that indeed genomic DNA was extracted from the untreated cells (Figure12A). Not only untreated cells but also, CBD and siRBBP6 treatment exhibited less or totally absent DNA fragmentation (Figure 12A).

Both the CBD and siRBBP6 treatment showed little to no smearing but a faint DNA band, as compared to the untreated and co-treatments. Apparent DNA fragmentation represented by the intensity of the band and smearing can be seen in the siRBBP6-CBD co-treatment cells (Figure 12A). This is suggestive of apoptosis. However, the appearance of smearing may also represent necrotic events (Matassov *et al.,* 2004). Thus, microscopy was subsequently conducted to assess the mechanism of action which siRBBP6-CBD co-treatment uses for cell death induction.

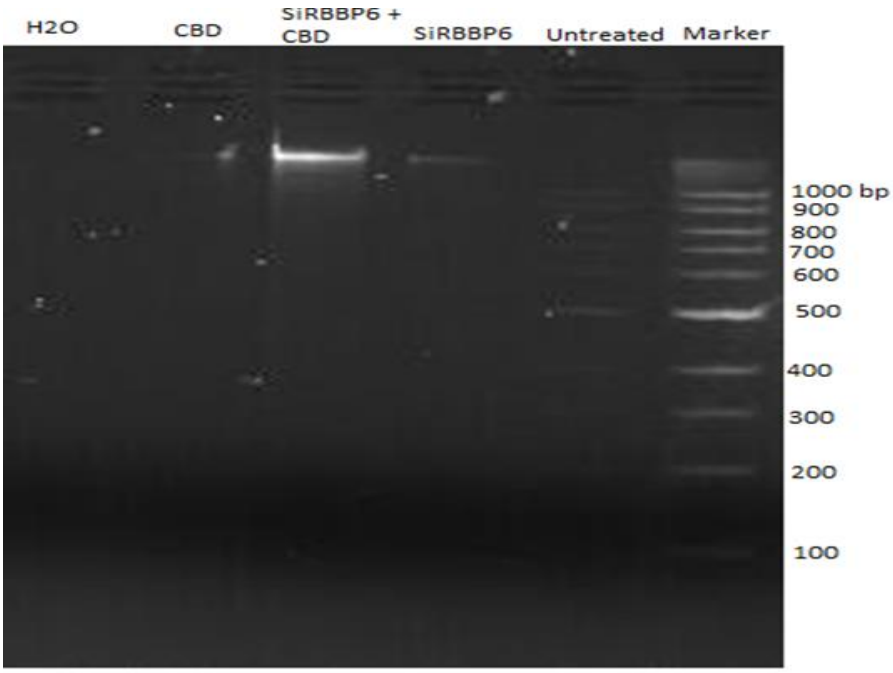


Figure 12A: The 1% agarose gel electrophoresis for visualizing DNA fragmentation of ME 180 cells. From the left, lane 1 is the water blank, followed by CBD treatment, lane 3 is the co-treatment with siRBBP6-CBD. The siRBBP6 treatment alone (lane 4) followed by the untreated control (lane 5). Mw is known as a molecular weight marker (1000 bp) represented in the last lane

Microscopy

Microscopy is one of the techniques used to observe morphological change and early and late apoptosis. In the process of programed cell death phospholipid phosphatidylserine is found inside the plasma membrane to translocate to the extracellular environment. This results in phosphatidylserine being exposed, thus targeted by stains such as Annexin V. This dye possess a stronger affinity to bind to the external phosphatidylserine residues (Abbady *et al.,* 2017). The loss of membrane integrity and external phosphatidylserine residues emerge at the early stages of apoptotic cell death. Thus, Annexin V is used to identify and stain the apoptotic cells at earlier stages while PI detects late apoptosis (Xu *et al.,* 2017).

In Figure 12B untreated cells did not uptake any dye hence the cells are not visible. The dye bind to the DNA of the dead cells and fluorescence microscope is used for observation. siRBBP 6 showed 60 % of dead cells after staining. siRBBP 6 showed early apoptosis on the cells, only Annexin V was able to detect the dead cells while PI did not show a significant result. Cells treated with CBD showed a significant cell death almost 90% of the cells were dead, as already stated in the MTT assay CBD is highly sensitive hence we observed high number of dead cells in the CBD treatment and also the shape of the cells were shrinking suspecting that the cytoplasm is dense the sizes are small.

In the co- treatment, 60% of cell death after knockdown of RBBP6 and CBD. siRBBP6 and CBD showed early apoptosis and morphological change were cells are smaller and shapeless. According to the result obtained, combinational therapy few cells reached early apoptosis which shows that the transfection coupled with CBD had little effect on the cell death.

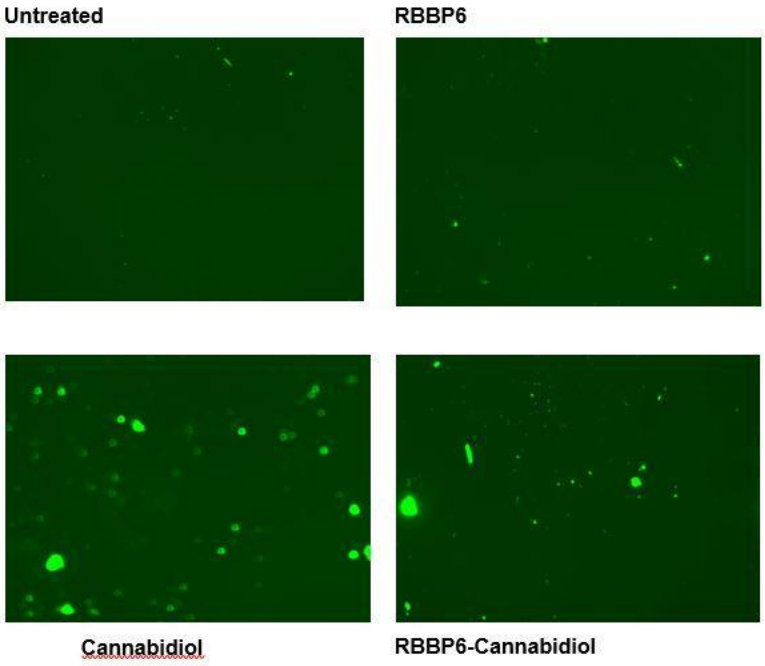


Figure 12B: Morphological features accompanied by apoptosis detection in ME-180 cervical cancer cells. Stained with a florescence Annexin V dye and Propidium Iodine after a 24-hour treatment with 0.5µg/ml CBD. Annexin V is shown by a light green stain

4.2.2 Real- time qPCR

To examine whether silenced RBBP6 was down-regulated in cervical cancer cell line ME-180 following the transient transfection, the real-time qPCR was used. Subsequently, the effect of CBD and co-treatment on gene expression of RBBP6, MDM2 and p53 was also evaluated by real-time qPCR. A house-keeping gene GAPDH was a reference gene to measure gene expression for the following genes RBBP6, p53 and MDM2. Figure 13 A, B and C represents the gene expressions of RBBP6, p53 and MDM2, respectively. The expression of target genes (RBBP6, p53 and MDM2) were evaluated in untreated cells, siRBBP6, siRBBP6+CBD and CBD treated cells (Figure 13A-C). In cells transiently transfected in siRBBP6, successful gene silencing was seen. The relative expression ratio of 0.03, indicated of a down-regulation in expression levels of RBBP6 (Figure 13A). The silencing of RBBP6 seems to have a similar down-regulation effect on MDM2, expression ratio of 0.01 (Figure 13B). This is in contrast to p53, which displayed a relative expression ratio of 0.1 (Figure 13C), approximately 7-10 fold higher than expressed RBBP6 and MDM2, respectively.

The CBD treated cells showed a successful downregulation of RBPP6 with a relative expression ratio of 0.23 (Figure 13A) and MDM2 also displayed a successful downregulation with a relative expression ratio of 0.02 (Figure 13B) whilst p53 was upregulated with relative expression of 0.6 (Figure 13C)

In the co-treatment, RBBP6 showed an increase expression levels with an increment of expression ratio of 0.15 (Figure 13A) and a successful downregulation of MDM2 with expression ratio of 0.02 (Figure 13B) in contrast with p53 displayed a successful upregulation with an expression ratio of 0.6 (Figure 13C). RBBP6 and MDM2 are negative regulators of P53

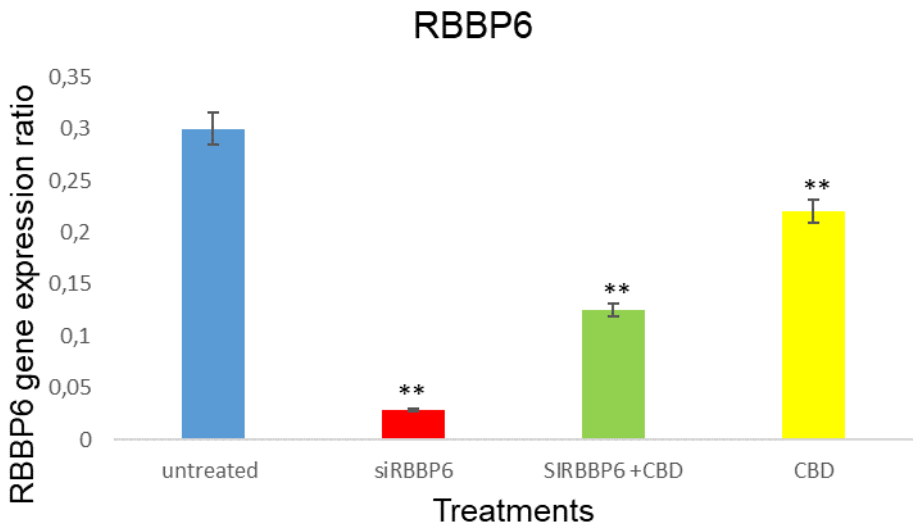


Figure 13 A: Gene expression analysis of RBBP6 in ME-180 cells following various treatments. The treatments with siRBBP6 and CBD were achieved after 24 hours and a co-treatment was allowed to occur over 48 hours. Data are expressed as the mean ± SD and **p<0.01

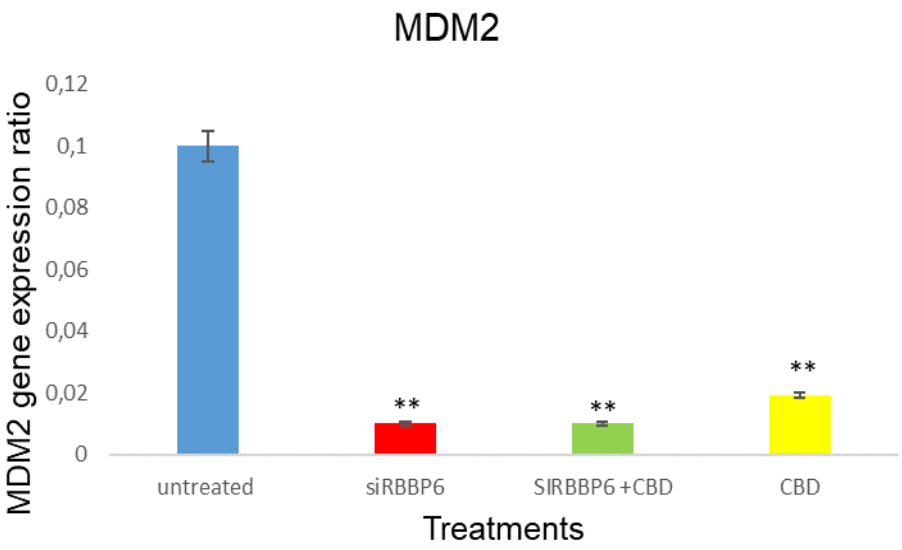


Figure 13 B: Gene expression analysis of MDM2 in ME-180 cells following various treatments. The treatments with siRBBP6 and CBD were achieved after 24 hours and a co-treatment was allowed to occur over 48 hours. Data are expressed as the mean ± SD and **p<0.01

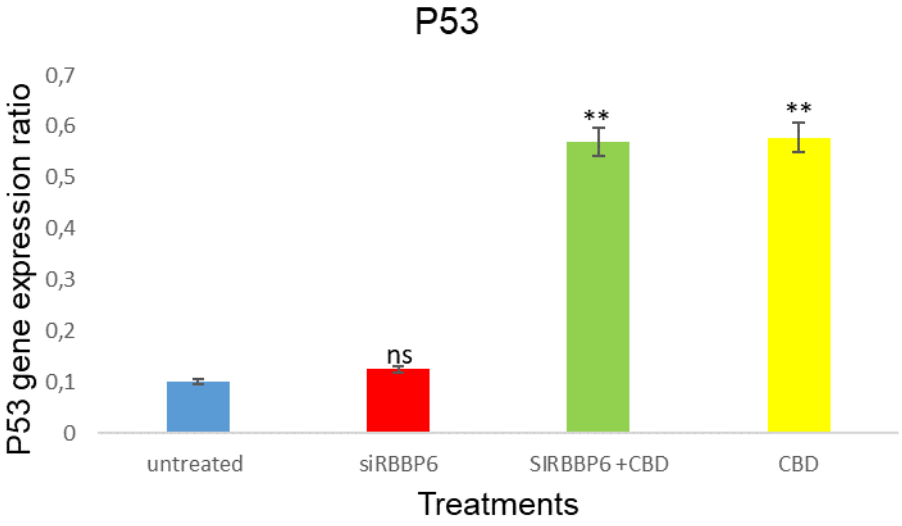


Figure 13C: Gene expression analysis of RBBP6 in ME-180 cells following various treatments. The treatments with siRBBP6 and CBD were achieved after 24 hours and a co-treatment was allowed to occur over 48 hours. Data are expressed as the mean ± SD and **p<0.01

4.2.3 TRAP Assay

In cancer cells, telomerase activity helps maintain telomere length and thereby by passes senescence and provides enhanced replicative potential. This study attempted to establish whether siRBB6, CBD and siRBBP6 and CBD induced apoptosis will result in reduced telomerase activity. Heat and non-heat treatments were used to visualize telomerase activity on agarose and native gel electrophoreses (Figure14A-B).

Figure 14A which shows amplification of telomeric repeats in the positive control (obtained with the kit), siRBBP6, CBD and co-treatment (Figure 14A, Lane B-E) shows no significant change between the telomerase activity of the treatments and the control. The same trend is also seen in the heat treatments (Figure14A, Lane F-H). Figure 14B shows a significant telomerase decrease in all the heat treatment from lane B-C ,telomeric repeats in controls (obtained from the kit) such as tsr8**,**primer mix**,** 10x TRAP buffer unexpected showed an increase in telomeric repeats as compared to the treatments.

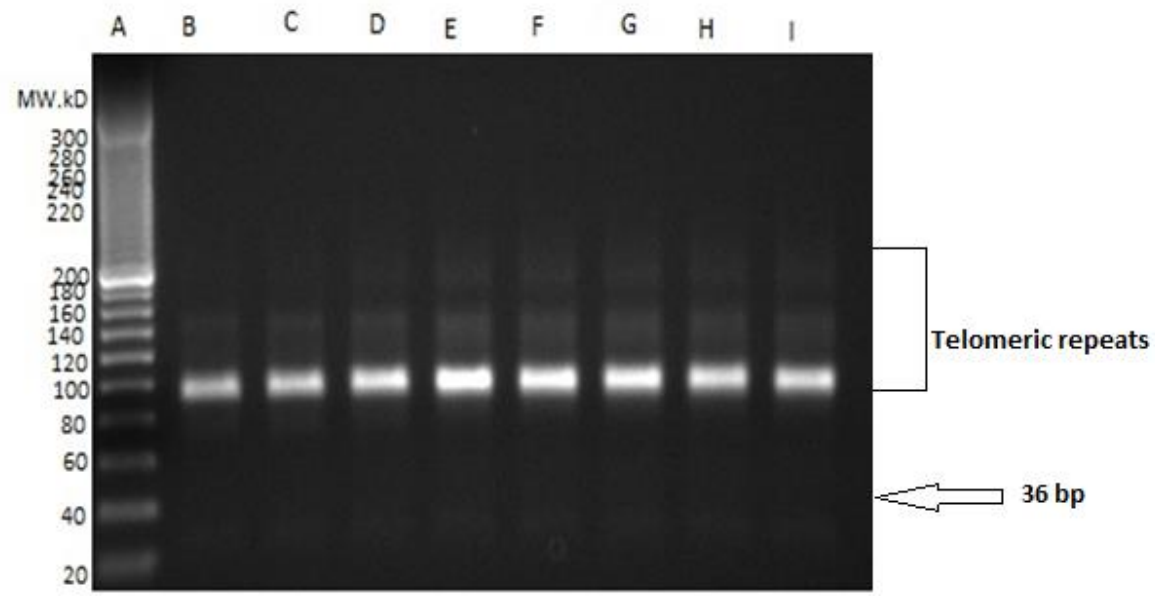


Figure 14A: Telomerase activity was measured using an agarose gel electrophoresis. Lane A represent a 320kb precision plus maker. Lane B positive control cells provided with the kit, C untreated, D siRBBP6, Lane E siRBBP6+CB D, Lane F CBD with negative heat treatment while

Lane G represent positive control cells + heat treatment, Lane H untreated cells + heat treatment, Lane I siRBBP6+ heat treatment.

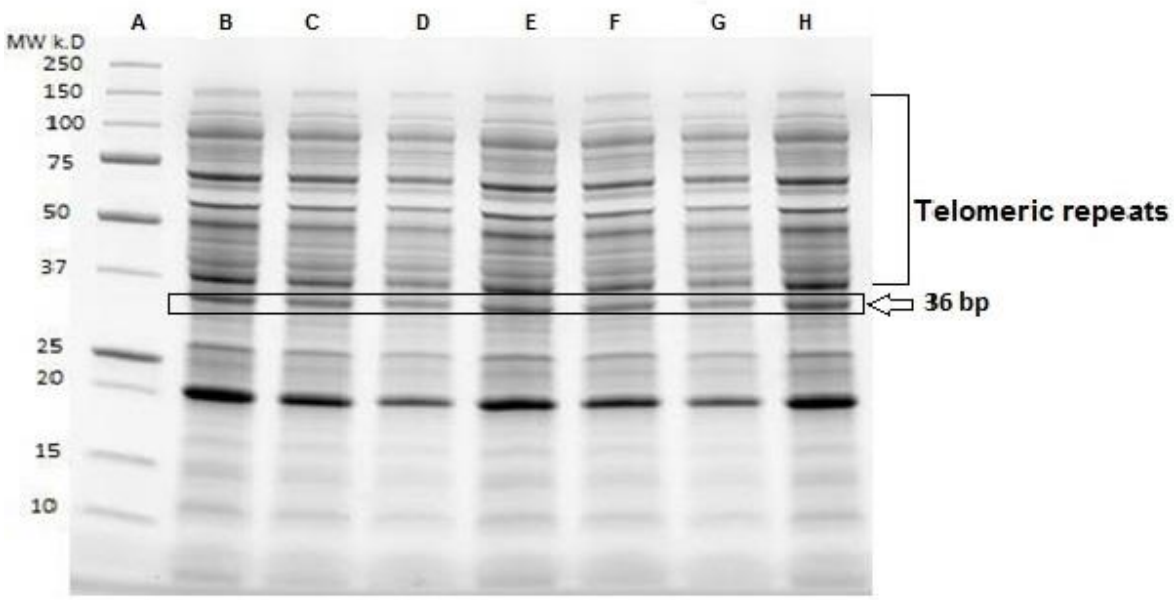


Figure 14B: Lane A represent a 320kb precision plus maker. Lane B siRBBP6+CBD heat treatment and CBD heat treatment C, Lane D, Lane E and F TSr8, Lane G primer mix, Lane H 10x TRAP buffer

4. 3 Discussion

The major gynaecological disorder of cervical cancer accounts for over 60% of this public health burden, mainly affecting women in developing countries of the world (Sankaranarayanan *et al.,* 2009). RBBP6 is abundantly produced in numerous cancers, where high expression serves as a biomarker and correlates with reduced survival and increased metastasis. The observation that RBBP6 interact with p53 tumour suppressor has attracted major investigation evaluating its potential as a cancer biomarker (Li *et al.,* 2000; Gao and Scott, 2003). P53 is an important regulatory protein that generates cellular responses such as cell cycle arrest, DNA repair, senescence and apoptosis in response to cellular stress (Speidel, 2015). In most human cancers, however, P53 pathway is often found to be defective. This could be due to either mutation or through aberrant regulation by MDM2 and RBBP6 (Speidel, 2015). The p53 found in many cervical cancer cell lines is inactivated rather than mutated. It is speculated that the negative regulator of p53, RBBP6, is involved in this deregulation (Koivusalo *et al.,* 2006). This research was

undertaken to evaluate the therapeutic potential of combination therapy (siRBBP6 and CBD) and to assess telomerase activity on cervical cancer.

CBD is one of main constituent of *Cannabis sativa* which has long been viewed as a toxic plant with psychotropic harmful effects. However, with more intent scientific inquisitions, a plethora of phytochemicals in *C. sativa* are showing excellent therapeutic efficacy in both *in-vitro* and *in-vivo* cancer cells (Chakravarti *et al.,* 2014). Indeed, the benefits of phytochemicals such as CBD for treating various human malignancies is increasingly being realized. CBD has also been demonstrated to possess anti-oxidant properties and to antagonise proliferation and inflammation (Ligresti *et al*., 2006; Scott *et al*., 2014).More evidence for potent anti-proliferative effects of CBD, especially in mediating cell cycle arrest and apoptosis in breast and prostate cancer cells, as well as glioma tumours has been provided by researchers all over the world (Sharma *et al.,* 2014; Scott *et al.,*2014; Marcu *et al.,* 2010)

4.3.1 Cell proliferation

In this project, MTT colorimetric technique was conducted to evaluate the ability of CBD to induce cell proliferation on ME-180 cervical cancer cells. In principle the techniques implore the ability of viable cells to reduce MTT dye to formazan crystals by dehydrogenases which is a sign of mitochondrial cell viability. Thus, a pronounced signal intensity of staining cells or high absorbance reading indicates excess amounts of viable cells (Bahuguna *et al.,* 2017). Indeed, the study corroborated this well accepted theory as untreated and DMSO cells which were included as negative and vehicle controls, respectively, displayed almost 100% viability. The data obtained for untreated cells was not statistically significant ($p>0.05$), however, DMSO which is not toxic at lower concentration and has for decades been used to preserve the integrity of viable cells represented statistically significant viability of cervical cancer ME-180 cells ($p< 0.01$(Figure 11).

To gain a full perspective of the anti-proliferative effect of CBD, positive control camptothecin, was used for comparison. As observed in Figure 11, 0.5µM of camptothecin resulted in 20% cell viability of cervical cancer ME-180 cells. The results were expected, as camptothecin is an accepted anticancer agent because of its ability to induce anti-tumour properties. Camptothecin is able to stabilize the DNA topoisomerase

I complex and cause breakage in the DNA molecule; Consequently, inhibiting DNA unwinding and relieving torsional stress during DNA transcription in the S-phase of the cell cycle (Chu *et al.,* 2014).

It was also observed that CBD is strongly sensitized toward ME-180 cervical cancer cells, in exhibiting cytotoxicity to approximately 70% of cells in concentrations of 0.5 µg/mL to 3 µg/mL. It was reported that CBD rather than the whole plant extract of C. sativa halt cell proliferation of ME-180 cervical cancer cells (Lukhele and Motadi, 2016). The finding from this study suggest that 0.5 µg/mL of CBD reduce the viability of cervical cancer ME-180 cells. This is in line with previous observations, which have shown that lower concentrations of CBD reduce the viability of various cancer cells and tumours (Turkekul *et al.,* 2018; Motadi and Lukhele, 2016; Sharma *et al.,* 2014; Shrivastava *et al.,* 2011). This study reports a much stronger inhibition than Lukhele and Motadi (2016), however, the lowest concentration 0.5µg/mL CBD was maintained for subsequent analyses as was the case in Lukhele and Motadi (2016). It is possible that the data obtained may lead to speculation that CDB is toxic to ME-180 cervical cancer cells, thus, it is being recommended that in light of this increased sensitization, future studies should optimize the IC50 of CBD in order to reinforce that CBD is not toxic to cancer cells. The results obtained however, lead to the hypothesis that co-treatment of CBD and siRBBP6 has the potency of inducing apoptosis in cervical cancer ME-180 cell line, and to further elucidate the mechanism of action that influenced the mode of cell death

4.3.2 Mode of cell death analysis

A successful strategy for obtaining an ideal anti-cancer agent include selecting compound(s) that selectively induce cell death in cancer cells by apoptosis without harming normal/healthy neighbouring cells. There is an evidence linking siRBBP6 and CBD with reduced cancer progress and apoptosis of cervical cancer (Moela and Motadi, 2014; Lukhele and Motadi, 2016). Since combination, therapy has shown desirable therapeutic potential in various cancers and has potential of inducing apoptotic signalling. This study further elucidates whether CBD in combination with siRBBP6 has potency of inducing apoptosis in cervical cancer ME-180 cell line. Furthermore, the mechanism of action that influenced the mode of death induced was elucidated.

Cell death occurs via apoptosis and necrotic pathways. Apoptosis is a controlled biological event leading to characteristic cell changes and death (Kanduc *et al.,* 2002) and necrosis, which is accompanied by morphological changes in lethally injured cells (Fink and Cookson, 2005). Apoptosis undergoing cells can be characterized by cellular and nuclear morphological changes including cell shrinkage, nuclear and DNA fragmentation. This study used DNA fragmentation to evaluate the apoptosis induction following treatment with siRBBP6, CBD and co-treatment (Figure 12A). Apoptotic cells produce DNA fragments between 180-200bp as a result of the endonuclease cleavage of chromatin (Matassov *et al*., 2004). From this study, there was no significant DNA fragmentation observed in untreated, CBD and siRBBP6 treated cervical cancer ME-180 cells (Figure12A). The slight DNA fragmentation observed was from co-treated cells with siRBBP6 and CBD. It is not clear how the co-treatment produced apoptotic bodies, although (Matassov *et al*., 2004), further indicated that the appearance of a prominent band on top of a gel is a good indicator for DNA laddering as it represents semi-fragmented particles of the DNA indicating incomplete apoptotic fragmentation (Matassov *et al.,* 2004). Furthermore, the increase on DNA fragmentation of the cells co-treated with siRBBP6 and CDB, as seen by signs of smearing is in contrast to the siRBBP6 and CBD only treatment, which showed faint bands accompanied by no smearing or laddering (Figure 12A). This data seems discrepant, however, it is being postulated that perhaps in the co-treatment, CBD induces a re-expression of RBBP6. As a consequence, the apoptotic pathway signalling become restored in ME-180 cells and the restored RBBP6 seem central to apoptosis induction.

DNA fragmentation is not the only technique that can be used to present apoptosis, microscopy is also often used to confirm programmed cells induction in response cellular stimuli, and in this case, combination therapy treatment in human cervical cancer cells (Figure 12B). Since the early nineteenth century, microscopy was used as the technique for observing specific morphological changes in viable versus dead cells. This is due to the fact that many morphologically distinctive changes alterationg in the phenotype of the cell occur during the apoptosis pathways (Radogna *et al.,* 2015). In this study, Annexin V fluorescence dyes were used microscopy to assess morphological changes in metastatic cervical cancer ME-180 cells to validate the form of cell death induced by treatments exploited. Fluorescence microscopy was conducted to further observe apoptosis in terms of size and shape characteristic. Annexin v dye is known to bind to phosphatidylserine

(PS) translocated from the cytoplasmic face of the membrane to the surface of the cell for determining early. Based on the results obtained in Figure 13B untreated cells revealed that the cell membrane was still intact and the cells were still viable hence, they did not uptake annexin V dye this means untreated cells are Annexin v and PI negative. In the rest of treatments, cells were able to uptake Annexin v .This proposes the induction of apoptosis, since it can only bind to externalized PS residues.

The results obtained are in correlation with a study conducted by Lukhele and Motadi in 2016 that revealed significant results of apoptosis in SiHa cells treated with CBD at an IC_{50} of 3.2 µg\ml, cell size was reduced and there was a morphological change in Annexin v/ FITC. As for untreated cells, apoptosis was not detected and this shows that these untreated cells are unable to undergo apoptosis without treatment. We further suggest that CBD and siRBBP6 as treatments act as external death signals whereby they bind to Fas receptor to transfer the treatment into the cells, death-inducing signalling complex becomes activated whereby they will automatically activate caspases that are responsible for apoptosis.

Often, tumorigenesis develops from the cells' immortalization. Indeed, cell death is important for cell cycle, which inhibits the division of aging, mutated and unhealthy cells. Apoptosis is the general mechanism by which cell death regulates cancer development. Some of the hallmarks of apoptosis are phospholipid phosphatidylserine residues, increased activity of caspase enzymes and the nuclear DNA fragmentation in the cells (Shrivastava *et al.,* 2011). Cell death stimulated by various treatments can be measured by assessing the activity of caspase-3 and -7. Thus, it is being recommended that in addition to DNA fragmentation and microscopy, future studies intending to elucidate whether CBD in combination with siRBBP6 has potency of inducing apoptosis in cervical cancer ME-180 cell line should quantify caspase-3 and -7 activity.

4.3.3 Gene expression analyses of CBD and siRBBP6 treated and CBD and siRBBP6 co-treated ME-180 cervical cancer cells

RNA interference is an important technique for targeting, directing and suppressing opportunistic gene expression without posing any effects on the expression of neighbouring and other genes on the mRNA (Kim and Rossi, 2007). In this gene knockdown process, a small interfering RNA (siRNA) molecules is introduced into the

cells to degrade specific mRNA and silence gene activity (Aagaard and Rossi, 2007). Previous studies have shown that CBD did not silence RB but it re-expressed the gene and its functional activity was restored resulting in upregulation of other pro-apoptotic gene (Kim & Rossi., 2007). In this study ME-180 cells were transfected with siRBBP6 and co-treated with CBD for the purpose of knocking down RBBP6 and measuring gene expression of the coupled treatment. Based on the data presented a successful silencing or knockdown of RBBP6 in ME-180 using RNAi interference was observed. However, unexpectedly in a combinational treatment, gene expression levels were increased in Figure 13A, this might be an indication of one if the isoforms that may had an Impact to the increment of the expression level or consistent with previous observation RBBP6 is restored by CBD treatment (Kim & Rossi., 2007). Amplifying each of the RBBP6 variants will provide important insight, if considered for future studies. Furthermore, MDM2 gene expression showed a successfully down-regulation in the treatment of siRBBP6, siRBBP6+CBD and CBD. It can be deduced that silencing with an interference and treating with CBD has a positive effect in reducing the gene expression of RBBP6 and MDM2 in cancer.

A tumour suppressor gene P53 was successfully upregulated in combinational treatment of siRBBP6+CBD. In the treatment of siRBBP6 there was no significant change in the expression of p53 relative to untreated cells this indicates that the treatment alone was unable to upregulate p53. RBBP6 serves as a negative regulator of p53 while MDM2 also promotes the degradation of p53 by ubiquitination. Based on this, the reason behind level of expression of these gene, a down-regulation in RBBP6 and MDM2 leads to an upregulation in p53. Previous study stated that MDM2 is mostly up-regulated in numerous cancer cells (Tang *et al.*, 2012; Wade *et al.*, 2013); therefore, an up-regulation of MDM2 might have not been induced by any of the treatments. This study suggests that the treatment for this experiment induced a level of p53 expression. An increase in p53 expression contributes to cell cycle to prevent cell death or genetic mutation by repairing genes in the G1 phase. Furthermore p53 is dependent on a medicinal plant such as CBD in order to be activated in mostly cancer cells however we further elucidate that medicinal plants have the potential to halt cell cycle arrest. Moreover, it is evident from a previous study that the co-treatments are supposedly influential in triggering the blockage in cell cycle at G1-S phase transition and CBD mediated-apoptosis in PC3 cells because, they observed a slight upregulation of p53 expression in co-treatment CBD with siRB; and a distinct increament in the expression of p53 is observed in cells administered with p53 in

combination with CBD. The study further suspected that these might have been due to re-expression of the genes activity or was restoration of transcriptional activities (Indovina *et al*., 2015).

4.3.4 TRAP assays

Previous studies have revealed that telomerase activity is one of the most problematic and unclear assay whereby most researchers who are interested in this assay find it difficult to obtain their results. Several authors who had studied the relationship between p53 and telomerase activity obtained contradictory results (Kusumoto *et al, 1999*; Maxwell *et al, 1997*; Righetti *et al,* 1996). This technique has been applied by many researcher groups and tends to be complicated and inaccurate (Sharma *et al*.,1995; Ogretmen *et al.,*2001;Moon *et al.,*2010;Kasiappan *et al.,*2012). This study aimed to assess the impact of telomerase activity on cervical cancer progression following co-treatment of siRBBP6 and CBD. However, preliminary data showed no significant change in all the non -heat treatments, control and heat treatment in Figure 14A. The results presented clearly indicates that telomerase is somehow complex. Preceding studies have investigated telomerase activity in the context of p53 where they showed that an over expression of p53 in immortalized endothelial cells does not affect telomerase activity (Maxwell *et al.,* 1997).However, another study revealed that p53 gene contributed to shortening of telomerase in a p53-negative H1299 human non-small cell lung cancer cell line (Mukhopadhyay *et al.,*1998).

Based on the relationship of p53 and RBBP6 it is clear that RBBP6 is a negative regulator of p53. This implies RBBP6 is downregulated leading to a decrease in telomerase activity.in Figure 14B in the combinational treatment and CBD we observed a decrease in the telomeric repeats with 4 and 3 base pairs respectively indicating a decrease from a 6 increment base pairs. In support of the shortening of telomerase by p53 it is safe to reveal that when telomeric repeats becomes short it indicates telomerase activity is decreasing as we already know the function of telomerase activity is to continuously add telomeric repeats to maintain its length (Xiao *et al.*, 2004). The majority of preceding studies suggests that the association with shortening of telomerase with age *in vivo* and with lifespan *in vitro* were done in fibroblast to detect if telomerase could be detected (Allsopp *et al.,*1992).However ,the results obtained revealed that telomerase could not be detected in the extracts of fibroblasts cell culture (Harle-Bachor *et al.,*1996).In addition

another study proposes that targeting telomerase activity during cancer therapy may have consequences for normal tissue renewal and healing due to lack of specificity towards tumour cells.(Belair *et al.,*1997).Interestingly, it is evident that telomerase activity is dependent on telomere length and chromosomal instability. However, researchers demonstrated that normal cells such as fibroblast do not have telomerase activity. Therefore in conclusion we deduce that telomerase activity is only detected in cancer cells and study of telomerease during cell cycle should assist to further elucidate the contradiction or complicated issue.

Chapter 5: Conclusion

This study widely elucidated the functions and relationships of RBBP6 gene knockdown with MDM2 and p53 in relations to cell proliferation, apoptosis, cell cycle and telomerase activity whereby we successfully observed a gene knockdown of RBBP6 and MDM2 and an upregulation of p53 as expected, also observed apoptosis in siRBBP6+CBD and other treatments and a positive telomerase activity with a decreased telomerase activity. We can further deduce that in this study the main investigation that was based on the prediction of telomerase activity responding to a combinational treatment was observed whereby a conclusion was reached that suggests a co-treatment of siRBBP6+CBD has a positive effect on telomerase activity by decreasing telomerase activity ,knocking down RBBP6 and inducing apoptosis in cervical cancer cells. In conclusion, it is possible that the activity of p53 can be correlated to some extent to telomerase activity. But p53 expression is cancer cell line dependant and in the case of cervical cancer, it is inactivated. This may have impacted telomerase activity and co-treatment of siRBBP6 and CBD apoptosis does not seem to be related to telomerase activity.

Chapter 6: Reference

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Annexures

Appendix A

Preparation of media

Glucose free media preparation

500 ml DMEM/MEM

10% FBS

1% Penicillin/ Streptomycin

Store at 4ºC

Preparation of buffers

10 x TBE buffer

Dissolve 180 g Tris and 55 g Borric acid in 900 ml distilled H_2O

Add 40 ml O.5 M Na_2EDTA (pH 8) (alternatively use 9.3 g Na_2EDTA)

Adjust to 1 L

Store at room temperature.

1 x TBE buffer

Dissolve 10,8g Tris and 5.5g Borric acid in 900ml distilled H_2O

Add 4ml O.5M Na_2EDTA (pH 8) (alternatively use 9.3g Na_2EDTA)

Adjust to 1 L

Store at room temperature.

TRAP assay Buffers

1X CHAPS Lysis Buffer

10 mM Tris-HCl pH 7.5

1 mM $MgCl_2$

1 mM EGTA

1.1Mm Benzamidine

5 Mm βmercaptoethanol

0.5% CHAPS

10% glycerol

10X TRAP Reaction buffer

200 mM Tris-HCl ,pH 8.3

15 mM $MgCl_2$

630 mM KCl

0.5% Tween 20

10mM EGTA

GEL preparations

10% Native PAGE gel electrophoresis

Stacking gel

Tris HCl pH 6.8	4.3 ml
BIS acrylamide	670ul
APS 20%	5ul
TEMED	10ul

Resolving gel

Tris- HCl pH 8.8	5.90ml
Bis-Acrylamidee	4ml
APS 20%	100ul
TE MED	10uL

Protocols

Cell detachment

Old media was discarded and cells were washed twice with 2 ml of 1X PBS. Three millilitres of 1X trypsin was added to the cells and further incubated at 37°C under 5% CO2 for a few minutes until cells have detached from the surface of the flask. Ten millilitres of supplemented DMEM media was added to the cells to deactivate the action of trypsin.

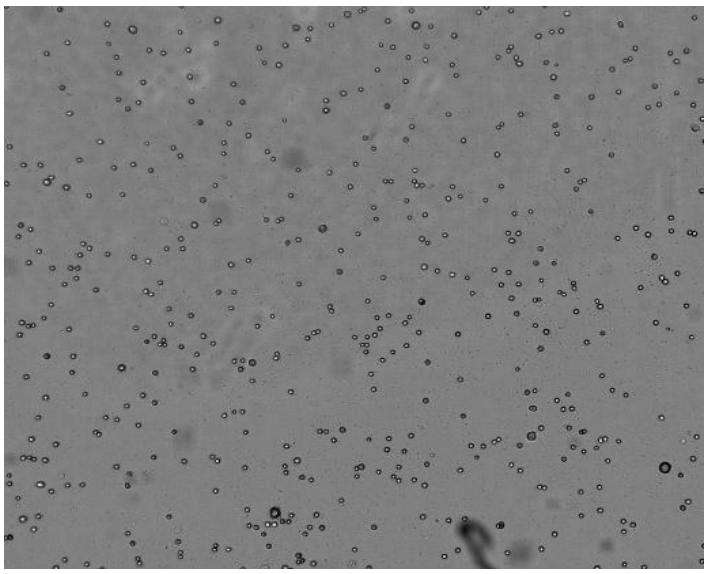

Figure A2: Million cells in a cell counter in 10ul

Appendix B

Description: Mtt assay

Drug: cannabidiol (CBD) [ug/ml]

Drug/Combo	Dm	m	r
CBD	1.8E-17	0.01708	0.16608

CI values at:

Combo	ED50	ED75	ED90	ED95

Data for Fa = 0.5

Drug/Combo	CI value	Dose CBD

CBD 1.8E-17

Dose-effect graph

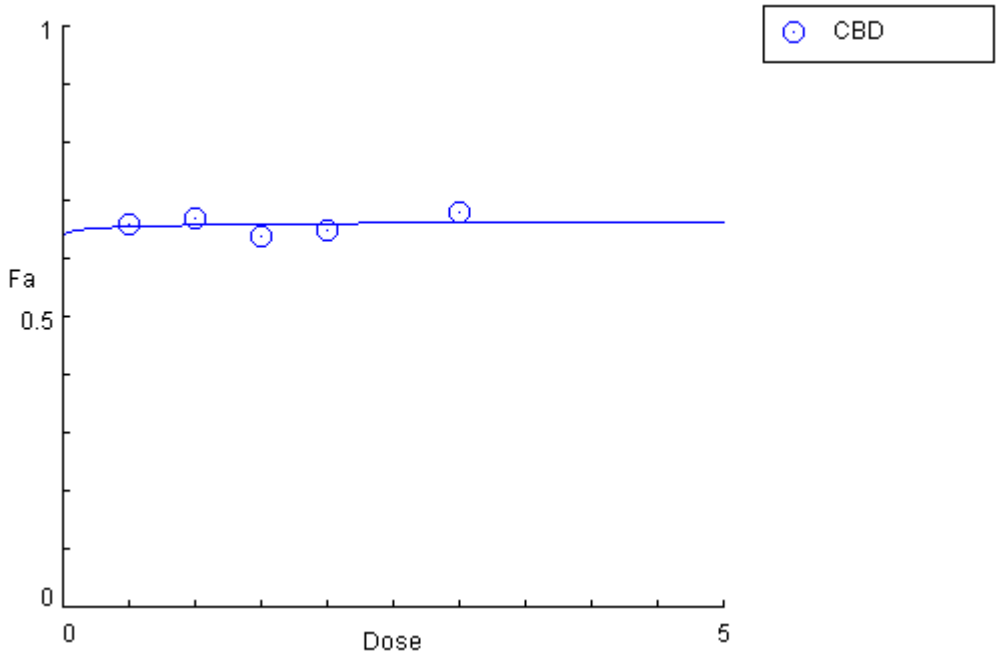

Figure B1: Median-effect plot

Appendix C

The concentration of cDNA and graphs

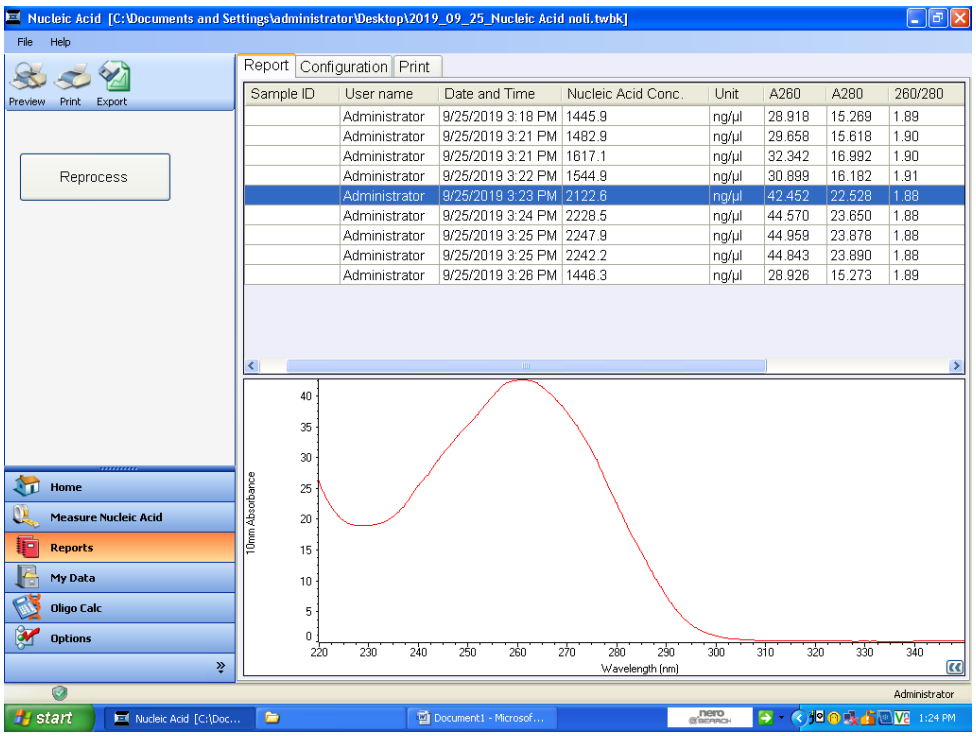

Figure C1: Untreated 2122.6 ng/ml ration A260/280 1.88

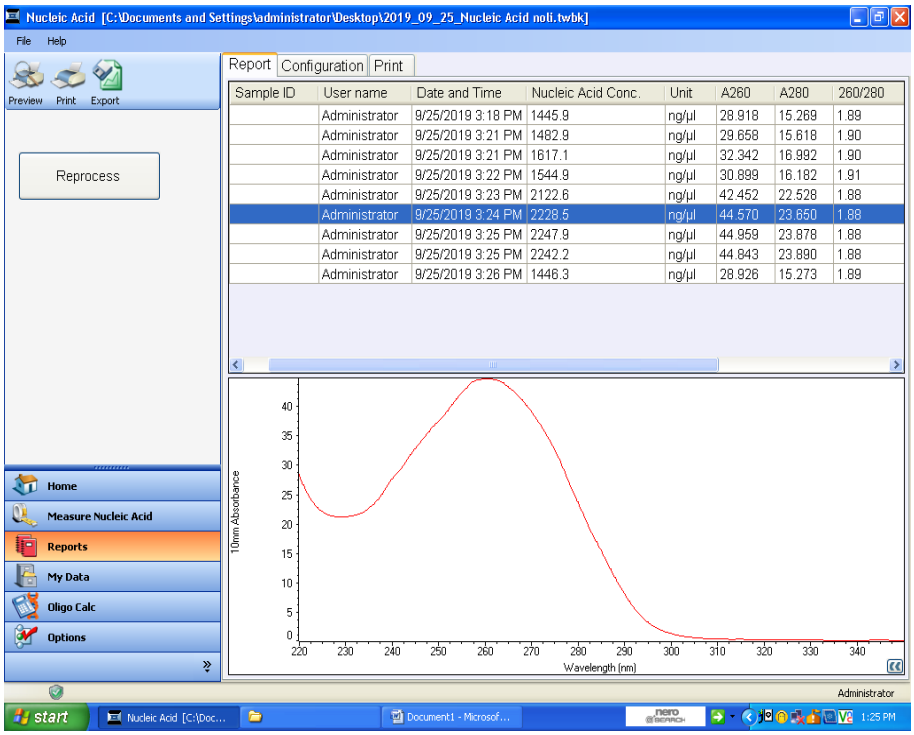

Figure C2 :SiRBBP6 2228.5 ng/ul ratio A260/280 1.88

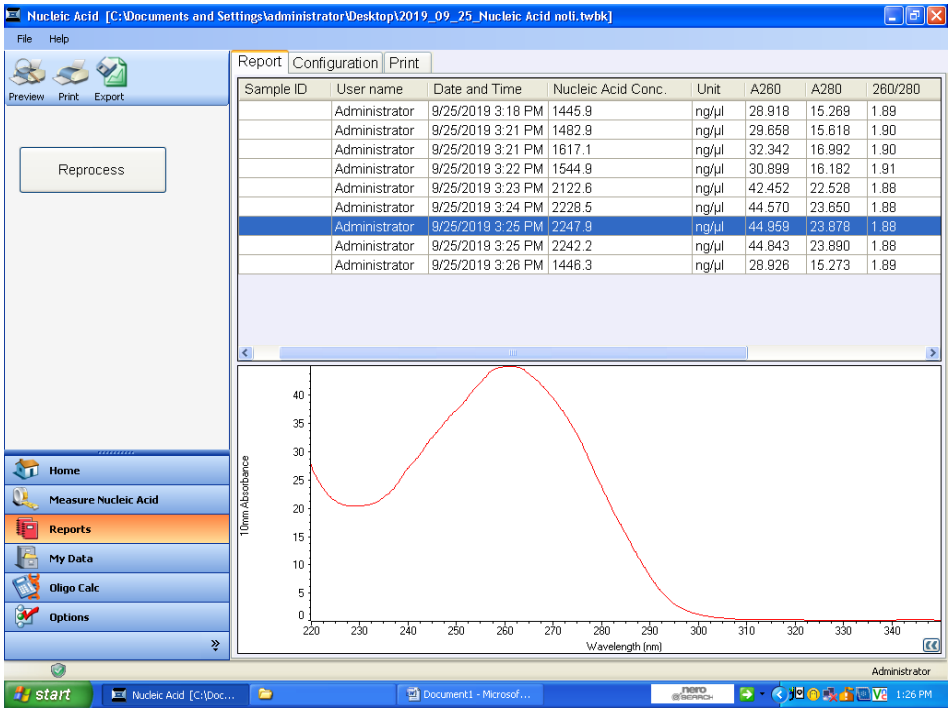

Figure C3: SiRBBP6 + CBD 2247.9 ng/ul ratio A260/280 1.88

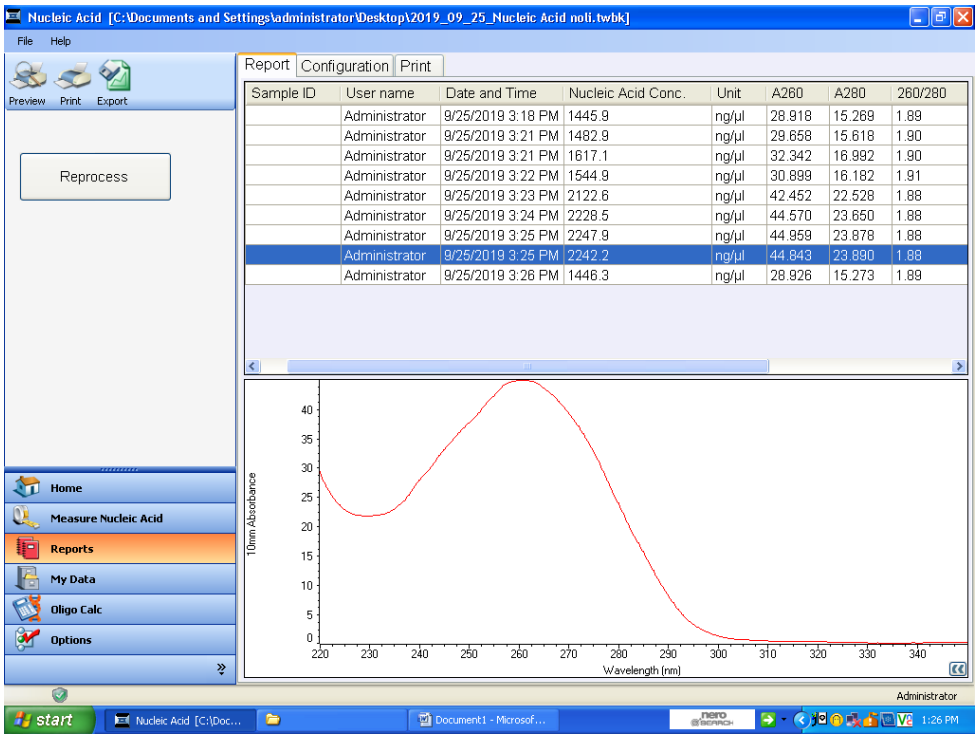

Figure C4: CBD only 2242.2 ng/ul ratio A260/280 1.88

Statistics of new cases and deaths for cancers including all cancers in 2018

CANCER SITE	NUMBER OF NEW CASES (% OF ALL SITES)	NUMBER OF DEATHS (% OF ALL SITES)
Lung	2,093,876 (11.6)	1,761,007 (18.4)
Breast	2,088,849 (11.6)	626,679 (6.6)
Prostate	1,276,106 (7.1)	358,989 (3.8)
Colon	1,096,601 (6.1)	551,269 (5.8)
Nonmelanoma of Skin	1,042,056 (5.8)	65,155 (0.7)
Stomach	1,033,701 (5.7)	782,685 (8.2)
Liver	841,080 (4.7)	781,631 (8.2)
Rectum	704,376 (3.9)	310,394 (3.2)
Oesophagus	572,034 (3.2)	508,585 (5.3)
Cervix uteri	569,847 (3.2)	311,365 (3.3)
Thyroid	567,233 (3.1)	41,071 (0.4)
Bladder	549,393 (3.0)	199,922 (2.1)
Non-Hodgkin lymphoma	509,590 (2.8)	248,724 (2.6)
Pancreas	458,918 (2.5)	432,242 (4.5)
Leukaemia	437,033 (2.4)	309,006 (3.2)
Kidneys	403,262 (2.2)	175,098 (1.8)
Corpus uteri	382,069 (2.1)	89,929 (0.9)
Lip, oral cavity	354,864 (2.0)	177,384 (1.9)

Brain, nervous system	296,851 (1.6)	241,037 (2.5)
Ovary	295,414 (1.6)	184,799 (1.9)
Melanoma of skin	287,723 (1.6)	60,712 (0.6)
Gallbladder	219,420 (1.2)	165,087 (1.7)
Larynx	177,422 (1.0)	94,771 (1.0)
Multiple myeloma	159,985 (0.9)	106,105 (1.1)
Nasopharynx	129,079 (0.7)	72,987 (0.8)
Oropharynx	92,887 (0.5)	51,005 (0.5)
Hypopharynx	80,608 (0.4)	34,984 (0.4)

Optimization of primers, the primers were designed and purchase from Inqaba :

	Primers
(a) GADPH	Forward – 5 ′ –AAG GTG GGA GTC AAC GGA TT –3’ Reverse – 5 ′ –CTC CTG GAG ATG GTG ATG G –3’
(b) RBBP6	Forward – 5 ′–ACA TCT CCC TCT GCG ACT T –3’ Reverse – 5 ′–TAG GAA TCA GAG CAT TAT CAT CAG T–3’
(c) TP53	Forward – 5 ′–CCA GGA CTT CCA TTT GCT TTT G –3’

	Reverse – 5 ′– CTT ACA TCT CCC AAA CAT CCC T – 3’
(d) MDM2	Forward – 5 ′–TGG GCA GCT TTG AAG CAG TTG–3’ Reverse – 5 ′– CAG GCT GCC ATG TGA CCT AAG A– 3’