Validating the use of p53-cannabidiol co-treatment as endogenous biomarker for treatment of cervical cancer

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Declaration

I, PHELADI LAZY KGOMO, declare that this dissertation is my own work and has not been submitted at any other institution before for any examination purposes.

Signature of candidate: *P. L. KGOMO*

Date: 30th April 2019

Abstract

Cervical cancer remains the most diagnosed gynaecological malignancy in women worldwide. The lack of resources and high HIV/AIDS incidences occurring in Sub-Saharan Africa are assumed to be some of the contributing factors to the annual increase in the mortality of cervical malignancies. Loss of p53 function seem to be the common characteristic in the cervical cells of many patients with cervical cancer. During cervical tumorigenesis, the p53 tumour suppressor gene becomes degraded by the activity of HPV 16 and 18, thus, allowing the uncontrolled cell division. Despite the p53 restoration and expression in cervical cancer, the p53 gene therapy is resisted due to nonspecific nature of the drugs to the cancer cells. The existing therapy is known to affect normal cells in patients suffering from cervical cancer leading to the developments of phenotypic side effects in patients. As a result, the implementation of natural compounds in the treatment of cancer seem to provide an attractive field that would result in a reliable therapeutic agent against many cancers. In the current study, p53 targeted therapy was used in combination with cannabidiol compound for treatment of metastatic cervical cancer. In the study, the aim was to inhibit growth of cervical cancer with minimal toxicity to humans and this was achieved by conducting the MTT assay, real-time PCR, caspase 3/7 assay, DNA fragmentation and morphology analysis through fluorescence microscopy.

The results have shown that the cannabidiol compound inhibited the ME-180 cells in a concentration-dependent manner with an IC50 of 3µg/ml. The cotreatment of p53-cannabidiol seemed to induce the over-expression of p53 in the ME-180 cells which further facilitated the cell cycle arrest as was confirmed by the knockdown of CDK2. The results from caspase 3/7 assay and DNA fragmentation implicated that the p53-cotreatmet has not only arrested cell cycle but also stimulated the apoptotic cell death in the cells. The apoptotic cell death induced by the p53cannabidiol cotreatment was confirmed through analysis of the cell morphology which implied to be that of apoptosis as cell shrinkage and other apoptotic properties were observed. Overall, the p53-cannabidiol compound is the recommended therapeutic tool with potency to treat human cervical cancer.

Keywords: TP53, cannabidiol, apoptosis, cell cycle, cervical cancer, P53-cannabidiol cotreatment and cytotoxicity.

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Research outputs

Poster presentation

Pheladi L. Kgomo and Lesetja R. Motadi. Screening of *Sutherlandia frutescens* and selected compounds in SiHa human cervical cancer cell line. SASBMB-FASBMB Conference. Potchefstroom, July 2018.

Abbreviations

APAF-1	Apoptotic Protease Activating Factor-1
APC	Anaphase Promoting Complex
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine Triphosphate
ATR	Ataxia-Telangiectasia and Rad3-related
BAK	BCL2 Antagonist/Killer
BAX	BCL2-Associated X Protein
Bcl-2	B Cell Lymphoma 2
BE	Elution Buffer
BID	BH3 Interacting-Domain Death Agonist
CBD	Cannabidiol
CDK	Cyclin Dependent Kinase
cDNA	Complementary Deoxyribonucleic Acid
Chk2	Checkpoint Kinase 2
CIP/KIP	CDK Interacting Protein/ Kinase Inhibitor
CKI	Cyclin Dependent Kinase Inhibitor
Cyt C	Cytochrome C
DED	Death Effector Domains
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNTPs	Deoxynucleotide
DISC	Death-Inducing Signaling Complex
EDTA	Ethylenediaminetetraacetic Acid
FADD	Fas-Associated Death Domain Protein)
Fas-L	First Apoptotic Signal- Ligand
FAS-R	First Apoptotic Signal- Receptor
FBS	Foetal Bovine Serum
GADD 45	Growth arrest and DNA damage inducible
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
IC_{50}	Inhibitory Concentration
INK4	Inhibitor of CDK4
kDa	Kilodalton
MDM2	Mouse Double Minute 2

$MgCl_2$	Magnesium Chloride
mRNA	Messenger Ribonucleic Acid
MTT	3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide.
MYT1	Myelin Transcription Factor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PLK1	Polo-Like Kinase 1
Rb	Retinoblastoma
RBBP6	Retinoblastoma Binding Protein 6
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TBE	Tris-Borate-EDTA
THC	Tetrahydrocannabinol
TNF	Tumor Necrosis Factor
TRADD	TNF Receptor-Associated Death Domain
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAIL	TNF-Related Apoptosis-Inducing Ligand
ORF	Open Reading Frame
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling

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CHAPTER 1: INTRODUCTION

This section is comprised of the elaboration on the cervical cancer tumorigenesis and the statistical data implicating this type of cancer as a nightmare in South Africa and Sub-Saharan Africa as a whole. It further briefs the significance of the study and the detailed problem statement that led to the research interest of the present study. The aim and several objectives acquired to achieve the desired aim of the study have been fully described on this chapter.

1.1. Cervical cancer and cervical tumorigenesis.

Cervical cancer is 2nd most prevalent gynecological malignancy in South Africa ranking after breast carcinoma and remains the most common malignancy in South African women of 15-44 years (LaVigne et al., 2017). Bruni et al. (2017) recorded 7 735 incidences and 4 248 death events of cervical cancer reported yearly in South Africa by 2012. This carcinoma remains the nightmare amongst women living in the sub-Saharan Africa with an estimated 8,652 new cases occurring annually and this prevalence is correlated with high HPV infections (Bruni et al., 2017). Except the delayed diagnosis and dearth of treatment facilities, studies claim that high mortality of cervical carcinoma in Southern Africa is associated with HIV/AIDS endemic since HIV/AIDS positive women possess suppressed immune system and the progression of HIV infection is associated with the excess unregulated apoptosis (Arbyn et al., 2011; Moela et al., 2016). Cervical cancer is the predominant HPV linked cancer in women worldwide and about 20.2 million females of over 15 years

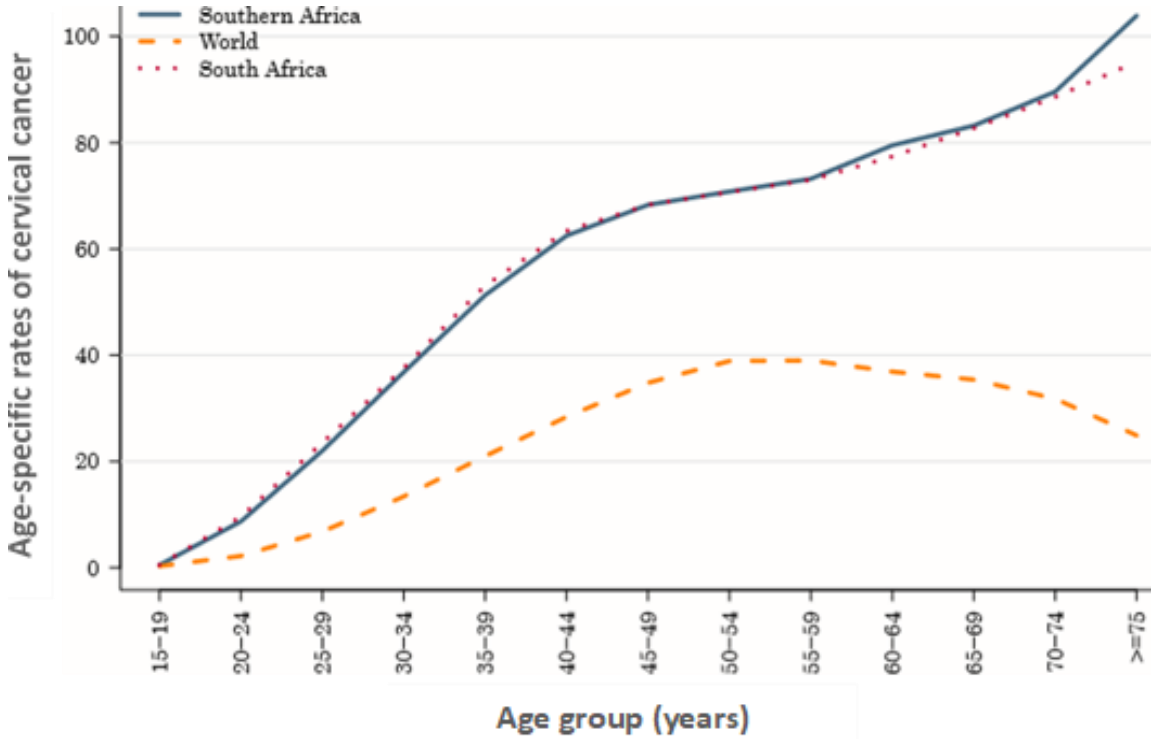

Figure 2.1. Assessment of cervical cancer cases based on age between South Africa, Southern Africa and worldwide (Bruni et al., 2017).

are vulnerable to cervical carcinoma. Globally, cervical cancer is the 3rd most frequent malignancy in females ranking after breast and colorectal cancer malignancies with an estimated 0.5 million new incidences and 0.25 million deaths in 2012 (Duenas-Gonzalez et al., 2014; Bruni et al., 2017). It is recognized that despite the current standard treatments for controlling cervical cancer and available HPV vaccines, about 50% of reported cervical cancer incidences resulted in death by 2012 (Moodley, 2009; Duenas-Gonzalez et al., 2014).

According to Hoste et al. (2013), cervical cancer develops overtime from a precursor lesion in the cervix and can be diagnosed by cervical cytology. There are two types of cervical malignancies namely adenocarcinomas and squamous cell carcinomas. Squamous cell carcinomas are one of the most common gynaecological malignancies composed of cells that are recognizably squamous but vary in either growth pattern or morphology and form from the cells in the exocervix (Colombo et al., 2012; Gulati et al., 2013). Adenocarcinomas form from the mucus-producing gland cells of cervix and they form in the endocervix. HPV infections are associated with over 90% of cervical cancer cases and are revealed to degrade p53 and pRb proteins leading to tumorigenesis in a cervix (Hengstermann et al., 2001).

In cervical carcinogenesis, the p53 is commonly disrupted by human papilloma virus (HPV) protein E6. About 96% of cervical carcinomas are caused by HPV infections, especially HPV 16 and -18 subtypes (Colombo et al., 2012). HPV -16 and -18 consist of E6 and E7 oncoproteins that interact specifically with tumor suppressor proteins, p53 and Rb respectively leading to the disruptions of their biological functions (Vishnoi et al., 2016). These proteins (p53 and pRb) are reported to be participate in apoptosis induction and regulating cell division by either inhibiting G0-G1 or G1-S phase transitions (Baldi et al. 2011; Giarre et al. 2001; Kumar et al. 2014). In cervical cancer, the interaction of HPV-E6 oncoproteins and p53 lead to degradation of p53 and HPV-E7 oncoproteins inactivate the Rb protein (Scheffner et al., 1991; Munger and Howley, 2002). However, the mechanism of inactivation of these tumor suppressor proteins by HPV oncoproteins remains unclear and poorly studied.

1.2 Problem statement

The p53 based gene therapy is the crucial targeted therapy in cervical cancer however the toxicity associated with the therapy is too high, thus potentiates the research interest in implicating the use of p53 gene therapy in combination with plant derived compounds (hence plant-derived compounds exhibit no harm to human health) to inhibit cervical cancer cells with minimal toxicity.

To date the treatments such as surgery, radiotherapy and chemotherapy are the most common therapies used to treat cervical cancer. Although these therapies have been used for ages, they are still not clinically contenting therapies due to their non-specificity that causes several phenotypic side

effects to the patients exposed to them. In spite that these therapies also target the normal cells, they still remain the recommended potential therapeutic targets in treating many different cancers worldwide. Hence, such limitations led to the research interest in production of novel therapeutics originating from natural compounds with reduced toxicity at low costs.

1.3 Significance of the study.

The current study will elucidate the molecular impact of cannabidiol in combination with TP53 gene expression in cervical cancer with hope of identifying it as an ideal therapeutic agent for metastatic human cervical cancer. It will benefit the researchers on the characterization and understanding of the mechanisms implemented by the TP53-cannabidiol co-treatment on eradicating the primary ME-180 cervical cancer cell line.

1.4. Research aim

In this study, we aim to investigate and elucidate the effect of p53 up-regulation and cannabidiol co-treatment in metastatic human cervical cancer cell line ME-180.

1.5. Research objectives

i. To determine the cytotoxic effect of cannabidiol compound on cervical cancer cells using MTT assay.
ii. To upregulate p53 levels in ME-180 human cervical cancer cells through transient transfection.
iii. To quantify the expression levels of TP53 gene using RT-PCR following p53-cannabidiol co-treatment.
iv. To investigate the mode of cell death induced by co-treatment using the DNA fragmentation and caspase 3/7 assay.
v. To analyze the cell morphology following gene manipulation and cannabidiol co-treatment using fluorescence microscopy.

CHAPTER 2: LITERATURE REVIEW

2.1 Cell cycle and cell cycle regulation as disrupted pathways in carcinogenesis.

Cell cycle is a sequence of events that occurs to duplicate components of the parent cell and to divide into two equal daughter cells (Barnum and O'Connell, 2014). Van den Heuvel (2005) discovered that these cell cycle events steps are comprised of interphase, mitotic phase and cytokinesis. Weber et al. (2014) reviewed that the phases of cell division are divided into Gap0 (G0), Gap1 (G1), Gap2 (G2), Synthesis (S) and Mitosis (M) phase and the first four phases are classified as interphase. For the cell to divide precisely, the cell needs to undergo each phase and checked without any interruption or mutations before it proceeds to next phase of the cell division (Barnum and O'Connell, 2014). During the G0 phase, which is regarded as quiescent stage, the cells are at their resting state and some of the cells maintain this state throughout their whole lifespan, hence this phase is entered by the non-duplicating cells as not all the cells duplicate (Dominguez-Brauer et al., 2015; Van den Heuvel, 2005). The cell enters G1 phase, whereby the proteins needed for the chromosome replication are synthesized and the cell grows maximally at this stage and some of the cell organelles are produced which result in the increase in volume of cytoplasm (Berridge, 2014). The chromosome duplicates its DNA via semiconservative replication during the S phase and the cell resumes the growth in the G2 phase and prepares the cell for division whilst the mitochondria continue to divide, hence the G2 phase is regarded as the holding time phase which allows the synthesis of the relevant proteins for mitosis phase and is referred to as checkpoint control mechanism (Weber et al., 2014). After the G2 phase the cell enters the mitosis phase, whereby the replicated chromosome is segregated into two genetically identical nuclei and the phase is preceded by cytokinesis which allows the completion of cell division and production of two daughter cells (Weber et al., 2014; Dominguez-Brauer et al., 2015). It has been reported that when the cell leaves cell cycle, it enters four possible cell fate i.e. senescence, apoptosis, differentiation or cell proliferation through the aid of signaling systems activated (Barnum and O'Connell, 2014).

2.2 Cell cycle regulation

Cell cycle regulation is one of the major important fundamental cellular process that controls cell proliferation by implementing the checkpoint control mechanisms to check the transitions of cell division (Kastan and Bartek, 2004). The cell cycle checkpoints are the biochemical switches that safeguard the integrity and fidelity of major events occurring in cell division (Barnum and O'Connell, 2014). The regulation of cell cycle does not only provide a successful cell division but also allows the generation of daughter cells with non-mutated DNA/ conserved parent DNA which prevent the cancer development (Visconti et al., 2016).

2.3 Key regulators of cell cycle.

For a cell to duplicate efficiently, the cell cycle process needs to be regulated. van den Heuvel (2005), reviewed that the key regulators of cell cycle are cyclins and cyclin dependent kinases (CDKs); positive and negative phosphorylation of CDKs; CDK inhibitory proteins; Rb/E2F pathway and Protein degradation. Cyclin dependent kinases/ cyclins play a crucial role in regulation of transcription, cell cycle progression and other biological functions such as metabolism (Peyressatre et al., 2015). Cyclin dependent kinases are often inhibited by cyclin dependent kinase inhibitors (CKIs) and other cellular proteins (Besson et al., 2008). The stability between activation and inactivation of CDKs controls the progression of cell division from G_1 into S phase, and from G2 to M, through regulatory mechanisms that are preserved in eukaryotes (van den Heuvel, 2005).

2.3.1 Cyclins and cyclin-dependent kinases.

The action of cyclins is mediated by the association with cyclin-dependent kinases (CDKs) as the individual cyclins lack the enzymatic activity (van den Heuvel, 2005). Cyclins regulate the cyclin dependent kinases (CDKs) by forming the complex with specific CDKs and this association is responsible for control of cell cycle progression at each phase (Kastan and Barte, 2004). Hence, the formed complex possesses heterodimeric kinases that not only participate in cell cycle regulation, but also in regulation of transcription and other major biological processes such as metabolism, angiogenesis, proteolysis, DNA damage repair, etc. (Peyressatre et al., 2015). During the beginning of mitosis, the dephosphorylated cyclin B/ CDK1 complex coupled with Polo-like kinase 1 (Plk1) in the nucleus are employed to initiate the segregation of parent cell into two daughter cells (Palou et al., 2010). The Cyclin B/CDK1 association further inhibit the anaphase-promoting complex (APC) that is used in the metaphase arrest (Visconti et al., 2016). The cyclin-A associate independently with CDK1 and CDK2 whereby the cyclin A/CDK2 complex coupled with the cyclin E/CDK2 promote the G1 progression and the initiation of DNA replication in the S phase (Barnum and O'Connell, 2014). Peyressatre et al. (2015) revealed that the cyclin A prepares and directs the activation of Cyclin B/CDK1 association at the centrosome and in nucleus during G2 phase. It's further discovered that at the later stage, the cyclin A associates with CDK1 which is later replaced by cyclin B coordinating the entry and progression in mitosis. Cyclin D associates individually with CDK4 and CDK6 to regulate the progression through G1 phase thus stimulating the phosphorylation of pocket protein family members of Retinoblastoma i.e. p107, p130, p105 (van den Heuvel, 2005). Certainly, the mutations of CDKs subsequently lead to cancer formation (Do et al., 2013).

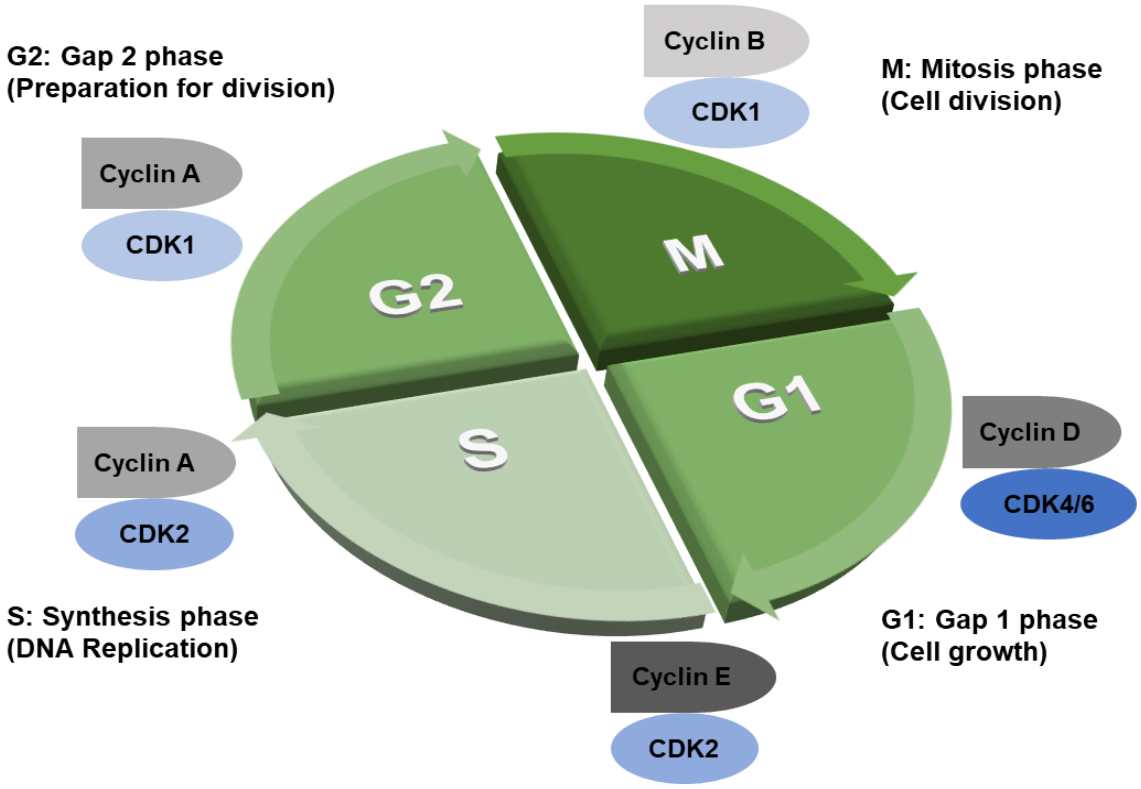

Figure 2.1. The distinguished roles of various associations of cyclin/CDKs complexes at each phase of cell cycle. CDK1 interacts with cyclin A and cyclin B at G2 and mitosis phase respectively; CDK2 associates with both cyclin E and cyclin A at the synthesis phase; Both CDK4 and CDK6 interacts with cyclin D during the G1 phase of cell cycle (Peyressatre et al., 2015).

2.3.2 Positive and negative phosphorylation of CDKs.

Phosphorylation of Cyclin Dependent Kinases (CDKs) can either be beneficial or inhibitory to the cell division (Barnum and O'Connell, 2014). Cyclin Dependent Kinases of most eukaryotes are regulated through phosphorylation and dephosphorylation of certain significant residues such as threonine and tyrosine residues (Palou et al., 2010). It was discovered that in most cases the activation of CDK/cyclin complexes is usually averted by the Wee1/Myt1 phosphorylation, however, the Cdc25 family counteracts the Wee1/Myt1 phosphorylation and regulate the suitable period for activation of CDK (Visconti et al., 2016). That is, the excessive mutations of WEE1 regulate the CDK-1 though the Cdc25 gain of function might cause the premature activation of CDK/Cyclin complex in the G1 phase, therefore the Inhibitory phosphorylation allocates for developmental regulation of CDK activity (Potapova et al., 2011).

2.3.3 CDK inhibitory proteins.

CDK inhibitory proteins are implemented to inhibit the functioning of individual CDKs and cyclin/CDK complexes in cell cycle (Kastan and Barte, 2004). The cyclin-dependent kinase inhibitors (CKIs) are classified into two distinct families, the INK4 and the CIP/KIP family depending on the CDKs inhibited (Palou et al., 2010). Hence, the monomeric CDK4/6 and cyclin D/CDK4/6 complex which regulate the G1 progression is inhibited by the family of the INK4 namely $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ whereas the CDK2/ cyclin A/E complex which controls the G1 and S phase progression is inhibited by the CIP/KIP family consisting of p21, p27 (KIP1) and p57 (KIP2) (Peyressatre et al., 2015). p21 expression which is highly induced by p53 due to DNA damage, acts as cyclin dependent kinase inhibitor of CDK2/cyclin E association to arrest cell cycle at G1 phase (Berridge, 2014). On the other hand, p27 regulates both cell division progression and cell death pathway depending on the binding and levels of p27 present in a particular cell. In a case when levels of p27 are high, the CDK5/p53 binds p27, E2F1 and DP1 in the nucleus thus inactivating cell cycle but when p27 is degraded the CDK5/p53 returns back to the cytoplasm to inactivate the cell death pathway (Satyanarayana et al., 2008; Wang et al., 2015). Wang et al (2015) further assured that the G2/M arrest is stimulated by the other target genes of p53 such as GADD 45, 14-3-3σ and also p21 whereby these genes regulate/inhibit the Cdc25/cyclin B association. Hence the central role of CKIs is to mediate the cell cycle control through regulation of CDKs (Visconti et al., 2016).

2.3.4 Protein degradation.

Ubiquitination of proteins plays an important role in cell-cycle regulation as in most cancer cases the tumor suppressor proteins are degraded through ubiquitination pathway thereby leading to proliferation of cancerous cells (Zhuang et al., 2012). One of the E3 ubiquitin ligase, APC regulates the transitions of cell cycle by inducing the segregation of sister chromosome in the M phase (Peyressatre et al., 2015). The APC multi-subunit consist of APC4 subunit that is encoded by the emb-30 which is responsible for the metaphase-to-anaphase transition in mitosis and meiosis (van den Heuvel, 2005). The studies have further discovered that either the mutations or degradation of tumor suppressors lead to more than 50% of various cancers and the two known regulators of the main tumor suppressor protein p53 are mouse double minute 2 homolog (MDM2) and retinoblastoma binding protein 6 (RBBP6) (Moela and Motadi, 2016).

2.3.5 Rb/E2F pathway.

The E2F is a family of promoter-binding factors that facilitate process of transcription and this family consists of E2F1, E2F2, E2F3, E2F4, E2F5, E2F6 and E2F7 (Peyressatre et al., 2015). The retinoblastoma (Rb) pocket protein family contains the Rb/p105, p107 and p130/Rbl2 which plays a critical role in safeguarding the cell cycle through regulating the E2F family of transcription factors (Berridge, 2014). It is reported that during cell cycle regulation, the association complex produced

by the binding of the Rb family and the E2F family is needed for both activation and inactivation of the transcription of various genes whereby the Rb/p105 functions as the activator whereas the p107 and p130/RbI2 function as repressor for transcription of the cell cycle control genes (Kastan and Barte, 2004). Many studies revealed that the cell is maintained at the quiescent G0 phase through repression of the E2F facilitated by the binding of the hypophosphorylated Rb pocket proteins-p107 and p130/RbI2 thus inhibiting transcription (Berridge, 2014). However, when the cyclin D/CDK4/6 complex phosphorylates Rb, the E2F1-E2F3 becomes derepressed thus allowing the transcription of different cell cycle genes required for G1/S transition (Visconti et al., 2016). Hence only the E2Fs coupled with their associating partners DP1 and DP2 are responsible for the bona fide transcriptional regulation (van den Heuvel, 2005).

2.4 Cell cycle checkpoints.

Visconti et al. (2016) reviewed that there are four major cell cycle checkpoints known as G1/S checkpoint, S checkpoint, G2/M checkpoint and spindle-assembly checkpoint. The major role of cell cycle checkpoint is to safeguard the DNA replication and to retain the stability and fidelity of the cell division (Potapova et al., 2011). Do et al. (2013) further revealed that the other central role that cell cycle checkpoints exploit to safeguard the bona fide cell division is to induce apoptosis, senescence and mitotic catastrophe to maintain the fidelity of the genome. Apart from the cell fates mentioned, the cell cycle checkpoints induce the cell cycle arrest in response to DNA damage, therefore allowing the sufficient period for DNA repair (Dominguez-Brauer et al., 2015).

2.4.1 G1/S checkpoint.

The G1/S checkpoint functions to prevent the cells with damaged DNA from entering the S phase until the damaged DNA is removed or repaired, or the cell enters other cell fates i.e. senescence and apoptosis (Dominguez-Brauer et al., 2015). The G1 checkpoint is primarily dependent on the phosphorylation and activation of Checkpoint kinase 2 (Chk2), that is induced by the ATM kinase activated by the DNA damage (Potapova et al., 2011). Berridge (2014) emphasized that the Chk2 arrest the cell cycle at the G1 phase through two pathways. In the first pathway, the Chk2 hyperphosphorylates and inhibit the Cdc25A which is responsible for dephosphorylating the cyclin A/CDK2 and cyclin E/CDK2 complexes, thus halting the cell cycle at G1 phase. Berridge (2014) further reported that the other pathway exploited by Chk2 to regulate the G1 phase is through p53 phosphorylation and activation, of which p53 becomes stabilized due to its low affinity to MDM-2 (p53 negative regulator) leading to the induction of p21 that binds and inhibit the CDK2 therefore activating the transcription of the E2F-regulated genes such as cyclin E, cyclin A and Cdc25A that are required for initiating the DNA replication in the S phase.

2.4.2 S phase checkpoint.

The S phase checkpoint is activated by the DNA damage and is implemented to halt DNA replication process (Visconti et al., 2016). Once the DNA damage occurs during the S-phase due to hindered replication fork, double strand breaks or nucleotide excision/repair, the S-phase checkpoint is activated to stop the cell from duplicating the parent DNA (Segurado and Tercero, 2009). This DNA damage in the S-phase can be sensed by the ATMs and ATRs (Rad3-related) kinase that induces the degradation of Cdc25A halting the progression of S phase (Dominguez-Brauer et al., 2015). The significant role of this checkpoint is to delay DNA duplication during the S-phase in order to decrease the errors occurring during DNA replication (Palou et al., 2010). Genetic stability is one of the critical concepts in the cell division and failure to the processes that lead to stability of the genome might lead the cancer development, hence this checkpoint is exploited to acquire complete and precise DNA replication (Satyanarayan et al., 2008).

2.4.3 G2/M checkpoint.

ATR and Chk1 which are known to activate the DNA repair checkpoint, also trigger the G2/M checkpoint (Do et al., 213). The hallmark of G2/M checkpoint is to prevent the cells with damaged or incompletely replicated genome to precede to mitosis stage thus decrease the mis-segregation of the chromosome (Dominguez-Brauer et al., 2015). As Visconti et al. (2016) noted, the ATR inhibits the association of CDK1/cyclin B and activates the Wee1 kinase and inhibiting Cdc25 through Chk1, this way the G2/M ensures that the cells with damaged DNA do not proceed to the mitotic phase (Matheson et al., 2016). Potapova et al. (2011) further recorded that the activated Wee1 kinase arrest cell cycle at the G2/M checkpoint delaying the onset of mitosis of cells with damaged genome through inhibitory phosphorylation of Cdk1 thus permitting the DNA repair to take place. However, when the cell with irreparable DNA damage are forced to proceed to the mitotic phase, they are subjected to cell death or senescence (permanent growth arrest) through the mechanism called mitotic catastrophe (Peyressatre et al., 2015).

2.4.4 Spindle-assembly checkpoint.

The spindle assembly checkpoint is a mitotic checkpoint whereby the mechanism to protect the correct segregation of replicated genome is offered and is implemented by delaying the cell cycle into anaphase thus preventing the faults in chromosome duplication (Visconti et al., 2016). The spindle assembly checkpoint is the only checkpoint implemented during mitosis to prevent aneuploidy, which is the most common characteristic in aggressive tumors (Berridge, 2014). Spindle checkpoint ensures that all the chromatids are assembled precisely on the metaphase plate prior to chromosome segregation and this is achieved by inhibiting APC which initiates the separation of chromosome at anaphase segregation (Barnum and O'Connell, 2014). Hence the spindle checkpoint

shares the same goal with other checkpoints to control the DNA integrity and temporarily arrest the cell cycle to allow other effectors to correct the genomic mutations. However, this mitotic checkpoint is unique as it maintains the activity of CDKs whereas other interphase checkpoints maintain the inactivity of CKDs (Dominguez-Brauer et al., 2015). The spindle assembly checkpoint coupled with other checkpoints employ different mechanisms and pathways for the production of daughter cells that are genetically identical to their parent cells by ensuring the fidelity of chromosomal segregation (Dominguez-Brauer et al., 2015).

2.5 Apoptosis and apoptotic genes as current targeted therapy for treatment of various cancers.

Apoptosis is a programmed cell death that occurs when a damaged DNA is irreparable and it is known to eliminate the aging cells and the cells undergoing the senescent stage, however this mode of cell death plays a crucial role in carcinogenesis (Elmore, 2007). Apoptotic cell death is not only exploited to eliminate the senescent cells, but this mode of cell death is remarkedly known to separate the human embryonic fingers and toes during development (Hongmei, 2012). Although apoptosis remained the recommended cell death for centuries in eradicating cancers, the excessive apoptosis causes atrophy (Pfeffer and Singh, 2017). Prenek et al. (2017) reported that inadequate apoptosis in the tissues lead to uncontrolled cell division leading to cancer development. In most cancer cases, apoptosis absence seems to be the problem that lead to immortalization of the cells. Since cancerous cells do not undergo apoptosis, apoptosis remains the main target for anticancer therapy (Wang and Youle., 2009). Apoptosis is differentiated by specific morphological and biochemical events such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, apoptotic bodies, phagocytosis by neighboring cells, phosphatidylserine externalization, etc. (Ouyang et al., 2012). Besides apoptosis there are other two different modes of cell deaths in mammals namely autophagy and necrosis (Tamiru et al., 2017). Due to its phagocytotic and anti-inflammatory nature, apoptosis remains the advantageous mode of cell death over other modes of cell death stimulated in cell cycle (Xu et al., 2017).

Although the apoptotic pathways follow different separate routes to induce cell death, both pathways recruit caspases which are cysteine proteins that cleaves the cellular proteins to elicit cell death (Pu et al., 2017). The apoptotic caspases are classified in to two groups namely the procaspases (caspase 2, 8,9 and 10) and the effector caspases (caspase 3, 6 and 7) which are sometimes referred to as executioner caspases (Merghoub et al., 2017). Typically, the caspases occur in dormant state and the initiator caspases are activated by the formation of apoptosome whereas the executioner caspases are activated through cleavage by initiator caspase (Pu et al., 2017). Both caspases act as the proteases that cleaves the aspartic residues (Merghoub et al., 2017). Like any other pathway, the apoptosis is regulated particularly by the pro-apoptotic and anti-apoptotic genes. The proapoptotic genes involve the members of Bcl-2 family (Baig et al., 2016).

There are two common pathways that are demonstrated to activate apoptosis namely intrinsic mitochondrial pathway and the extrinsic death receptor pathway (Elmore, 2007). The discrete implementation of both intrinsic and extrinsic apoptotic pathways leads to recruitment and activation of the caspases to execute apoptotic cell death.

2.5.1 Intrinsic pathway.

Intrinsic pathway is categorized apoptotic pathway that gets activated by the intracellular signals developed by cellular stress e.g. hypoxia and occurrence of this particular pathway depends on the release of relevant proteins from mitochondria (Kabel et al., 2016). According to Takayama (2003), the mitochondrial participation and formation of apoptosome are the hallmarks of intrinsic pathway and is initiated and executed within the cell. During the intrinsic pathway, the internal stimuli triggers the binding of proapoptotic genes to the mitochondrial membrane thus stimulating the porousness of the membrane (Wang and Youle, 2009). Hüttemann et al. (2011) emphasized that the pores formed on the mitochondria alters the membrane potential and further triggers the release of intramitochondrial apoptotic proteins e.g. Cyt C, Smad and high temperature requirement protein A2 to the cytosol which acts as a death factor outside the mitochondria i.e. in the cytoplasm/nucleus. Usually during the mitochondrial pathway, the cytosolic cyt C bind to APAF-1 and inactive

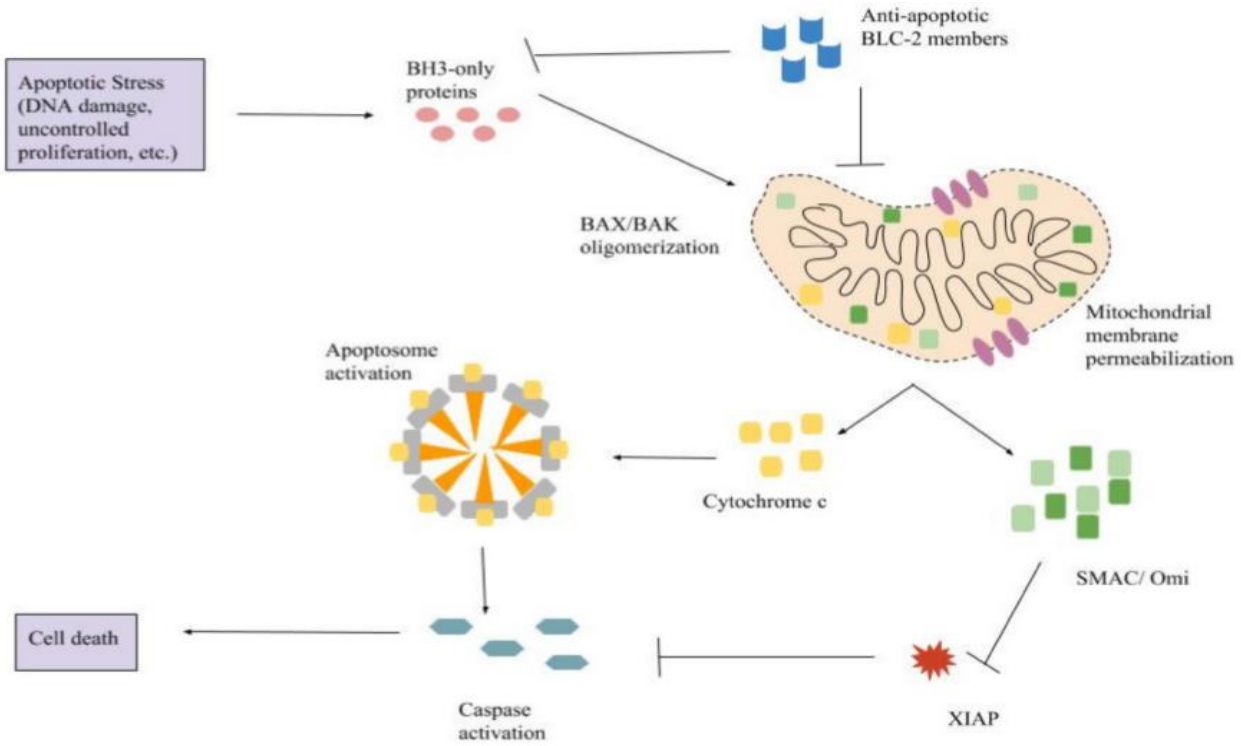

Figure 2.2. Representation of the intrinsic mitochondrial pathway of apoptosis. In response to apoptotic stress, BH3-only proteins are upregulated within the cell thus activating BAX and BAK which causes mitochondrial membrane permeabilization. Mitochondrial cyt C is released and interact with APAF-1, dATP and procaspase-9 to form apoptosome. Apoptosome activate the executioner caspases that cleave cellular proteins resulting in apoptosis (Pfeffer and Singh, 2018).

procaspase- 9 (initiator caspase) to develop apoptosome complex (Prenek et al., 2017). The recruited initiator caspase which becomes activated during formation of apoptosome and subsequently lead to the cleavage and activation of executioner caspase-3 which initiate the caspase cascade reactions that results in degradation of intracellular proteins leading to apoptosis induction of a cell (Ouyang et al., 2012). The common proapoptotic genes which are found to be crucial in regulating the intrinsic pathway are members of Bcl-2 family (Amaral et al., 2010).

In most cases, especially in terms of infected cells, the DNA damage act as apoptosis inducer to prevent proliferation of cells with mutated DNA thus safeguarding the integrity of a genome (Pfeffer and Singh, 2017). Nuclear fragmentation is commonly recorded characteristic of apoptosis especially the intrinsic apoptosis triggered by the translocation of the proapoptotic molecules from mitochondria into the nucleus and cytoplasm (Einsele-Scholz et al., 2016).

2.5.2 Extrinsic pathway.

One more apoptotic pathway whereby cell suicide is triggered by signals from other cells is known as the extrinsic pathway (Kabel et al., 2016). Extrinsic pathway is often designated a death receptor pathway which is activated by the extracellular ligands which tends to bind to the cell surface death receptors leading to generation of death inducing signaling complex (DISC) (Kabel et al., 2016; Pfeffer and Singh, 2017). So far other researchers emphasized that although the apoptotic signaling pathways are initiated at the plasma membrane, they induce the extrinsic pathway to the nucleus with the recruitment of the ligands (Prenek et al., 2017). Usually during extrinsic pathway, the death receptors comprised of Tumor Necrosis Factor (TNF) receptor superfamily situated on surface of the cellular membrane recruit specific proteins e.g. initiator caspase-8 to trigger the stimulation of caspases and compose apoptosis (Amaral et al., 2010). Some of the death receptors initiating the extrinsic pathway other than the TNF receptor gene family are the TNF-Related Apoptosis-Inducing Ligand (TRAIL) that also activate the death receptors in a cell with Death Domain and these cell death receptors are the Fas-Associated Protein (Hongmei, 2012). This signaling pathway is primarily activated by the binding of death ligands for instance Fas ligand (Fas-L), TNF and TRAIL to their corresponding membrane-bound death receptors to yield the Fas-R, TNF-R1, TRAIL-R1 association (Tamiru et al., 2017). The association of death ligands to their corresponding receptors recruit ligation of the adaptor protein FADD to Fas/FasR association or TRADD to TNF/TNFR in order to form DISC (Bank et al., 2017; Pfeffer and Singh, 2017). Hence the interaction of the adaptor proteins to their respective death ligand/ death receptor interaction is through the linker death domain (Kiraz et al., 2016). The resulting DISC binds and activate the initiator caspase through the death effector domain (DED) (Bank et al., 2017). The activated initiator caspases then activate the executioner caspase that will be implemented in the degradation of cellular proteins to accomplish the apoptotic cell death (Pfeffer and Singh, 2017). In extrinsic pathway, the procaspase-8 activates the proapoptotic BID which further activates BAK and BAX to continue to mitochondrial apoptotic

pathway (Kiraz et al., 2016; Bank et al., 2017). In most cancers carcinogenesis the cells evade apoptosis leading to division of abnormal cells, this causes apoptosis to remain the primary targets for anticancer drug development (Anita et al., 2014).

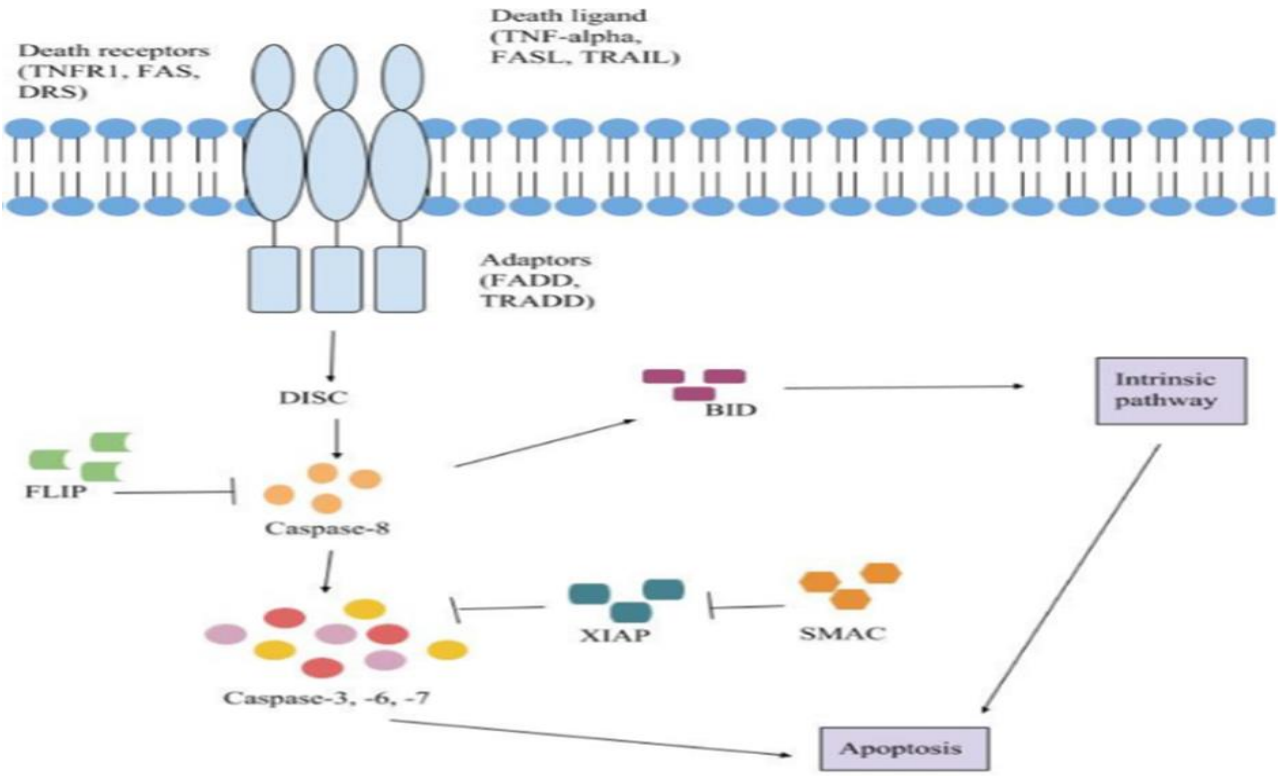

Figure 2.3. Schematic representation of the extrinsic pathway of apoptotic cell death. Extrinsic pathway is activated when death ligand binds to death receptor stimulating the binding of adaptor proteins. The interaction of adaptor protein with caspase-8 and -10 result in formation of DISC. The activated caspase-8 activates executioner caspases resulting in apoptosis; and BID which further activate BAX and BAK to induce intrinsic pathway (Pfeffer and Singh, 2018).

2.6 p53 regulation in cancer.

Ubiquitination of tumors suppressor genes is common in most of human malignancies (Amaral et al., 2010). Half of all cancer cases are associated with p53 degradation or rather p53 mutations (Wang and Sun, 2010). p53 degradation can be caused by virus invasion, however in normal cells p53 becomes target of ubiquitin-proteasome pathway carried out by genes such as RBBP-6 and MDM-2 (Peyressatre et al., 2015). It has been confirmed that MDM-2 is a negative regulator of p53 in cell cycle and in MDM-2 mediated p53 ubiquitination, the ring finger domain of MDM-2 ubiquitin ligase interacts with p53 for degradation of the p53 tumor suppressor protein (Amaral et al., 2010). Retinoblastoma binding protein-6 (RBBP-6) also mediate the ubiquitination of p53 and pRb in normal cells (Moela and Motadi, 2016). The RBBP6 ring finger domain interact with both p53 and pRb genes to degrade these proteins, however the mechanism used by RBBP 6 to disrupt the tumor suppressor

proteins is currently not known (Moela et al., 2014). These regulatory genes MDM-2 and RBBP6 are commonly known genes that are implicated in ubiquitination of essential proteins needed for regulation of cell cycle and apoptosis initiation in normal cells (Chappell et al., 2012). The ubiquitination of “guardian of the genome” triggers uncontrolled cell proliferation leading to cancer (Zhuang et al., 2012). Based on this, restoration of p53 activity in cancer cells accompanied by treatment with certain natural compounds remain the only hope to treatment of cancer and reducing side effects.

2.7 Role of p53 and associated genes in cell cycle.

p53 plays a crucial role in regulating cell growth by contributing to suppression of tumorigenesis and initiation of apoptosis through cell cycle arrest (Zhu et al., 2013). The p53 content naturally occurs at low quantity in the normal cells, but when a cell encounters genotoxic stress such as DNA damage, the p53 becomes upregulated in such cell and thus induces transcription and overexpression of repair genes such as p21 proteins that inhibit CDKs by forming p21-CDK-cyclin complex (Yoshida and Miki, 2010). Inhibition of CDKs lead to accumulation of hypo-phosphorylated retinoblastoma (Rb) protein that cause the inactivation of E2F which will result in the halt of cell growth at G1-to-S transition (Duronio and Xiong, 2013). This halting of cell cycle allows the damaged DNA to be

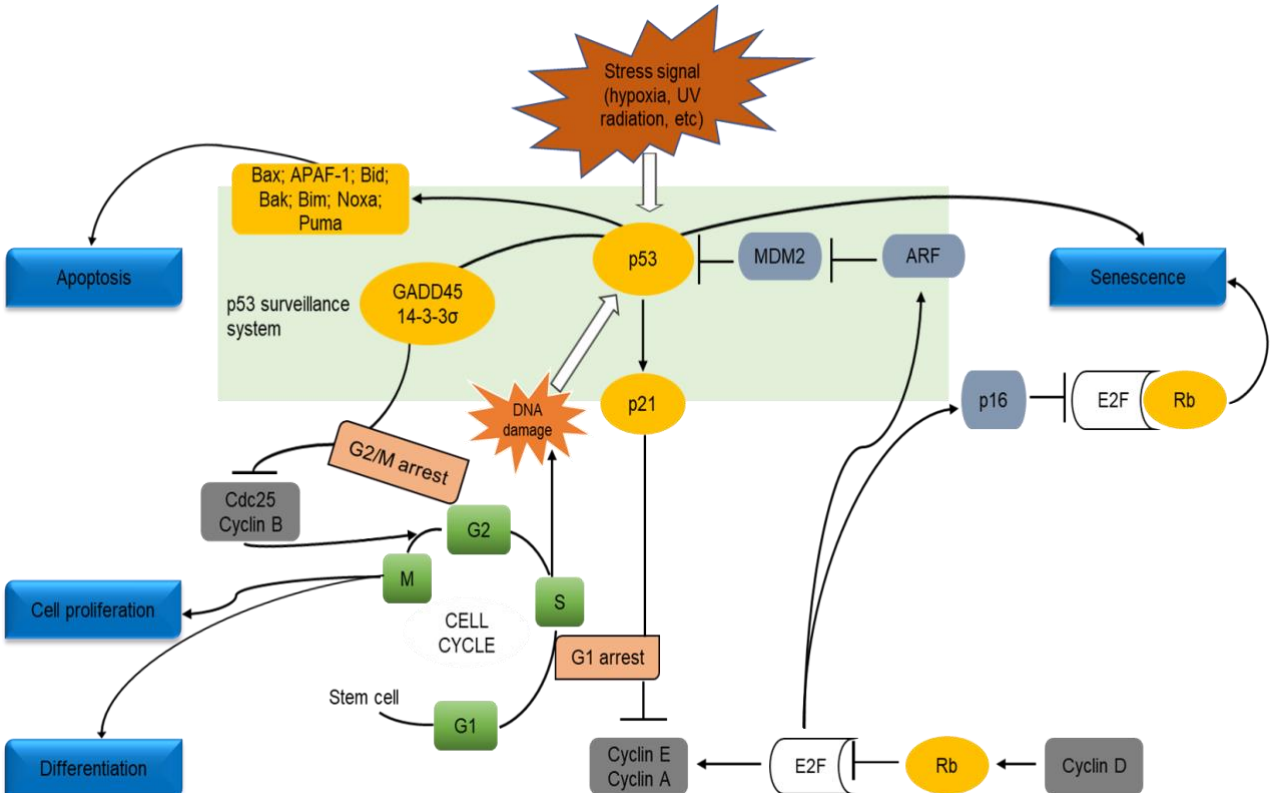

Figure 2.4. The overview of p53 surveliance. In response to stress signal, p53 pathway becomes activated to modulate cell fate. During DNA damage, p53 induces p21/GADD45/143-3σ to arrest cell cycle thus allowing the DNA repair to occur before the cell proliferation proceed; alternatively, the p53 can activate proapoptotic proteins to induce apoptosis (Berridge, 2014).

repaired and the cell cycle will resume following successful DNA repair however the cell apotosise if the DNA damage persist to prevent proliferation of cells with mutated/damaged DNA (Chappell et al., 2012).

2.8 p53-dependent apoptosis.

p53 plays a critical role in stimulating apoptosis in intrinsic as well as extrinsic pathway. It has been reviewed that p53 that is stabilized and activated by stress signals, arrest cell division at G1 and G2 phases to allow DNA repair and eventually induces cell suicide in case where damaged DNA is irreparable (Fridman and Lowe, 2003). In most cases the intrinsic pathway is accomplished by p53 activity, whereby p53 signal the BCL-2 pro-apoptotic family proteins to initiate apoptosis (Schuler and Green, 2001). The studies discovered that, in conditions where DNA is irreparable, the p53 protein activates the transcription of pro-apoptotic genes (e.g. Bax) to initiate apoptosis. Bax induces permeability of mitochondrial membrane thus allowing the release of cytochrome C from the mitochondrial inner membrane (Jürgensmeier et al., 1998). Upon its delivery to cytoplasm, the cytochrome C bind APAF-1 and ATP, followed by the binding with procaspase-9 to generate apoptosome (Zhuang et al., 2012). The formed apoptosome therefore cleaves the procaspase-9 into its active mode which subsequently activate the effector caspase-3 and -7. The effector caspase-3 and -7 carry out the cell degradation hence apoptotic cell death is accomplished (Bell and Megeney, 2017). In extrinsic pathway, the production of death receptors leads to apoptosis induction (Kabel et al., 2016).

2.9 P53 gene therapy and how it failed as a sole therapy.

The p53 gene therapy is in clinical trials for treatment of cancer and has been approved in other countries for treatment purposes (Chen et al., 2014). p53 is a 53 kDa polypeptide encoded by TP53 gene. Normally under healthy cellular conditions the p53 exists in low levels but as the cell experience some stress signals such as hypoxia, the p53 protein content increases in the cell so that it can induce the DNA repair genes (Wang et al., 2015). The p53 is commonly known tumor suppressor protein and is regarded as the guardian of genome since it possesses capability to arrest cell cycle and induces apoptosis after the cell encounter some stress such as DNA damage to maintain original code of parent DNA during the cell division (Yoshida and Miki, 2010). Although p53

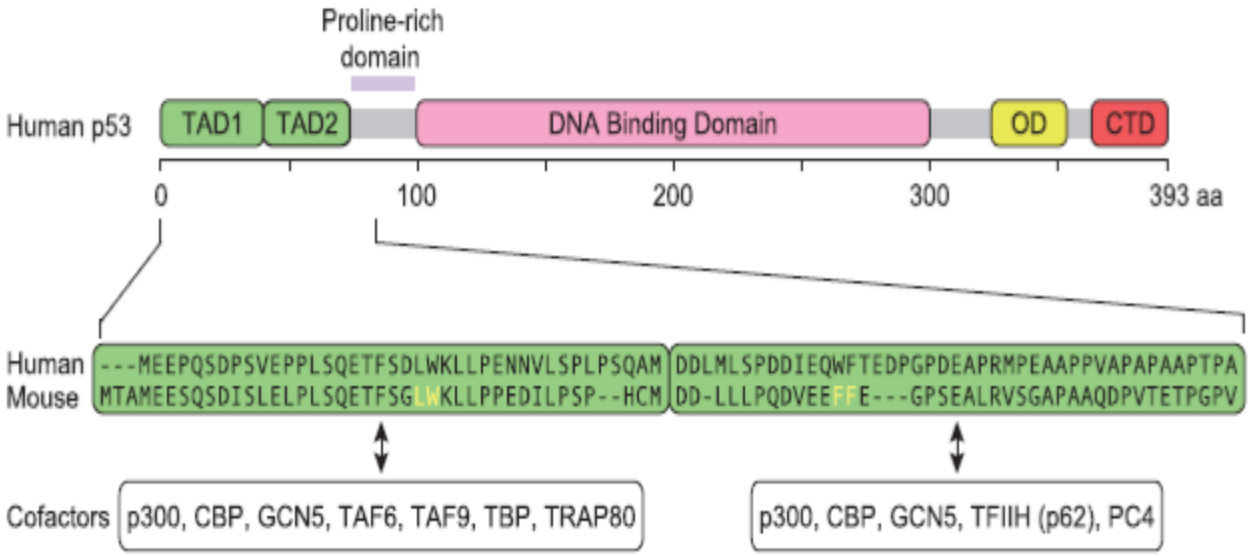

Figure 2.5. Association of eukaryotic p53 protein domains. Top: TAD (Transactivation domains) 1 and 2, DNA binding domain, OD (Oligomerization domain) and CTD (C-terminal domain). Bottom: Amino acid sequences of TAD1 and -2 in both human and mouse. Cofactors associating with the aligned residues are mentioned (Sullivan et al., 2017).

is regarded as genome guardian of the cell, excessive overexpression of p53 in the cell is not recommended in therapies as it result in critical genetic disorders and brings other toxicities to human health (Amaral et al., 2010).

Above all, p53 gene therapy is the recommended therapy for treatment of different types of cancers, although the efficiency of this therapy seemed to be disrupted by the poor symptoms that are experienced by the patients after each administration of the drug (Chen et al., 2014). The toxicity related to this therapy tends to be a challenge in the recovery of every cancer patient however the combinational therapy is recommended for the exploitation of p53 gene and chemotherapy or any other natural compound (Xiao et al., 2017). Besides the fact that p53 gene therapy is being used by other countries for treatment of certain cancers, p53 is still not a successful therapy due to the non-specificity nature of the drug as this lead to high toxicity on human health (Chen et al., 2014).

2.10 Combinational therapy.

By far, some of the common therapies used to treat human cervical cancers are chemotherapy and radiotherapy, however, the DNA damage caused by these two therapies to the normal cells limits the clinical efficacy. Therefore, recently the combinational therapy is exploited for treatment of various cancers in the hope of retaining the normality of the non-cancerous cells. Although chemotherapy is not considered the ideal therapy, in most studies the combinational therapy is implemented in the aim of eradicating the resistance of chemotherapy.Currently we aim to investigate effects of p53-canabidiol co-treatment on human cervical cancer cells by targeting

activation and expression of TP53 gene coupled with CBD treatment in metastatic ME-180 cells. Hence during the carcinogenesis of estimated 90% cervical malignancies, the p53 and pRb tumor suppressors are either mutated or degraded thereby inhibiting apoptosis and allowing the steady increase in division of cells with mutated genome (Hengstermann et al., 2001; Kalu et al., 2017). Many studies have implicated the targeting of p53 gene for treatment of cervical cancer as a promising and crucial therapy that need to be implemented in drug development for this particular cancer (Wong, 2011). Recently, several studies have stated that combinational therapy of p53 complementary DNA (cDNA) with chemotherapy or other plant extracts has promising therapeutic potential with little side effects in anticancer drug development (Sen et al., 2011). Notably, the plants have been utilized since ancient times for medicinal purposes, but recently the plant studies have yielded attention in medical science research due to low-lethal activity and has successfully led to developments of various novel therapeutics or the ingredients used thereof (Tiwari et al., 2011). Cannabidiol as a sole studied cannabinoid with anticancer properties on several cancers, it is reported to be the ideal phytochemical to be implemented in the combinational therapy because of the low toxicity it exhibits to healthy human cells (Velasco et al., 2016).

2.11 Cannabidiol as a potential compound for treating cancer.

In this study the Cannabidiol, secondary metabolite of *Cannabis sativa* compound will be used to elucidate anticancer properties and efficiency in combination with p53 gene therapy on human cervical cancer. Cannabidiol compound constitute 40% of the *Cannabis sativa* extracts and concentration of this compound depends on the geographical impact where the plant is originated (Bergamaschi et al., 2011). Several researchers have discovered that the cannabidiol possesses

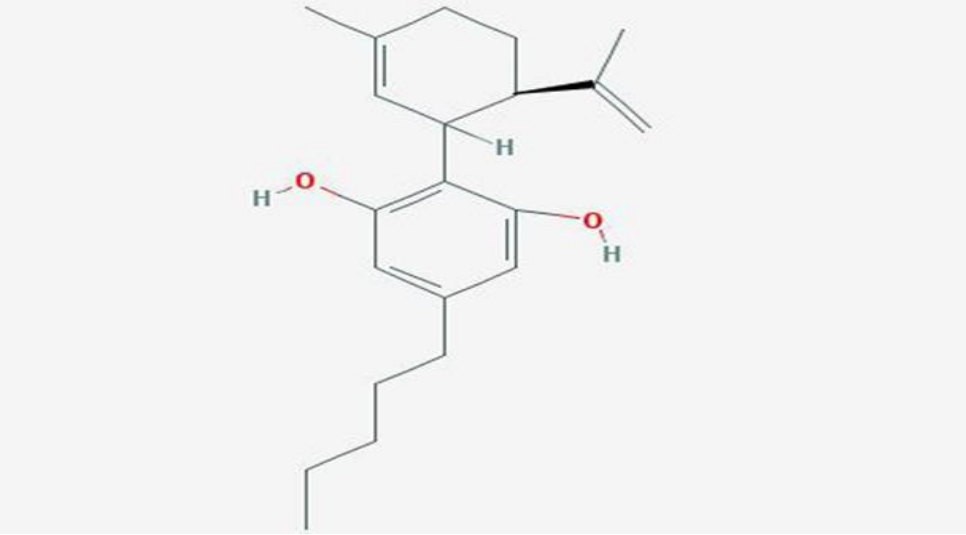

Figure 2.6 The molecular structure of Cannabis sativa derivative cannabidiol. Cannabidiol, C21H30O2, constitutes of 2,6-dihydroxy-4-pentylphenyl group, prop1-en-2-yl group and methyl group substituting cyclohexene (Pisanti et al., 2017).

anticancer properties against malignancies of prostate, lung, cervical and breast but none has reported their effect concurrently with tumor suppressor p53 in metastatic cervical carcinomas (Śledziński et al., 2018; Ramer et al., 2013; Sharma et al., 2014; Lukhele and Motadi, 2016).
Although the molecular mechanism of action in which the cannabidiol mediates to regulate the cell proliferation in the cervical cancer is yet to be fully characterized and understood, the cannabidiol remains the most recommended effective cannabinoid in the anticancer drug development (Ramer et al., 2013). Different studies have been conducted to treat various cancers using cannabidiol as therapeutic agent. Cannabidiol compound has found to elicit the antiproliferation and apoptosis in the breast cancer cell lines MDA-MB 321 and MDA-MB 436 (McAllister et al., 2007; Shrivastava et al., 2011; Pisanti et al., 2017). According to the study conducted by McAllister et al. (2007), about 2.0 µmol/L of cannabidiol has shown to stimulate the knockdown of Id-1 gene which is responsible for the metastatic, invasive and proliferative characteristic of MDA-MB 436 breast cancer cells. Shrivastava et al. (2011) further discovered that the compound cannabidiol induces the autophagy and apoptotic cell death via intrinsic pathway by reducing the mitochondrial membrane potential and allowing the translocation of BID and cytochrome c in the MDA-MB 321 breast cancer cells.

In other studies, the cannabidiol compound has been noted not only to be cytotoxic but to inhibit the tumor metastasis and elicit apoptosis in conjunction with tetrahydrocannabinol (THC) in glioma cells (Shrivastava et al., 2011). In the study conducted by Ramer et al., (2013), it was demonstrated that cannabidiol has successfully inhibited the proliferation of A549 lung cancer cells at the concentration of 3mmol.

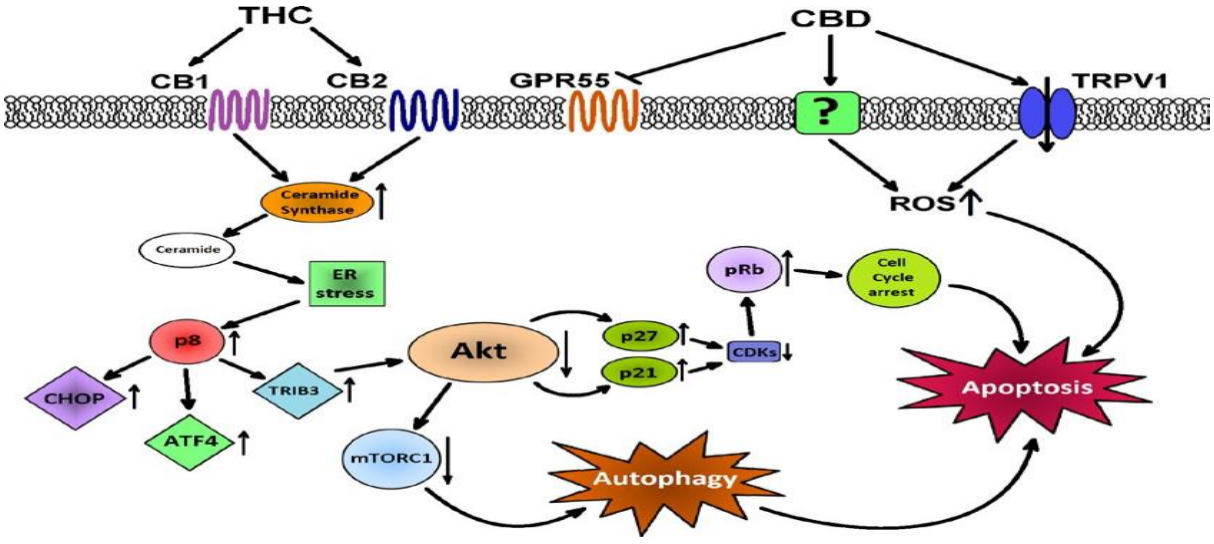

Figure 2.7. Currently discovered mechanisms of cannabinoids known to elicit cell death in cancer. Cannabidiol (CBD) induces apoptosis through generation of reactive oxygen species (ROS); Δ9-tetrahydrocannabinol (THC) interacts with cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) to activate ER-stress related pathway which further induce apoptosis and autophagy-mediated cell death through inhibiting AKT/mTOR Signalling (Śledziński et al., 2018).

Table 2.1. Overview of other pharmacological benefits of cannabidiol in humans.

Disease	Effects
Alzheimer's disease	Antinflammatory, antioxidant, antiapoptotic in in vitro and in vivo models of Aβ-evoked neuroinflammatory and neurodegenerative responses.
Parkinson's disease	Attenuation of the dopaminergic impairment in vivo; neuroprotection; improvement of psychiatric rating and reduction of agitation, nightmare and aggressive behavior in patients.
Multiple sclerosis	Improved signs of EAE in mice, anti-inflammatory and immunomodulatory properties.
Epilepsy	Anticonvulsant in vitro and in vivo; reduced seizures frequency in children and adults with treatment-resistant epilepsy.
Huntington's disease	Neuroprotective and antioxidant in mice transgenic models; no significant clinically important differences in patients.
Pain	Analgesic effect in patients with neuropathic pain resistant to other treatments.
Anxiety	Reduced social anxiety in patients.
Cancer	Antiproliferative and anti-invasive actions in a large range of cancer types; induction of autophagy-mediated cancer cell death; chemo preventive effects.
Nausea	Suppression of nausea and conditioned gaping in rats.
Inflammatory diseases	Antinflammatory properties in several in vitro and in vivo models; inhibition of inflammatory cytokines and pathways.
Rheumatoid arthritis	Inhibition of TNF-α in an animal model.
Infection	Activity against methicillin-resistant Staphylococcus aureus.
Inflammatory bowel & Chron's diseases	Inhibition of macrophage recruitment and TNF-α secretion in vivo and ex vivo; reduction in disease activity index in Chron's patients.
Cardiovascular diseases	Reduced infarct size through anti-oxidant and anti-inflammatory properties in vitro and in vivo.
Diabetic complications	Attenuation of fibrosis and myocardial dysfunction.

Note. Reprinted from Cannabidiol: State of art and new challenge for therapeutic challenges, by Pisanti et al., Retrieved April 09, 2018.

2.12 Other natural compounds targeting cell cycle and inducing apoptosis in anticancer drug development:

2.12.1 Vinca alkaloids

The first antineoplastic drugs to advance into clinical use were the constituents of *Catharantus roseus* plants (Cragg and Newman, 2005). This Madagascar periwinkle, *Catharantus roseus* possesses vinca alkaloids such as vincristine, vinblastine and their synthetic derivatives vindesine and vinorelbine which are essential constituents for cytotoxic properties of this plant (Widowati et al., 2013). Vinca alkaloids of *C. roseus* are potent inhibitors of cell proliferation and the mechanism of action for vinca alkaloids involves depolymerisation of mitotic spindle microtubules, preventing their assembly which causes mitotic arrest in metaphase thus inducing apoptosis (Stanton et al. 2011). Vinca alkaloids are believed to increase apoptosis by activating the production of high concentration of cellular tumor antigen p53 and cyclin-dependent kinase inhibitor 1 (p21), and by inhibiting Bcl-2 activity which lead to changes in protein kinase activity (Cupit-Link et al., 2017). These Vinca alkaloids are found to induce apoptosis in human neuroblastoma cell line SH-SY5Y and also act on M- phase (Santosh et al., 2018). Vincristine is used to treat Hodgkin's disease and paediatric cancers and its counterpart vinblastine is effective on non-Hodgkin's lymphoma, testicular and breast cancer (Zlatic and Stankovic, 2015). Both more recent semisynthetic analogs, vinorelbine and vindesine agents are used in combination with other cancer chemotherapeutic drugs for the treatment of variety of cancers, including leukemia, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma (Moudi et al. 2013).

2.12.2 Paclitaxel

The most commonly used plant-derived anti-cancer agent in clinical development is paclitaxel (Taxol), isolated from the bark of the *Taxus brevifolia* (Taxaceae) (Zlatic and Stankovic, 2015). The mechanism used by paclitaxel to inhibit proliferation of cancer cells involve the binding and stabilizing of microtubules, leading to mitotic arrest thus inducing apoptosis (Cupit-Link et al., 2017). Paclitaxel exhibit anticancer activity against metastatic breast, ovarian and non-small cell lung cancer, and has also shown activity against Kaposi sarcoma whereas its derivative Docetaxel is primarily used in the treatment of breast cancer and non-small lung cancer worldwide (Gascoigne and Taylor, 2009).

2.12.3 Camptothecin

Camptothecin is one of the common sources of clinically active anti-cancer agent and is isolated from *Camptotheca acuminate*. Camptothecin target topoisomerase I as its cellular sole antiproliferative target (Choene and Motadi, 2016; Li et al., 2017). There are currently more effective derivatives of Camptothecin, but the most common derivatives are Topotecan and Irinotecan (Liu et al., 2015). Topotecan is used for the treatment of ovarian and small cell lung cancers, while

Irinotecan is used for the treatment of colorectal cancers (Venditto and Simanek, 2010). These two Camptothecin derivatives inhibit the topoisomerase I enzyme which controls and modifies the topological state of DNA in many cellular metabolic processes (Zlatic and Stankovic, 2015).

2.12.3 Epipodophyllotoxin

Podophyllum peltatum major effective constituent, epipodophyllotoxin was used to synthesize etoposide and teniposide which are used to treat lymphomas and bronchial and testicular cancers (Zlatic and Stankovic, 2015). Etoposide is a topoisomerase II inhibitor stabilizing enzyme–DNA cleavable complexes leading to DNA breaks and is also effective against small-cell lung carcinoma (Cupit-Link et al., 2017).

CHAPTER 3: MATERIALS AND METHODS

This chapter is comprised of the materials and elaboration on the techniques implemented in the study to obtain the results. The basic principles and protocols followed in each technique are covered in this chapter. The techniques conducted in the study are cell culture, MTT assay, transient transfection- gene silencing and gene overexpression, RT-PCR, DNA fragmentation assay, caspase 3/7 assay and fluorescence microscopy. All these techniques were used to investigate the anticancer properties of the proposed targeted therapy in human metastatic cervical cancer cell line ME-180.

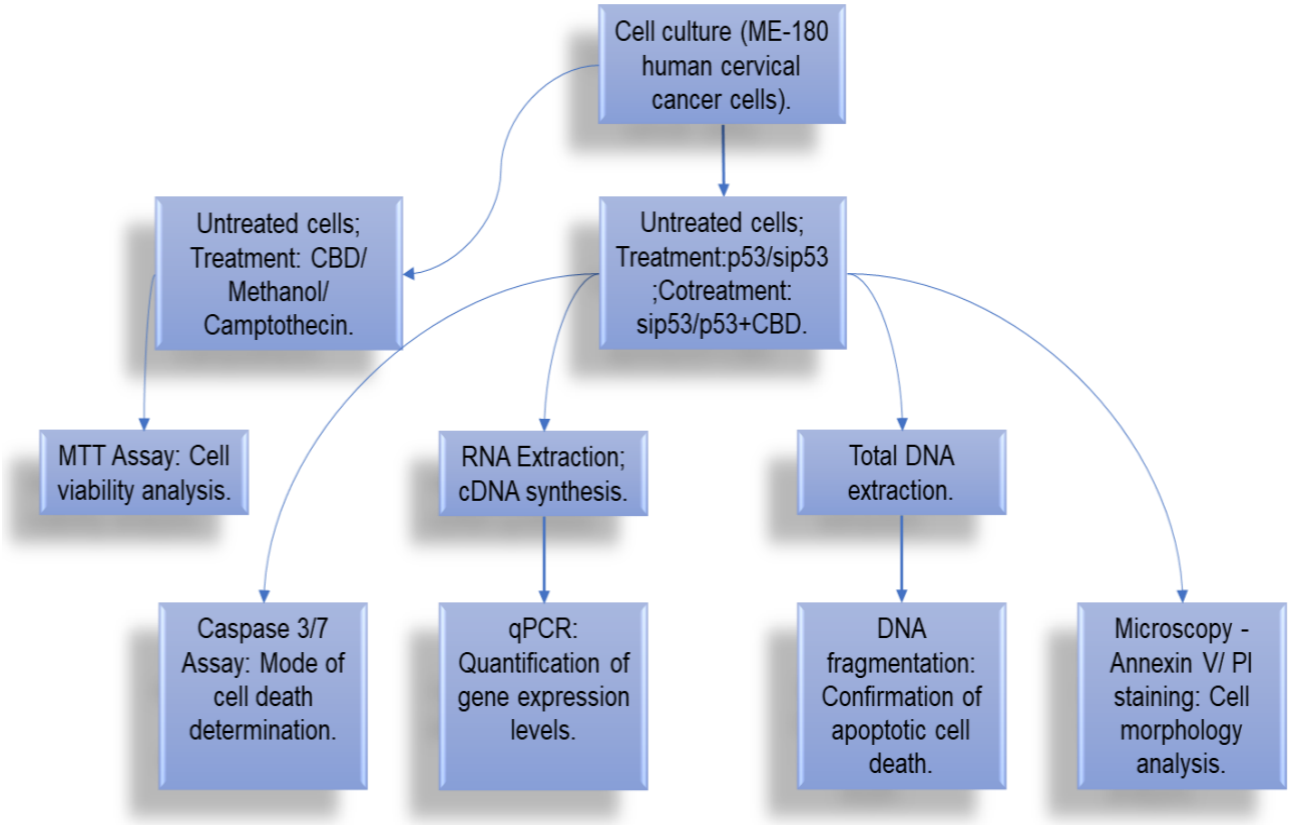

Figure 3.1. Overview of the methods implemented in the study. Different treatments were investigated for the anticancer effect on ME-180 cervical cancer cells by using MTT assay, qPCR, DNA fragmentation, microscopy and caspase 3/7 analysis.

3.1 sip53

The TP53 silencer (sip53) was purchased and used to knockdown the TP53 gene. This small interfering RNA of p53 gene was used throughout the study for evaluating the effect brought by the p53 knock down individually as well as in combination with cannabidiol compound.

3.2 TP53 cDNA clone

The TrueORF™ TP53 cDNA clone was purchased from the OriGene in the form of a dried plasmid. As instructed by the manufactures' protocol, the concentration of 100µM was prepared using the distilled water and stored at -20°C for future experimental use.

3.3 Primers

The pre-designed primers for the genes targeting cell cycle were bought from the IDT company. The primers of cell cycle targeting genes tumor suppressor p53, the p53 negative regulator MDM2 and the cyclin dependent kinase 2 (CDK2) were exploited in the current study to aid in the gene amplification for assessing both the cell cycle progression and the effect induced by cotreatment thereof.

3.4 Cell line and cell culture.

3.4.1 ME-180 cell line.

ME-180 cells were used throughout the study to determine the efficacy of p53 gene therapy concurrently with isolated cannabidiol compound. ME-180 cells are primary metastatic cervical cancer cells originated from 66-year-old Caucasian female (Scheffner et al., 1991). These cells are phenotypically adherent epithelial-like carcinomas (Romano et al., 2009). ME-180 cells are among the most common occurring primary metastatic cervical cancer cell lines worldwide.

3.4.2 Cell culture routine.

All cell culture was undertaken in lamina hood using aseptic technique to ensure sterility, and the equipment such as pipettes and tips were autoclaved before used. The cells were maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) for nutrients supply and 1% penicillin/streptomycin as antibiotic. The cells were incubated at 37 °C in the atmosphere of 5% carbon dioxide and 95% humidity. When the cells reached the confluency of ~70-80%, some of the cells were washed with 1X PBS and detached using 1X Trypsin (Gibco® 0.05% Trypsin-EDTA (1X)) to allow further continual growth through splitting and some were stored for the future use while some were used for the experimental purposes.

3.4.3 Cell counting.

Prior to plating for experiments, the cells were washed with 1X PBS and dissociated using trypsin (Gibco® 0.05% Trypsin-EDTA (1X)). The cells/ml were counted by adding 10µl of cells onto the dual chamber slide which was further inserted on the Bio-Rad TC20™ automated cell counter for counting of the cells. Approximately, the cell density ranging from $5x10^3$ to $1x10^6$ were used for the experiments conducted throughout the project.

3.4.4 Storage of cells

In case when more cells are cultured, the cells were washed, trypsinized and stored in cryovials with a freezing medium supplemented with DMSO on -80 ºC for future use.

3.5 Treatments and controls (cannabidiol, sip53 and p53).

3.5.1 Cannabidiol

Cannabidiol compound solubilized in methanol, of concentration 1mg/ml was purchased from Sigma and the working concentration of 10nM was used throughout the study to evaluate the effectiveness of the compound in combination with p53 on the ME-180 cervical cancer cells.

3.5.2 sip53 transfection

The p53 gene was silenced in the study for comparison with the other experimental and control samples. Confluent cells were washed with PBS and trypsinized to allow detachment from the flask. The cocktails for transfection were prepared and incubated separately for 10 minutes before mixed together. The transfection complex was prepared according to manufacturer's protocol and about 3ml of the master mix was added to each well containing cells (of density 1×10^6). After 4-hour incubation, about 3ml of growth medium (DMEM, supplemented with FBS and antibiotic) was added to each flask and further incubated for 24 hours to allow gene knockdown.

Table 3.1. Silencing master mix preparation. The first cocktail containing the RNAi for transfection complex.

Reagent	Volume
sip53	5µl
Opti-MEM	16ml

Table 3.2. Silencing master mix preparation. The second cocktail containing the transfection vector for transfection complex.

Reagent	Volume
Transfection reagent	30µl
Opti-MEM	32ml

3.5.3 p53 overexpression.

The p53 gene was expressed in ME-180 cells through liposomal transfection reagent. Briefly, ME-180 cells were plated in a 6-well plate for 24 hours to reach 50-70% confluency. A cDNA clone was mixed with transfection reagent in a 1:3 ratio (v/v) and incubated at room temperature for 10 minutes to allow DNA uptake by the transfection reagent. The cDNA clone/transfection reagent mixture was then added to each well that contained 500 µl of culture medium followed by gentle swaying of the plate to allow even distribution of the transfection mixture before incubating for 24 hours at 37˚C.

Table 3.3. Overexpression master mix preparation.

Tube 1	
Opti-MEM	250µl
Lipofectamine	11.25µl
Tube 2	
Opti-MEM	250µl
p53 cDNA	2µl
P3000 reagent	10µl

3.5.4 Co-treatment.

The cells seeded in the 6-well plates with either silenced/overexpressed p53 were incubated for 24 hours. After incubation the cells were treated with cannabidiol for further 24 hours. After the overall 48-hour incubation the cells were used for experimental purposes.

3.5.5 Positive and negative control- Camptothecin and methanol.

The Camptothecin of concentration 0.3µM was used as the positive control and other cells were left untreated throughout the study. Concurrently, the concentration of 0.3% methanol was used as the negative control throughout the study.

3.6 MTT Assay.

Principle

MTT assay is the cell viability assay that employs the use of yellow tetrazole (MTT reagent) which is reduced to purple formazan when entering viable cells. The MTT substrate which is used in cell culture and is usually allowed to stain the cells in duration of four hours, is soluble in 1X PBS or Isopropanol. A solubilizing solution such as DMSO is added to dissolve the insoluble purple formazan product into a colored solution. The quantity of formazan is directly proportional to the number of viable cells and is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. In this study, the MTT assay was used to measure the viability of metastatic ME-180 cells after treatment with methanol extract of *Cannabis sativa* and its main compound cannabidiol.

Experiment

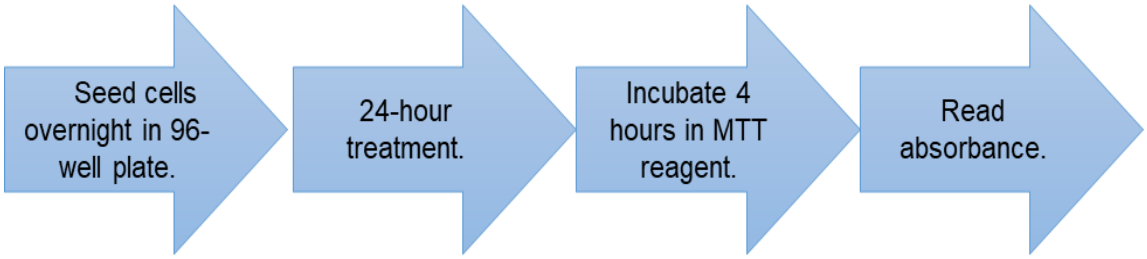

Figure 3.2. Workflow for the cell viability assay-MTT assay.

The counted cells at density of 5000 cells were seeded in each well of a 96-well cell culture plate in the final volume of 90µl per well; except for the wells for blank (which contained media only) and incubated overnight to allow cells to attach. After cells have attached, some wells were left untreated while other wells were treated at different concentrations of 0.5, 1, 1.5, 2, 2.5, 3µg/ml, and then incubated for 24 hours at 37 ˚C. The 0.03µM Camptothecin and 0.3% Methanol were both used as positive and negative controls respectively in the experiment. After treating cells for 24 hours, about 10µl of 5mg/ml MTT reagent was added in each well and incubated for 4 hours to allow the formation of formazan crystals. Following 4 hour- incubation, about 90 µl of DMSO was added to each well to dissolve the purple crystals, the absorbance was then measured at 570nm and the percentage of viable cells was calculated as follows:

$$\%\ Cell\ viability = \frac{Absorbance\ of\ treated\ cells - Absorbance\ of\ blank}{Absorbance\ of\ untreated\ cells - \ Absorbance\ of\ blank}\ X\ 100$$

3.7 DNA fragmentation.

Principle

Nucleosomal DNA fragmentation has been reported to be one of the common biochemical processes that usually signify the presence of apoptosis in cell(s), i.e. the presence of oligonucleosomal DNA fragmentation has been regarded by many scientists for ages as endogenous biomarker for apoptotic cell death (Martinez et al., 2010). Thus, DNA fragmentation can be used to elucidate the cytotoxic mechanism of the certain drug administered to the cells. In the present study, the fragmentation of nucleosomal DNA characteristic has been detected through agarose gel electrophoresis of isolated DNA from the cervical cancer cells treated with various therapies. Agarose gel electrophoresis is the common technique used to separate and purify molecules based on the electronic charge, shape and size of the molecule to determine the molecular weight (i.e. size) and purity of the macromolecules such as proteins and nucleic acids and this technique is highly affected by the concentration of the agarose gel exploited (Stellwagen, 2009). Hence, this technique is based on the principle that, when charged molecules are placed on the electric field they migrate towards their oppositely charged poles and eventually the size of such molecules can be determined (Stellwagen, 2009). In this study the agarose gel electrophoresis was used to evaluate the presence of the DNA fragmentation.

Experiment:

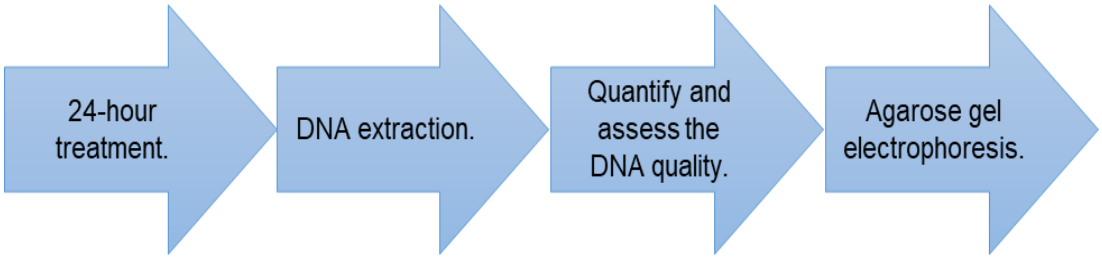

Figure 3.3. Workflow for DNA fragmentation.

DNA extraction: Following various treatments, the DNA was extracted from the ME-180 human cervical cancer cells using the Macherey-Nagel (Nucleospin) kit. Concisely, the cells were collected in a final volume of 200µl Buffer T1 and mixed with 25µl Proteinase K solution and 200µl Buffer B3. The sample was incubated for 15 minutes at 70°C. Following incubation, about 210µl of 96-100% Ethanol was added to the sample and vortexed vigorously. The precipitate was loaded in the column (NucleoSpin® Tissue Column) placed in the collection tube and spun at 11,000 x g for 1 minute. After centrifugation, the flowthrough was discarded and the column was placed back in the collection tube. The silica membrane was washed twice with 500µl Buffer BW and 600µl Buffer B5 consecutively while centrifuging for 1 minute at 11,000 x g and discarding the flowthrough following

each wash. To remove the residual ethanol on the silica membrane, the membrane was dried through centrifugation at 11,000 x g for 1 minute. The highly pure DNA was eluted by adding 60µl Buffer BE in a NucleoSpin® Tissue Column placed in 1.5ml microcentrifuge tube and incubated at room temperature for 1 minute. Then the tube assembly was spun at 11,000 x g for 1 minute. The extracted DNA was quantified to evaluate the purity and concentration of the isolated DNA using the nanodrop.

Agarose gel electrophoresis was conducted to assess the size of the DNA fragments produced by the treatments used in the study. About 2% agarose gel was prepared by dissolving 6g of agarose powder to 300ml of 1X TBE buffer in the microwave. The ethidium bromide was added towards the cooling of the gel. The gel was poured to the gel mould before it solidified, and the comb was assembled at the end of the gel and the gel could polymerize. The 1X TBE buffer was poured over the wells to cover the comb in the gel tank (Bio-Rad) and the comb was removed carefully from the solidified agarose gel. Each of the isolated DNA samples mixed with loading dye were loaded into each well. The molecular weight standard marker was loaded in the first well and electric field was applied. The gel was run for 60 minutes at 80 Volts.

3.8 Reverse transcription and cDNA synthesis using PCR.

Principle

Polymerase chain reaction (PCR) is advanced broadly used technique in molecular biology for amplification of DNA. This technique is commonly used by many scientists to amplify the gene of interest usually from genomic DNA in various experiments including recombinant DNA technology. The process occurs by exploiting either the RNA or cDNA as the start-up molecules to synthesis the desired gene of interest in large numbers. Incase whereby the cDNA probe is regarded as the starting material for gene amplification, the DNA is copied directly to amplify the gene of interest in large quantities (Ehtisham et al., 2016). And in other cases, the mRNA is used to synthesize and amplify the desired gene in the sample thus the mRNA would be transcribed into the cDNA using the reverse transcriptase enzyme. In exception to the cDNA/mRNA samples as start-up materials, the PCR assay requires several components to assist in amplification process and these include the recruitment of primers (reverse and forward primers or the oligo dt primers), magnesium chloride ($MgCl_2$), nuclease-free water, RNase inhibitor and reverse transcriptase (only when starting with mRNA for gene amplification). The assay involves numerous reaction steps and the number of cycles for amplification. The reaction steps for the assay exceptionally the reverse transcriptase PCR comprise the sample denaturation, annealing of primers, cDNA synthesis for certain number of cycles, reverse transcriptase inactivation and cooling of the sample.

Figure 3.4. Workflow for reverse transcription PCR assay (cDNA synthesis).

RNA extraction: The total RNA was extracted using the High Pure RNA Isolation Kit from Roche. Concisely, the cells were harvested in 200µl 1X PBS, lysed in 400µl lysis/-binding buffer and vortexed for 15 seconds. The sample was transferred to the High Filter Tube inserted in the collection tube and centrifuged the entire tube assembly at 8,000 × g for 15 seconds. Following centrifugation, the flowthrough liquid was discarded and the same filter was re-inserted in the collection tube. A solution of 90µl DNase incubation buffer thoroughly mixed with 10µl DNase I was pipetted into the top reservoir of the filter tube and incubated at 25°C for 15 minutes. After incubation, the sample (in the top reservoir of the filter tube) was washed with 500µl Wash Buffer I and Wash Buffer II successively and spun at 8,000 × g for 15 seconds after each wash. The flowthrough was discarded and the filter tube was re-inserted with the collection tube. The sample was washed for the third time with 200µl Wash Buffer II and centrifuged at a speed of 13,000 × g for 2 minutes to remove the residual Wash Buffer. Following spinning, the collection tube was discarded, and the Filter Tube was inserted in a new sterile microcentrifuge tube. To elute RNA, the amount of 50µl of Elution Buffer was added into the top of the Filter Tube and the tube assembly was span at 8,000 × g for 1 minute. The eluted RNA was stored at −80°C for the later analysis. The extracted RNA was quantified using the nanodrop.

cDNA synthesis: cDNA was synthesized using ImProm-IITM Reverse Transcription kit. Concisely, the reaction mixture was prepared by adding the reaction buffer, $MgCl_2$, dNTPs, RNase inhibitor, reverse transcriptase, oligo dt primers, RNA sample and nuclease-free water in the sterile PCR tube with adding the reverse transcriptase enzyme at the end. Hence the reaction mixture of about 20µl was prepared in sterile PCR tubes in the lamina hood using the RNA samples from various treatments. The tubes were vortexed and inserted in the T100™ Thermal cycler (Bio-Rad).

Table 3.4. Components for reverse transcription.

Reagent	Volume (µl)
Reaction buffer	2.5
$MgCl_2$	3
DNTPs	2.5
Oligo dt primers	2
RNase inhibitor	1
RNA sample	2
Reverse transcriptase	1
Nuclease-free water	6

Table 3.5. PCR thermocycler parameters for reverse transcription (cDNA synthesis).

Step	Temperature	Duration	
Initial denaturation	95 °C	5 minutes	
Denaturation	95 °C	30 seconds	
Annealing	60 °C	30 seconds	35 Cycles
Extension/Synthesis	72 °C	30 seconds	
Final extension	72 °C	10 minutes	
Cooling	4 °C	∞	

3.9 Real time Polymerase Chain Reaction (RT-PCR).

Principle

Real time Polymerase Chain Reaction (RT-PCR) is one of the quantitative methods used for several roles in molecular biology, including the precise quantification of manipulated gene (Valones et al.,

2009). PCR is the most sensitive molecular technique used to amplify the DNA fragment exponentially in a semi-consecutive manner in *in vitro* system. The quantification of the exponential amplification of a specific gene in PCR is possible, provided there are the known short sequences (primers) that can be forward or reverse primers (Ehtisham et al., 2016). The gene of interest which sometimes can be the manipulated gene, can be amplified more than million-fold and quantified at the same time at every cycle with the aid of a fluorescence dye usually the SYBR green dye (Mohammed et al., 2015). The successful amplification of manipulated gene is accomplished by the reaction of the following components: template DNA, oligonucleotide primers, *Taq* DNA polymerase, deoxy nucleoside triphosphate (dNTPs), buffer to maintain pH, divalent cations especially $Mg2^{+}$, monovalent cations such as 50 mM of KCl and fluorescent dye such as SYBR green (Joshi et al, 2011). qPCR is the most convenient DNA amplification method as compared to the conventional PCR in the way that during qPCR the amplified DNA fragment can be detected and quantified at every cycle rather than in conventional PCR where the results are obtained at the end of the whole reaction (Kavya, 2015). In this study, the amount of expressed/silenced p53 gene together with other apoptotic and cell cycle genes were quantified using the qPCR assay.

Experiment

Figure 3.5. Workflow for real time PCR.

The cDNA synthesized through reverse transcriptase was used for qPCR. The qPCR was conducted in reaction mixture containing cDNA, SYBR Green (SIGMA®) master mix and primers of different genes under the conditions recorded in **Figure B.1** (Annexure B).

Table 3.6. Cocktail preparation for RT-PCR for the total reaction volume of 10µl.

Reagent	Volume	Concentration
cDNA	2 µl	10 ng
Primer	1 µl	20 ng
SYBR Green master mix	5 µl	2X
Nuclease-free water	2 µl	-

3.10 Caspase 3/7 Assay.

Principle

Caspases are aspartic proteases that cleaves the protein at a specific site rich in aspartases. Caspase cascade activities result in apoptotic mode of cell death; hence this cascade involves the cleavage and activation of initiator caspase-9 by apoptosome which further activate the effector caspases that are responsible for degradation of the cellular proteins (Gurtu et al.,1997). In caspase 3/7 assay, the product formation quantified through luminescence, in which the product formation is proportional to the caspase activity, that is the amount of caspase 3/7 activity in the cells is relative to the amount of apoptosis yielded after treatment (Gurtu et al., 1997). Caspase 3/7 assay is a luminescent assay that quantify the activity of caspase 3 and 7, the assay offers the luminogenic caspase 3/7 substrate that contains the tetrapeptide sequence DEVD in a reagent enhanced for cell lysis, luciferase and caspase activity (Kumar, 2004). When the caspase Glo 3/7 reagent is added to the cells, the cell lyses subsequently followed by the cleavage of the substrate by the caspase producing the luminescent signal synthesized by the thermostable luciferase. The detectable luminescence is proportional to the quantity of the caspase activity present in the cells. In this study the assay was used to identify the mode of cell death induced by both co-treatments.

Experiment:

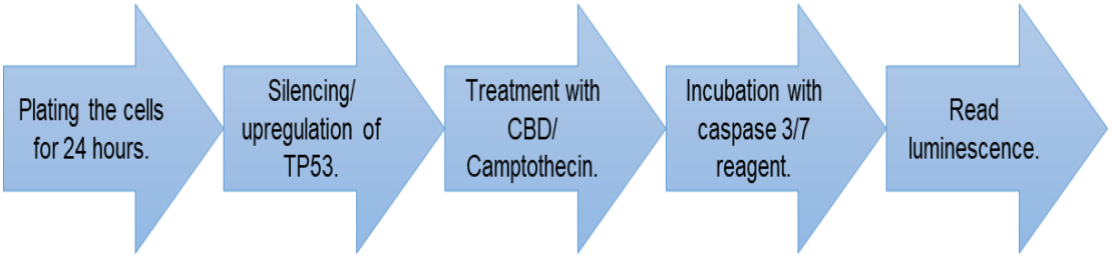

Figure 3.9. Workflow for caspase 3/7 assay.

After cotreatment with p53 and cannabidiol in a white 96-well luminometer plate at a total volume of 50µl, about 50µl of caspase 3/7 reagent was added to the cells and incubated for 2 hours. The luminescence was read and recorded. The assay was performed in duplicates and caspase activity was represented as a mean of Relative Light Units (RLU). The caspase 3/7 activity was calculated in RLU as follows:

$$RLU = Luminescence(sample) - Luminescence(blank)$$

3.11 Morphology analysis by fluorescence microscopy.

Principle

Morphological changes of the cell can be used as a biomarker of apoptosis. Apoptotic properties such as membrane blebbing, cell shrinkage, Nuclear fragmentation and chromatin condensation are some of the morphologies that can be examined under the microscope (Henry et al., 2013). During apoptosis the plasma membrane remains intact almost until the end, then loses asymmetry and integrity, which causes the phosphatidylserine residues located in the inner membrane to translocate to the cell surface in the exterior cellular environment (Gatti et al., 1998). Hence these residues can be recognized by certain stains such as Annexin-V, Diamidino Phenyl Indole (DAPI), propidium iodide (PI), etc. Annexin-V is a calcium dependent phospholipid protein sized 35-36 kDa that bind with high affinity to the phosphatidylserine residues located on the outer plasma membrane (Bernard et al., 2001). Researchers reported that this green fluorescence stain stains the cells at early stages of apoptosis by binding to the externalized phosphatidylserine residues of the plasma membrane (Abbady et al., 2017). Among other DNA stains, the stains such as DAPI and PI are regarded nuclear and chromosomal stains that detect apoptosis and emits blue and red fluorescence respectively by binding to the AT regions of DNA in the cells undergoing apoptosis at the later stages of cell death (Bernard et al., 2001; Krämer et al., 2016). The PI stain is only permeable to the damaged cells with poor membrane integrity and the dead cells (Krämer et al., 2016). The fluorescence microscopy is used for such analysis and detection of fluorescence.

Experiment:

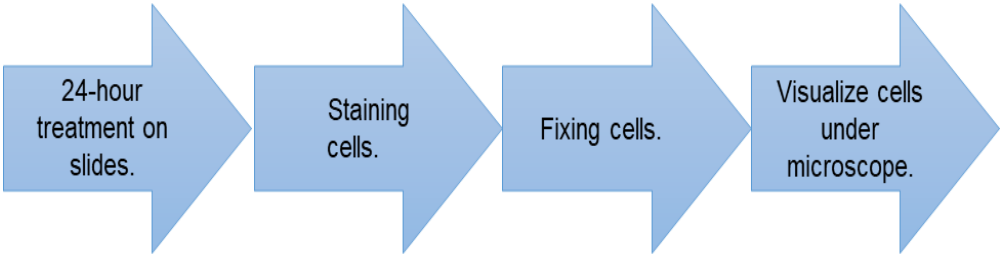

Figure 3.10. Workflow for fluorescence morphology analysis.

The cells were seeded on the coverslips in the six-well plates containing the DMEM growth medium for 24 hours to allow the cells to attach on the coverslip. Following the culturing, the cells were treated with various treatments including the p53-Cannabidiol cotreatment for 24-48 hours depending on the type of treatment applied to the cells while the other cells were left untreated. Once the cells were treated the media was removed, and the cells were washed with 1X PBS. The cells were then stained with Annexin V (ApoAlert®) and counterstained with Propidium Iodide (PI) according to the manufacturer's instructions. Briefly, the cells were resuspended in 200µl of 1X binding buffer (ApoAlert®) and further stained with 5µl Annexin V/FITC and 10µl PI at room temperature for 15 minutes in the dark. Following the staining process, the cells were fixed in the 2% formaldehyde. The coverslips of fixed cells were inverted on the glass slide and visualized under the fluorescence microscope (Euromex® Inverted Oxion microscope) for morphology analysis.

3.12 Statistical analysis

Experiments were performed in duplicates, and the data sets were expressed as mean ± standard deviation (SD). Data was analyzed using Student's t-test and statistical significance in contrast to untreated was regarded as $p \leq 0.05$.

CHAPTER 4: RESULTS

Introduction

The current chapter demonstrate the detailed results acquired from various experiments conducted in the aim of determining the antiproliferative, apoptotic and morphological characteristic of the treatments and cotreatments used in the study on the cervical cancer cell line ME-180.

4.1 Cytotoxic effect of cannabidiol on ME-180 cells.

To evaluate the effect possessed by cannabidiol compound on viability of primary ME-180 cervical cancer cell line, we conducted MTT assay. The experiments were conducted in duplicates and the methanol and Camptothecin were both used as negative and positive control respectively. Following the treatment with respective cannabidiol concentrations of 0.1, 0.25, 1.5, 2.0 and 3.0 µg/ml, the yielded percentage cell viability of 72.6%; 67.2%; 65.0%; 60.7% and 51.5% were obtained respectively (**Figure 4.1**). Following the statistical analysis, it was found that the p-values of methanol and cannabidiol concentrations of 0.1, 0.25, 1.5, 2.0 and 3.0 µg/ml were 0.0066; 0.0476; 0.0033; 0.0017; 0.0009; and 0,0002 respectively. As the p-values of 0.05 and lower were considered significant therefore all the cannabidiol concentrations of 0.1, 0.25, 1.5, 2.0 and 3.0 µg/ml were found

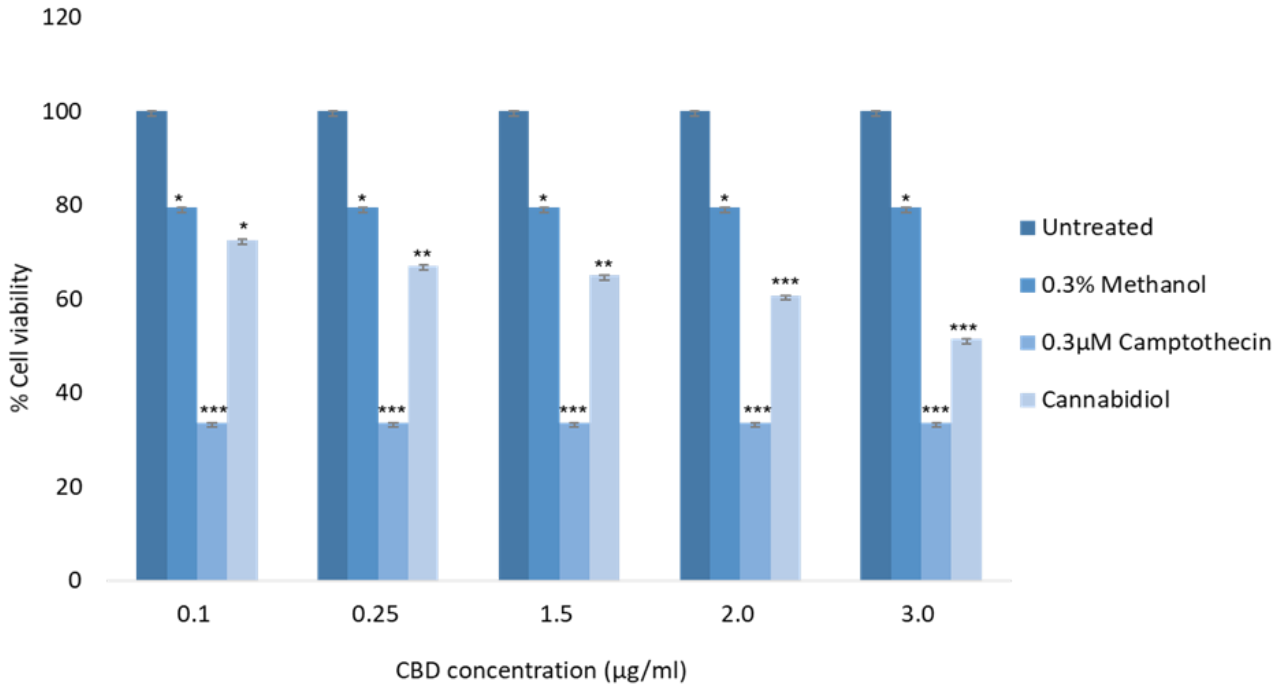

Figure 4.1. Evaluation of the cytotoxicity of CBD on ME-180 cervical cancer cells using the concentrations of 0.1, 0.25, 1.5, 2.0 and 3.0 µg/ml. The data is expressed as mean ± standard deviation and $^{ns}p \geq 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ representing the level of significance in contrast to untreated control.

to be more significant. The cannabidiol concentration that was found to induce the estimated 50% of the cell death was 3.0µg/ml which was regarded the inhibitory concentration throughout the study. The cytotoxicity effected by compound cannabidiol was found to be concentration dependent, as the cell death induced was increasing with increasing concentration of the compound. The methanol-treated cells gained the viability of 79,5% which clearly indicate that the cytotoxicity induced in the study by the CBD is not caused by the methanol as a solvent used to prepare CBD.

4.2 Quantification of p53 following the p53 gene manipulation

To examine whether the manipulated TP53 gene was upregulated in the ME-180 cervical cancer cells following the transient transfection, the real-time PCR was conducted. The cells were transfected with the p53 silencer and others with the TP53 gene while others were left untreated to serve as control. The RNA was isolated from the experimental cells and then quantified with nanodrop and the ratios of 1.8-2.0 were recorded from each RNA sample. The cDNA template for p53 amplification was synthesized for quantitative PCR for further quantitation of the p53 expression levels in the cells. The standard curve (**Figure B2.**) was created for the absolute determination of Log DNA concentration of the unknown samples using the obtained equation from the standard curve. The expression was calculated using the expression ratio normalized to untreated control

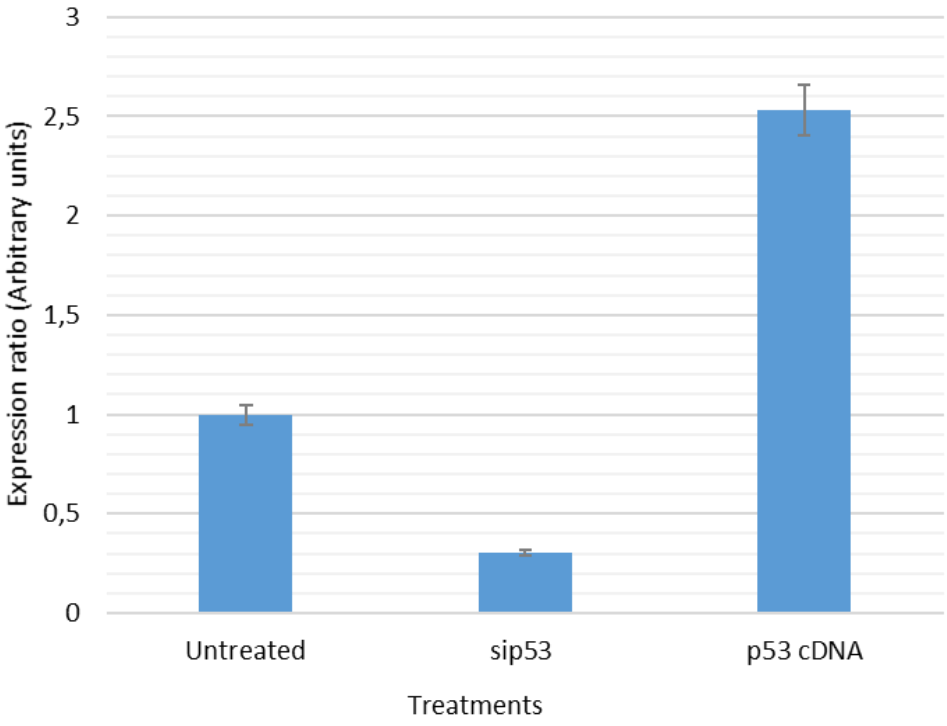

Figure 4.2. Quantification of the expressed p53 in the ME-180 cervical cancer cells following the p53 gene manipulation. The data is represented as geometric mean normalized to untreated control.

sample. Levels of TP53 following treatment with sip53 and p53 individually have shown different expression levels whereby TP53 treated cells have denoted the higher expression of p53 gene with expression ratio of 2.53. However, the sip53 treated cells earned the lower expression of p53 gene with a mean factor of 0.3 as compared to that of the control and the p53-overexpressed cells. As projected the p53 gene was expressed highly in the TP53 treated cells with 250% upregulation in contrast to the control cells whereas the ME-180 cells with downregulated TP53 have shown the knockdown of 30% (expression ratio of 0.3) in relative to control sample **(Figure 4.2)**.

4.3 Analysis of cell cycle genes.

Cell cycle regulation is the common disrupted cell machinery during the cancer development. In this study the cell cycle genes CDK2, p53 and its regulator MDM2 have been quantified to assess the fidelity of cell division. As demonstrated in **Figure 4.3**, the p53 gene manipulation in the ME-180 cervical cancer cells have produced distinct expression quantities of respective genes of interest (GOI). The MDM2 gene was highly overexpressed by approximately 376% (3.76) in the cells with

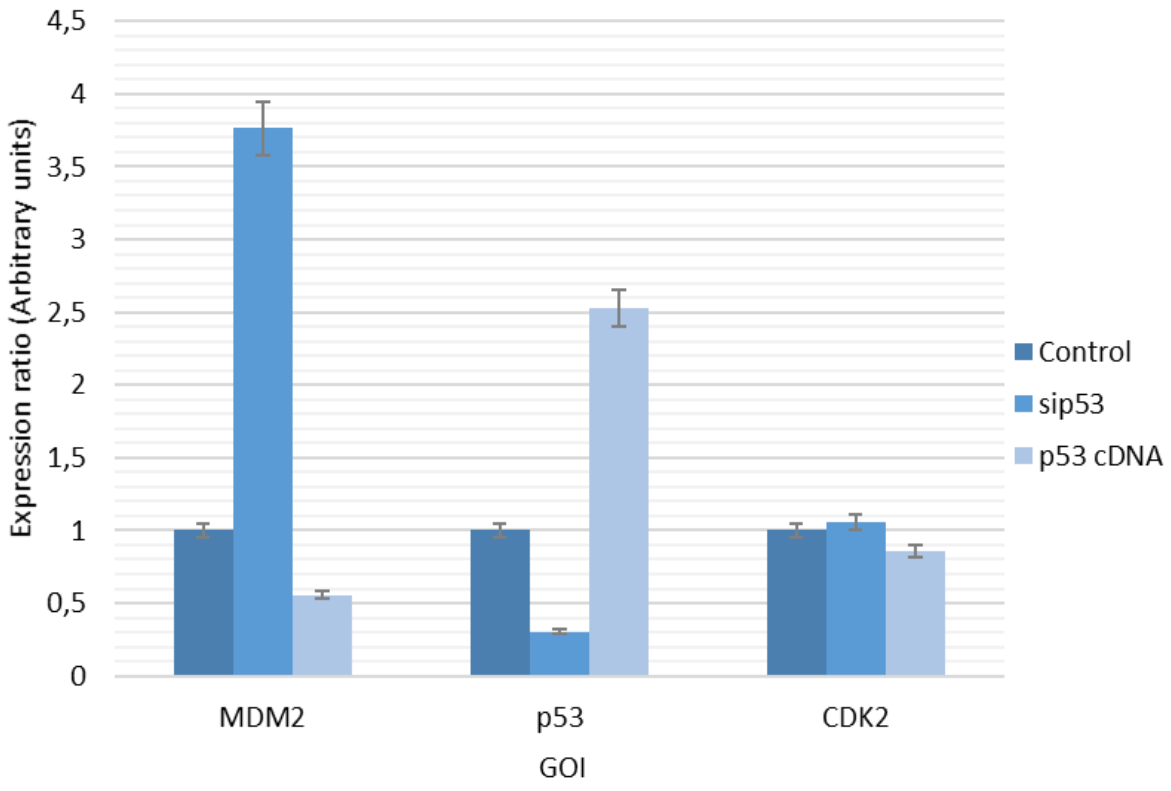

Figure 4.3. Expression levels of gene of interest (GOI) MDM2, p53 and CDK2 following the p53 gene manipulation in the human cervical cancer cells. The data was reported as geometric mean normalized to the untreated control.

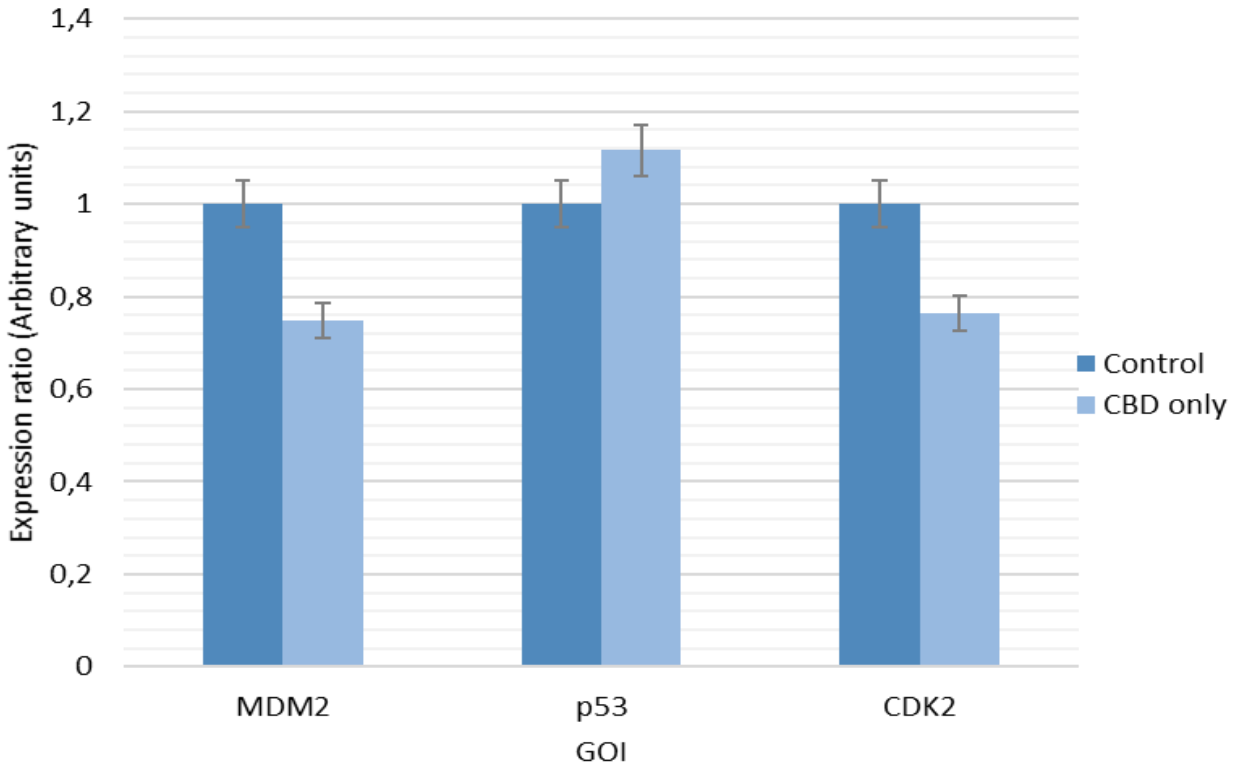

Figure 4.4. Expression levels of cell cycle genes MDM2, p53 and CDK2 following the treatment of the cells with compound cannabidiol.

silenced tumour suppressor p53 gene and downregulated by 50% in the cells with overexpressed p53 gene. Relatively to the control, the cells with overexpression of p53 as treatment have shown to upregulate the expression level of p53 by 253% and knocked down the MDM2 by 55% **(Figure 4.3)**. In other case the cells with silenced p53 as treatment have shown to decrease the expression of TP53 to 30% and increased that of MDM2 by 376%. The CDK2 levels in both the downregulated and upregulated p53 cells seemed not to be affected as the expression was slightly changed by the respective treatments. The treatment of ME-180 cells with cannabidiol compound for 24 hours seemed to enhance the expression of p53 to 110% and reduced that of MDM2 and CDK2 gene to 75% and 76 % respectively **(Figure 4.4)**. In contrast to untreated control, expression of cell cycle genes in **Figure 4.4** showed a slight difference in the expression levels of the gene of interest.

Despite individual treatment of the cells with p53 or sip53 and cannabidiol separately, the cells were cotreated with both p53 and cannabidiol for further assessment on the impact of the p53-cannabidiol cotreatment on the growth of cervical cancer cells. From **Figure 4.5**, we noted that the p53-cannabidiol cotreatment significantly decreased the expression of MDM2 and CDK2 to 62% and 74% respectively while cotreatment with sip53-cannabidiol showed no expression changes on the levels of MDM2 and CDK2 in the cells. The detected p53 expression was upregulated by 454% in

the cells cotreated with p53 and cannabidiol whereas in the sip53-cannabidiol cotreated cells the p53 gene level was downregulated to 60%.

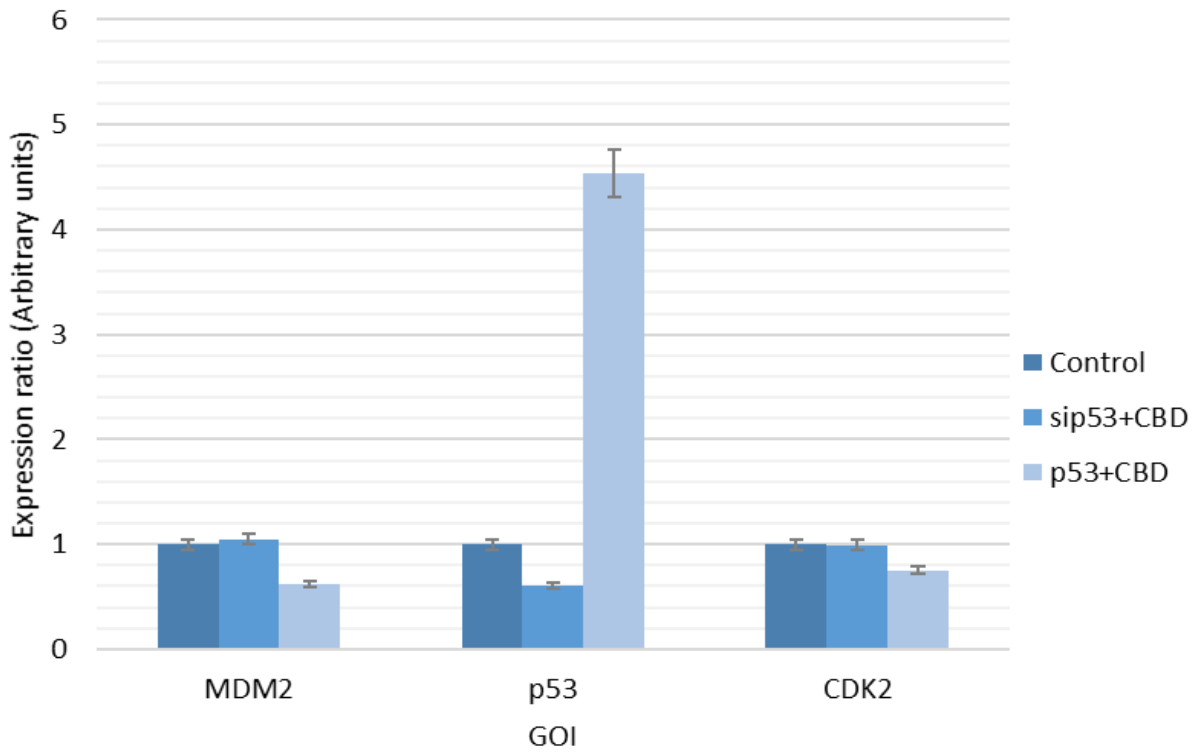

Figure 4.5. The expression levels of cell cycle genes MDM2, p53 and CDK2 following co-treatment with TP53 cDNA and cannabidiol compound.

4.4 Mode of cell death induced by p53 manipulation, cannabidiol compound and p53-cannabidiol co-treatment.

4.4.1 Detected caspase 3/7 activity in the ME-180 cells.

Activity of caspases in the cells is the hallmark of apoptosis and plays a crucial role as apoptotic biomarker in research of anticancer therapy developments (Pu et al., 2017). After administering caspase 3/7 substrate to the treated cells (including co-treated cells), the luminescence was detected from the cells. The detected luminescence which was read in Relative Light Units (RLU) was proportionally regarded as activity of executioner caspase-3 and -7. The caspase activity of the samples for untreated, Camptothecin, CBD, sip53, p53, sip53 cotreated with CBD (sip53+CBD) and p53 cotreated with CBD (p53+CBD) were found to be 21 638, 39 610, 23 543,12 027, 34 887, 11 070 and 49 723 RLU respectively (**Figure 4.6**). The caspase activity of the cells treated with both sip53 and sip53+CBD was noted to be extremely lower than the rest of the treatments used in the experiment. The caspase activity of p53 and the p53+CBD treated cells was found to be relatively higher, with p53+CBD being the highest of all the treatments used. As expected, the caspase activity

of the untreated cells was proportionately low since there is little to no apoptosis occurring in those cells. Activity of caspase-3 and -7 of Camptothecin and Cannabidiol treated cells were moderately high and this imply that these plant-derived compounds do possess some apoptotic properties however the activity of the executioner caspases of cotreatment with p53 noted to be higher than the individual treatments.

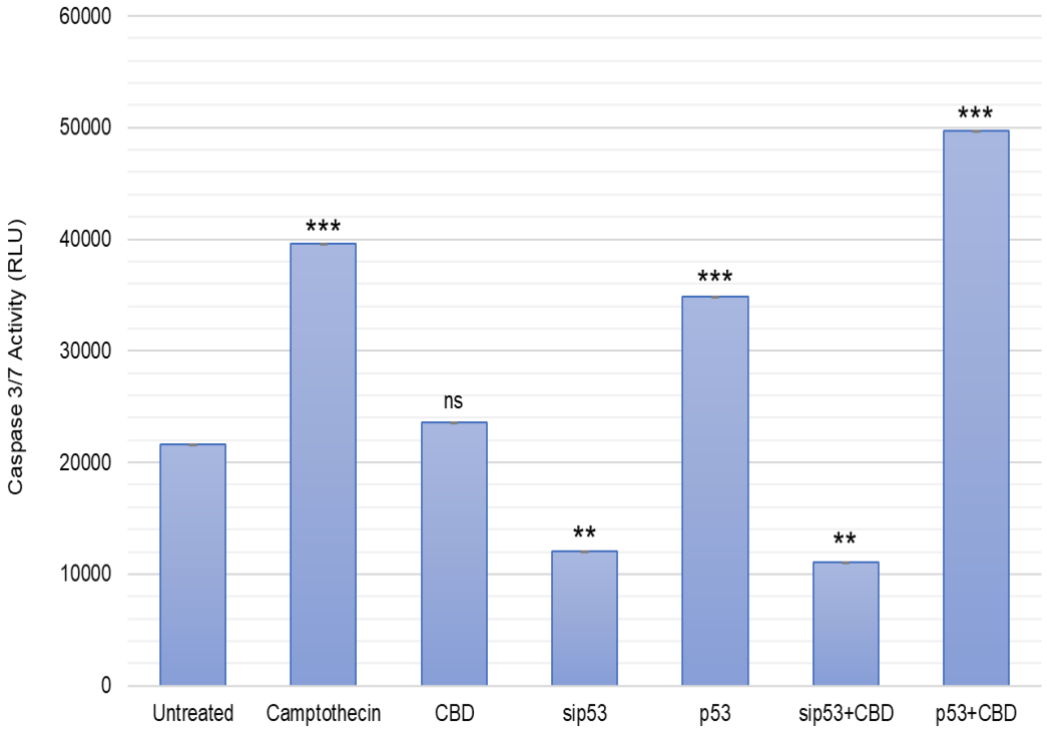

Figure 4.6. The activity of executioner caspase 3 and 7 in the ME-180 cells following the treatment with Camptothecin, cannabidiol (CBD), sip3, p53 cDNA, sip53+CBD cotreatment and p53+CBD cotreatment. The untreated and Camptothecin were both used as the negative and positive control respectively. The data was expressed as mean ± standard deviation and $^{ns}p \geq 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ representing the level of significance in contrast to untreated control. The caspase activity was measured in relative light units (RLU).

4.4.2 Nuclear DNA fragmentation.

DNA fragmentation has been reported to be one of the basic morphological features displayed by the cells undergoing apoptotic cell death. Usually during apoptosis chromatin condenses, and the nuclear DNA fragmentation occurs in response to catalytic cleavage that is usually accomplished by caspases (Kijima and Mizut, 2019). The nucleosomal DNA fragmentation takes place in the later stage of apoptotic cell death thus, it's used to detect apoptosis especially late apoptosis in the cells (Martinez et al., 2010). During apoptosis the catalytic aspartases degrade the cellular proteins to kill the cells to generate the small oligonucleosomal DNA fragments commonly identified as the apoptotic bodies, which are usually recognized by phagocytes and are engulfed by these phagocytes

and are engulfed by these phagocytes during the phagocytosis process to evade resulting in the inflammation (Martinez et al., 2010; Anita et al., 2014).

Fragmentation of nucleosomal DNA can be detected using the DNA fragmentation techniques such as the DNA agarose or polyacrylamide gel electrophoresis, TUNEL assay and can also be visualized using the microscopy as described in the study. According to Ioannou and Chen, (1996), the fragmentation of nucleosomal DNA is identified by DNA smearing/ laddering on the gel. In the current study the fragmentation was assessed from the isolated DNA treated variously to analyse and to check the presence of whole or fragmented DNA through implementation of agarose gel electrophoresis to identify mode of cell death induced by the treatments thereof. From the results obtained we observed the absence of DNA fragmentation from both treated and untreated cells. The molecular weight of the DNA from cells treated with TP53, cannabidiol and TP53+cannabidiol cotreatment (Lane 4, 5 and 7) seemed to be estimated to 200bp, however that did not suggest the presence of DNA fragmentation as there was no DNA laddering nor smearing observed on the gel (Figure 4.7). As noted in the electrophoretogram (Figure 4.7), there was no apparent DNA

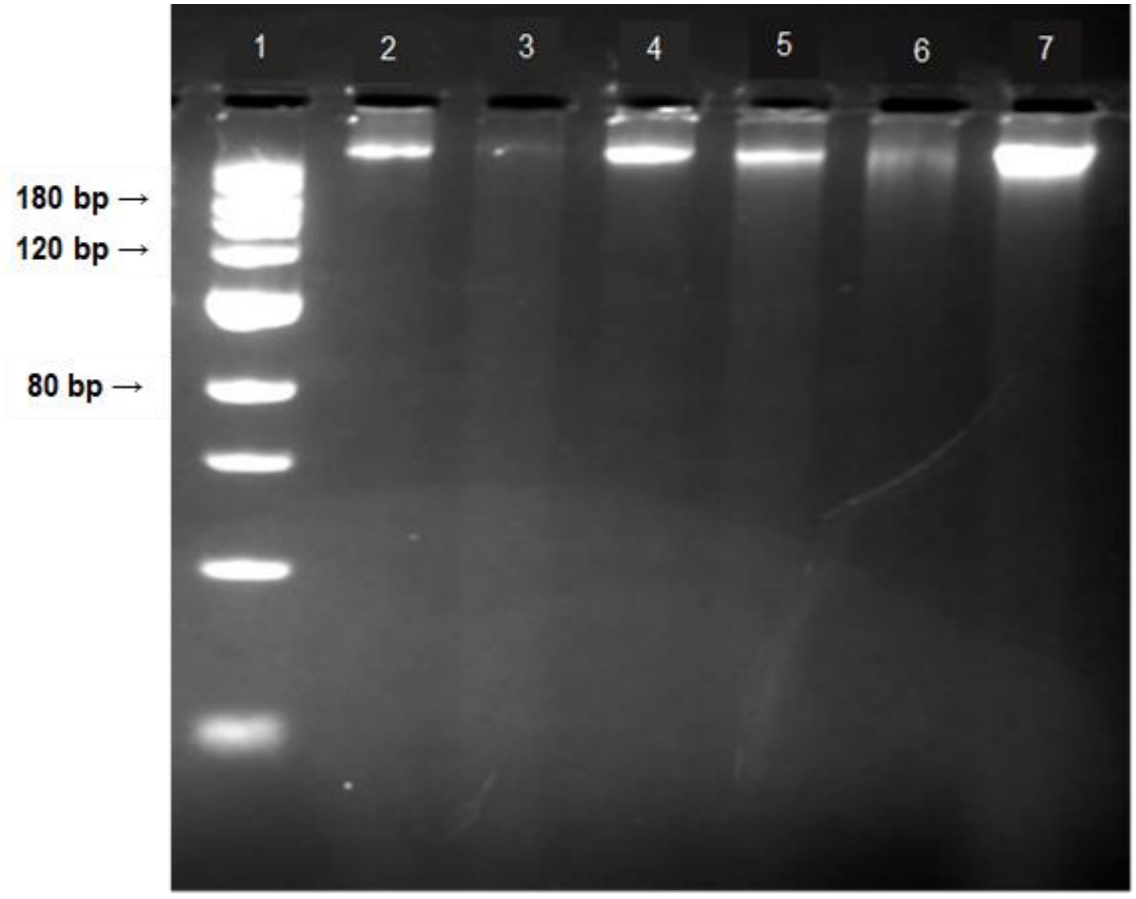

Figure 4.7. Determination of DNA fragmentation following treatments used to treat ME-180 cells in order to analyze amount of apoptosis induced by various treatment. The contents of the lanes were as follows: 1-Genetic marker. 2- Untreated cells. 3- sip53-treated cells. 4- TP53-treated cells. 5- Cannabidiol treated cells. 6- sip53+Cannabidiol cotreated cells. 7- TP53+Cannabidiol cotreated cells.

fragmentation in the control and experimental cells implicating that none of the treatment used in the study induced nucleosomal DNA fragmentation.

4.5 Morphological properties effected by the treatment of either p53 or cannabidiol and p53-cannabidiol cotreatment on the ME-180 human cervical cancer cells.

Apoptosis functions in embryonic development and the maintenance of tissue homeostasis (Radogna et al., 2015). During apoptosis the phospholipid phosphatidylserine which usually reside in the inner plasma membrane becomes translocated to the exterior environment and exposed phosphatidylserine becomes target of the stains such as Annexin V which possess the elevated binding affinity to the externalized phosphatidylserine residues (Abbady et al., 2017). The loss of membrane integrity and exteriorization of the phosphatidylserine residues emerge at the early stages of apoptotic cell death hence Annexin V is used to identify and stain the apoptotic cells at earlier stages (Xu et al., 2017).

In this study the morphology of the cells was examined using the fluorescence microscopy following the staining of cells with Annexin V and PI stains and only the stained cells were considered apoptotic cells. In **Figure 4.8**, **Figure 4.9** and **Figure 4.10**, it was observed that lot of cells undergoing apoptosis were treated with the p53, cannabidiol and cotreatment p53-cannabidiol as the cells exhibited loss of membrane integrity and most of them detached from adjacent cells especially the p53-cannabidiol cotreated cells (**Figure 4.10** from **g)-i)**). The clearer morphology of apoptotic cells with loss of membrane integrity, cell detachment from its substratum and neighboring cells and cell shrinkage were demonstrated in **Figure 4.10** with cells cotreated with p53-cannabidiol.

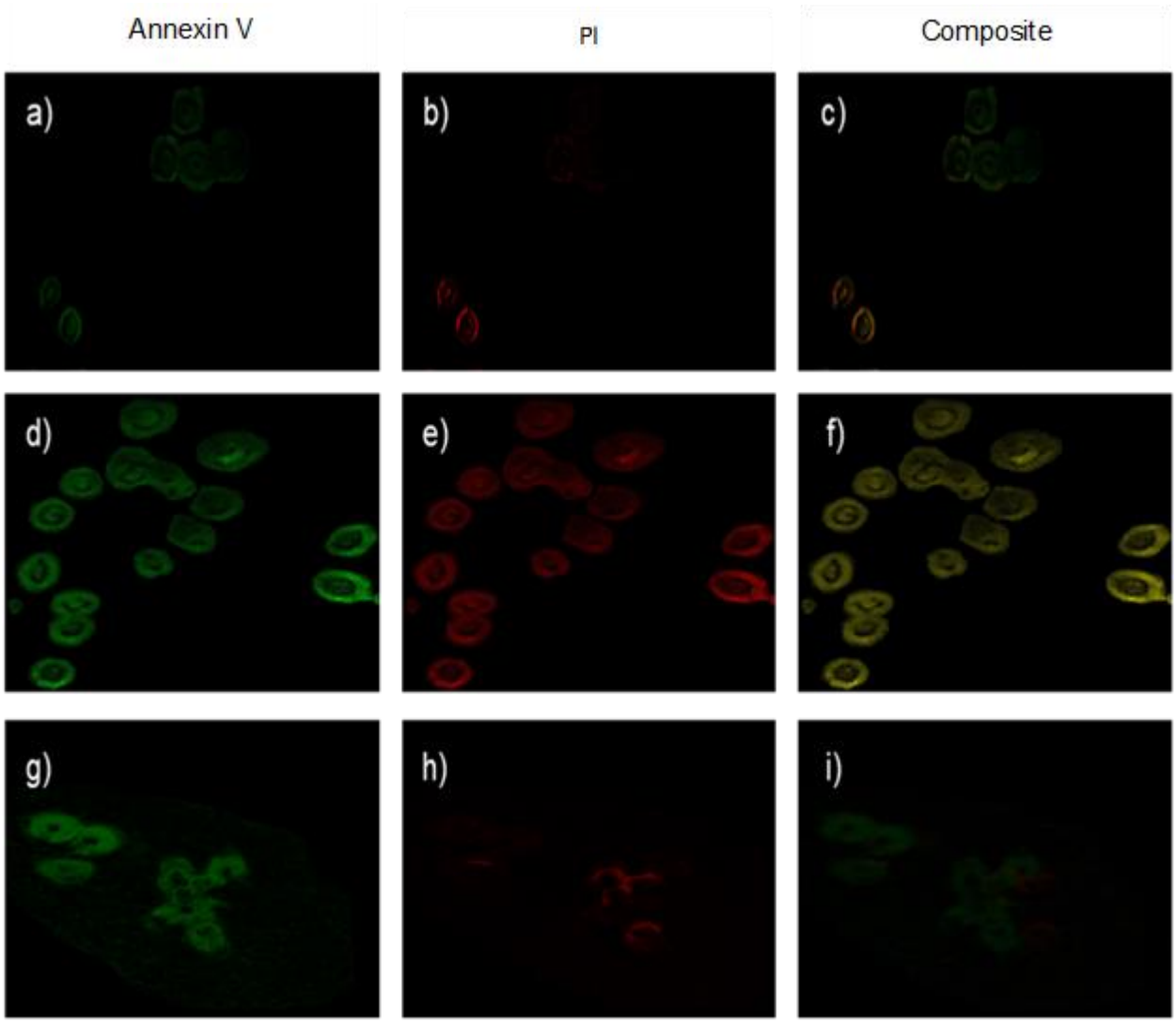

Figure 4.8. The fluorescence micrographs obtained following the 24-hour post TP53 gene manipulation after staining with the nucleic acids' apoptosis-detecting stains Annexin-V and PI which emits green and red fluorescence respectively. The micrograph labelled **a)** & **b)** Untreated ME-180 cells stained with Annexin V and PI respectively; **c)** A composite micrograph of ME-180 untreated cells stained with Annexin V and PI emitting fluorescence from both stains; **d)** & **e)** ME-180 cells stained with Annexin V and PI following the TP53 gene over-expression; **f)** A composite micrograph of TP53 overexpressed ME-180 cells stained with Annexin V and PI; **g)** & **h)** ME-180 human cervical cancer cells counterstained with Annexin V and PI following 24-hour knockdown of TP53 gene using the sip53. Hence **c)**, **f)** & **i)** exhibit the composite micrographs of counterstained cells that emit both the respective fluorescence of stains employed.

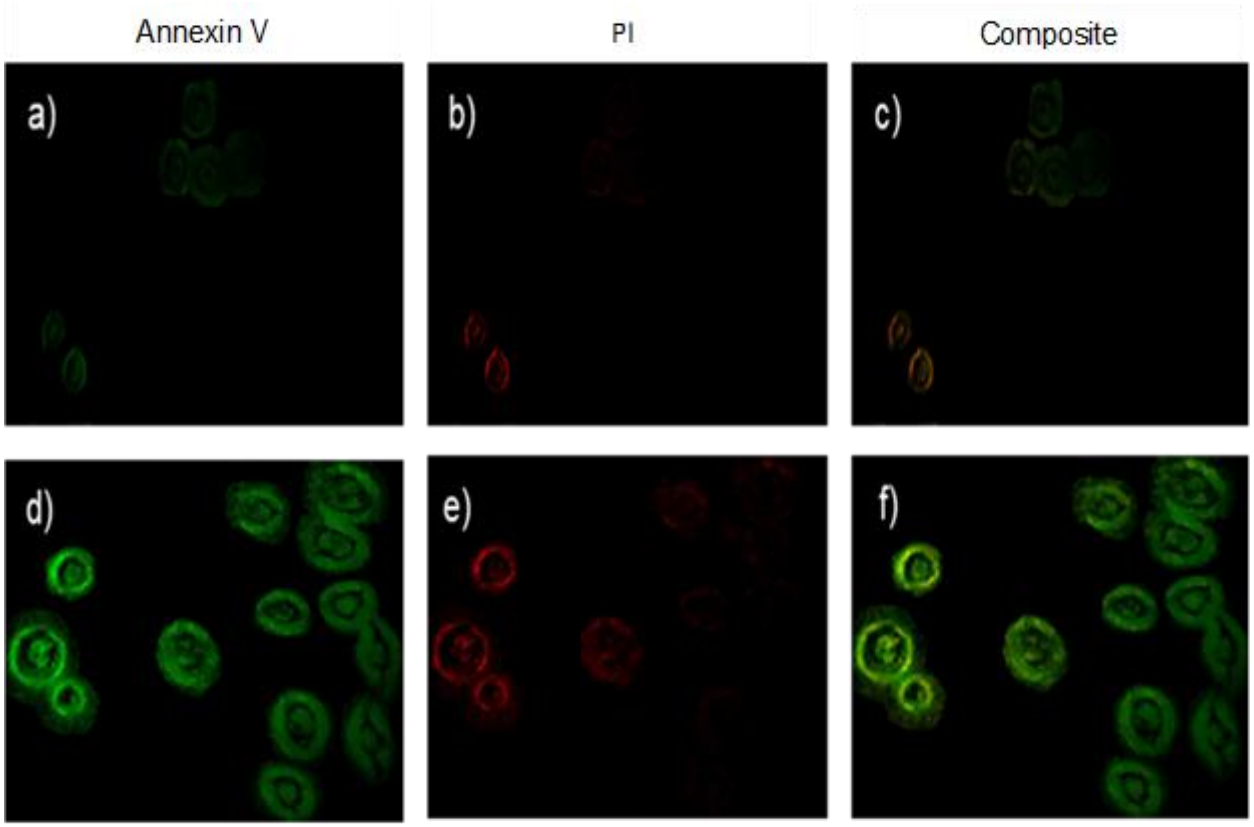

Figure 4.9. The fluorescence micrographs obtained following the 24-hour post cannabidiol treatment after staining with the nucleic acids' apoptosis-detecting stains Annexin-V and PI which emits green and red fluorescence respectively. The micrograph labelled **a)** & **b)** and **c)** Untreated ME-180 cells stained with Annexin V and PI respectively; **d)** & **e)** ME-180 cells counterstained with Annexin V and PI following the 24-hour exposure to cannabidiol compound; Hence **c)** and **f)** exhibit the composite micrographs of counterstained cells that emit the respective fluorescence of both stains employed.

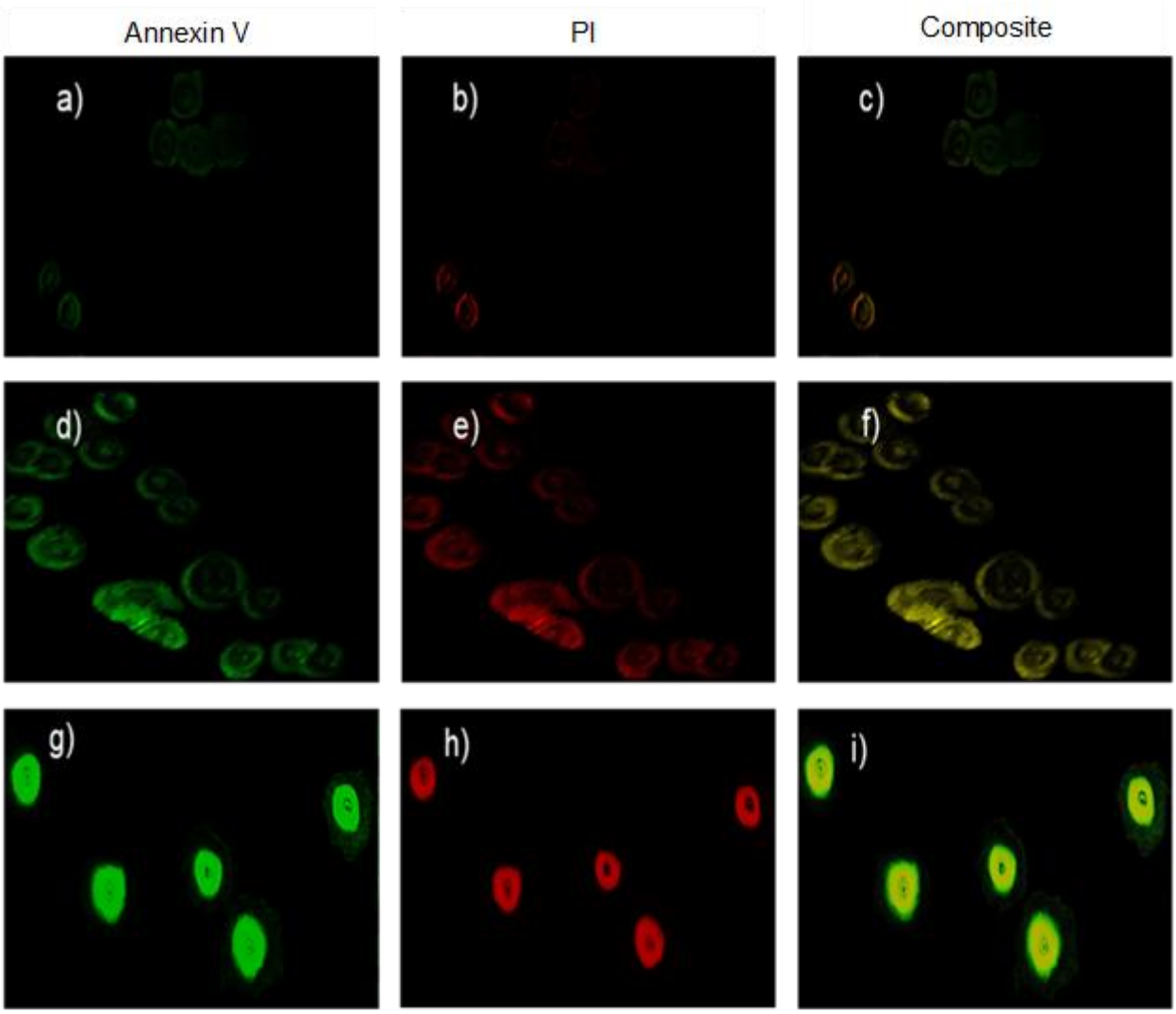

Figure 4.10. The fluorescence micrographs obtained following the 48-hour post TP53 gene manipulation and cannabidiol cotreatment after staining with the nucleic acids' apoptosis-detecting stains Annexin-V and PI which emits green and red fluorescence respectively. The micrograph labelled: **a)** & **b)** Untreated ME-180 cells stained with Annexin V and PI respectively; **c)** A composite micrograph of Annexin V and PI counterstained untreated ME-180 cells emitting fluorescence from both stains; **d)** & **e)** ME-180 stained cells with Annexin V and counterstain PI following the sip53-cannabidiol cotreatment; **f)** A composite micrograph of Annexin V and PI counterstained ME-180 cells emitting fluorescence from both stains following the sip53-cannabidiol cotreatment **g)** & **h)** ME-180 human cervical cancer cells counterstained with Annexin V and PI following the 48-hour cotreatment of TP53 overexpression and cannabidiol compound; **i)** A composite micrograph of the counterstained ME-180 cells cotreated with p53 and cannabidiol compound; Hence **c), f)** & **i)** exhibit the composite micrographs of counterstained cells that emit the respective fluorescence of both Annexin V and PI stains. The visuals were taken from the fluorescence microscope and analyzed using ImageJ software.

CHAPTER 5: DISCUSSION AND CONCLUSSION

5.1 Cytotoxicity analysis

In the present study, MTT assay was utilized to evaluate cytotoxicity induced by compound cannabidiol on the ME-180 human cervical carcinoma cells. Different concentrations of 0.001; 0.1; 0.25; 1.5; 2.0 and 3.0 µg/ml were used for cytotoxicity evaluation and the MTT reduction assay was conducted in replicates with the p-value of below 0.05 regarded significant. We noted that the cell viability obtained from the treatment of respective cannabidiol concentrations (in **Figure 4.1**), concentration of 3 µg/ml of cannabidiol implied to be the recommended inhibitory concentration with the estimated cell viability of 51.5%. We further noticed that the cell viability was significantly higher with 76.5% when the cells were subjected to 0.001 µg/ml cannabidiol. Furthermore, cannabidiol compound was distinguished to significantly inhibit the viability of metastatic cervical cancer cells highly at concentration of 3.0 µg/ml with resulted percentage viability of 51.5%.

However, the cytotoxicity of the cannabidiol on the metastatic human cervical cancer was noticed to be concentration dependent. Thus, in the present study cannabidiol seemed to be more cytotoxic at the higher concentrations used but rather less cytotoxic at lower concentrations. The treatment of the cells with methanol as the negative control at a concentration of 0.3% has gained the highest cell viability of approximately 80% suggesting that the cytotoxicity induced in the study was not as a result of methanol solvent in cannabidiol compound, specifically the methanol tends to exhibit no effect on viability of cervical cancer cells in humans as a sole treatment. As expected the treatment of the cells with 0.3µM Camptothecin has resulted in low cell viability. The Camptothecin is known to be the effective cytotoxic and antiproliferative compound in various cancer cell types (Venditto and Simanek, 2010).

5.2 Elucidated effects induced by the p53 upregulation, cannabidiol and p53-cannabidiol cotreatment on expression of cell cycle genes p53, MDM-2 and CDK2 in ME-180 cervical cancer cells.

During malignant tumorigenesis cell division of cells with mutated genome proceed to take place leading to the uncontrolled cell proliferation of malignant neoplasia (Satyanarayana et al., 2008). The significance of the successful cell division is to maintain and safeguard the integrity of the genome (Palou et al., 2010). Therefore, the cell division is the most delicate process to be monitored and considered in development of anticancer therapeutics. In present study, cell proliferation was evaluated through the expression analysis of the cell cycle genes CDK2, MDM2 and p53 by real time PCR. Remarkably, MDM2 gene functions as negative regulator of tumour suppressor p53 that induces either cell death, halting of cell division or the DNA repair to maintain content cell division (Kiraz et al., 2016).

Quantification of cell cycle genes is important in advancement of anticancer therapy. In present study we realized that cannabidiol treated cells increased expression of p53 in ME-180 cells than that of the control and the MDM2 gene. As anticipated, the levels of MDM2 as negative regulator of p53 was decreased by p53 upregulation, cannabidiol compound and the administration of the p53-cannabidiol cotreatment in the ME-180 cells (**Figure 4.3, Figure 4.4** & **Figure 4.5**). The inhibition of MDM2 in the cells allow the expression of TP53 tumour suppressor gene which facilitate the transcription of the downstream proteins such as p21 to inhibit the activity of CDKs (including CDK2) and association of CDKs and cyclins (such as CDK2/cyclin-E association) subsequently leading to halt of cell division at G1-S transition (Bačević et al., 2017). From the current study, we recognized that the silencing of TP53 gene in the cancer cells seem to induce the upregulation of the negative regulator MDM2 (**Figure 4.4**) inferring the sip53 as the agonist to the cervical carcinoma therefore not to be considered in any development of anticancer therapy.

Due to the knockdown of MDM2, the expression levels of p53 was elevated through restoration of the tumour suppressor gene in the ME-180 cells which could've been intensified by both p53 overexpression in the cells and administration of cannabidiol treatment to the cells (**Figure 4.3** & **Figure 4.4**). Moreover, p53 gene was highly expressed in the cotreated cells as compared to the other treatments implicating the p53-cannabidiol cotreatment as the most reliable form of therapy for restoration of p53 in the primary metastatic cervical cancer cells (**Figure 4.5**).

The previous studies reported that the expression of p53 activity diminishes the association of the CDKs and cyclins thereby arresting the progression of the cell division that is mostly cell cycle termination is associated with the loss-of-function of CDKs' activity. The CDK2 expression induces the association of CDK2 and cyclins facilitating the phosphorylation of cyclins thereby activating the cyclin functioning to allow the advancement of cell division. As a result, we assessed the expression of the CDK2 following the cannabidiol administration and the p53-cannabidiol cotreatment in the cervical cancer cells. From **Figure 4.5,** we observed that the p53-cannabidiol cotreatment downregulate the CDK2 expression in the ME-180 cells. The acquired results suggest that the respective downregulation of CDK2 could have been associated with the p53 stabilization caused by either cannabidiol treatment or the elevation of p53 expression through p53-cannabidiol cotreatment consequently halting the cell cycle progression. Moreover, these results suggest that the cotreatment of p53-cannabidiol on cervical cancer cells mediate the inhibition of cervical tumorigenesis through the p53 restoration and stabilization by diminishing the MDM2-mediated ubiquitination and degradation in the cells.

Generally, the arrest of cell cycle is recommended for discovery of cancer treatment since the cell cycle arrest allows the adequate period for DNA repair and stimulation of cell death in particular the programmed cell death in carcinoma cells. Therefore, stimulation of cell cycle arrest by the targeted therapy is highly recommended.

5.3 Mode of cell death analysis

In most cases, the tumorigenesis develops as a result of the cells' immortalization. Cell death is one of mechanisms required in cell cycle to inhibit the division of unhealthy cells such as aging cells and cells with mutated and damaged DNA. Typically, the recommended mode of cell death to regulate human carcinogenesis is apoptosis which is characterized by the translocation of phospholipid phosphatidylserine residues, caspase activation and the breakdown of the nuclear DNA in the cells (Shrivastava et al., 2011).

Activation and involvement of caspase pathway has known to be the hallmark of apoptosis for ages (Pu et al., 2017). Caspases, especially the executioner caspases are usually quantified in cellular assays to measure the amount of apoptosis induced in the *in vitro* and *in vivo* studies especially when investigating the anticancer properties of certain pharmacological drug administered for development of therapeutics for research purposes (Bell and Megeney, 2017). Currently we demonstrated the type of cell death stimulated by several treatments through quantification of effector caspase-3 and -7 as biomarkers of apoptosis.

Response of caspase activity in the ME-180 cells remains treatment dependent and from the current study conducted, we revealed that activity of executioner caspase 3 and 7 is highly increased by the cotreatment of cannabidiol compound and p53 upregulation. This data implicates that the p53-cannabidiol cotreatment elicited apoptosis in ME-180 cells through activation of caspase pathway specifically by exploiting the executioner caspase 3 and 7. Despite the moderate activity of caspases from the cells treated with cannabidiol and TP53 separately, the activity of effector caspases has decreased significantly in the cells with silenced p53 only and silenced p53 together with cannabidiol cotreatment thus implying that the apoptosis was not induced by the treatments that involved any of the knockdown of p53 in the cells. Our data supports the findings that expression levels of p53 as tumor suppressor protein in ME-180 cells respond positively to the cannabidiol treatment of approximately 3.4µg/ml by inducing apoptosis as potential target for development of anticancer therapeutics (Lukhele and Motadi, 2016).

The recruitment of effector caspases which eventually lead to execution of the intracellular proteins following the caspase cascade whereby in the current study the effector caspase 3 and 7 are recruited and exploited is very vital for the accomplishment of apoptosis pathway. The recruitment of caspase cascade is stimulated by the activation of procaspases through cleavage by the binding of APAF-1 and ATP subsequently forming the complex apoptosome. Along the caspase cascade the apoptosome complex as the activator thereof initiate the series of caspase recruitments including the executioner caspases to complete the apoptosis process.

Apoptotic cell death is not only presented by the increase in activity of caspases in cells, consequently in present study DNA fragmentation (**Figure 4.7**) was conducted to attest the apoptosis

induced by the ideal targeted therapy in human cervical cancer cells. According to Matassov *et al*, (2004), the cells undergoing apoptosis produces the nucleosomal DNA fragments of between 180-200 bp following the endonuclease cleavage of chromatins. From the study we observed that there was no DNA fragmentation present from the untreated cells and the cells treated with sip53 only. The slight DNA fragmentation was observed from cells treated with sip53+cannabidiol and this is assumed to be as a result of the cannabidiol compound present in the cells. Conversely, the cells treated with cannabidiol, TP53 and the cotreatment TP53+cannabidiol have shown the increased nucleosomal DNA fragmentation of the fragment estimated to 200bp with the p53-cannabidiol cotreated cells exhibiting the highest nucleosomal DNA fragmentation. Hence the nucleosomal fragment produced by these treatments demonstrate the apoptotic bodies generated by respective treatments thereof.

5.4 Morphology analysis

Since the nineteenth century, scientists discovered that the apoptotic pathway produces the specific phenotypic morphological properties on the cells that can be distinguished from other modes of cell death and live cells. While there are other techniques that can be used to detect apoptosis in molecular biology, microscopy is only technique that can be used for assessing the morphology of the cells undergoing apoptosis. There are different events that occurs during the apoptotic pathways and these events elicit the alterations on the morphology of the cells.

Morphological analysis of the cells have been one of the accurate techniques to confirm the viability of the cells and in some instances to examine the effect induced by treatment on the cells and this technique acquire the microscopic implementation for visualization and analysis (Radogna et al., 2015). Thus, in activation of apoptotic cell death, the cells undergo the morphological changes that are usually detected in microscopy. There are several phenotypic features that are observed to differentiate the live cells from the cells undergoing apoptosis or any other form of cell death. Among other morphological features shown by apoptotic cells, loss of plasma membrane symmetry features occurs usually at the early stages of apoptosis leading to the translocation of the phospholipid phosphatidylserine from the inner plasma membrane to the outer cellular environment (Riccardi and Nicoletti, 2006). The externalized phosphatidylserine molecules then attract the binding of Annexin V proteins implicating the mode of cell death as apoptotic. Therefore, the binding and staining of Annexin V on the cells signals the loss of plasma membrane integrity thereby implicating apoptosis as the induced programmed cell death in the cells regardless of the treatment applied to the cells. In current study the recruitment of Annexin V and counterstain PI in fluorescence microscopy were used to assess morphological features of metastatic cancer cells to substantiate form of cell death induced by treatments exploited. The fluorescence microscopy showed the distinct morphologies post the individual treatments and cotreatments of the cells. The morphological properties of the cells form one of many characteristics of the cell death including the apoptotic cell death. The distinct

properties indicated by the cells following different treatments confirmed the apoptotic cell death induced in the cells co-treated with p53 and cannabidiol.

5.5 Overall effect of the implemented p53-cannabidiol cotreatment on the ME-180 cervical cancer cells.

Although our findings suggest that the metastatic human cervical carcinoma-derived cell line is sensitive to both cannabidiol and p53-cannabidiol cotreatment, the ME-180 cells emerge to be more susceptible to cotreatment than to the individual treatment of cannabidiol. In spite the apoptotic cell death induced by cotreatment we further discovered that the cotreatment implemented in the study didn't only induced apoptosis but was able to halt cell proliferation in metastatic cervical carcinoma-derived cell line. The programmed cell death induced by the potentially validated cotreatment was confirmed through the visualization and analysis of microscopic morphology of the cells. The visualization of ME-180 cells under the fluorescence microscope clarified the observed morphology as that of the cells undergoing apoptosis. From the **Figure 4.9 d)-f)** and **Figure 4.10 g)-i)**, the observed fragmentation of nucleosomal DNA, cell shrinkage, membrane blebbing, detachment of the cells from the adjacent cells and the apoptotic bodies demonstrated the apoptotic cell death induced by both the 3.0µg/ml cannabidiol treatment solely and p53-cannabidiol cotreatment. Our data suggest that co-treating the cells with p53 and the compound cannabidiol induces the efficient mode of cell death that is preliminary consistent in the anticancer therapy development. The morphology analysis was not only confirming the apoptosis induction but the detection of the apoptosis presence at both the early and later stages was identified by the observed staining of Annexin V and the PI counterstain from microscopy (**Figure 4.8**, **4.9** and **4.10**). Hence Annexin V and PI only penetrate the cells with poor membrane integrity undergoing apoptosis.

The antiproliferative effects of p53, cannabidiol and p53-cannabidiol cotreatment were detected from the acquired results at the cannabidiol concentration of 3µg/ml for period of 24 hour-treatment and 48-hour cotreatment **(Figure 4.3).** The apoptotic cell death elicited by the p53 and cannabidiol treatments as well as the p53-cannabidiol cotreatment was elucidated in the current study through the morphology analysis and the DNA fragmentation obtained from the study. However, the 48-hour p53-cannabidiol co-treatment dose induced more apoptosis than the individual treatment of cannabidiol and p53 obtaining the highest caspase activity of 49 723 RLU (**Figure 4.6**).

Nucleosomal DNA fragmentation and quantification of cell cycle genes indicate that p53-cannabidiol cotreatment induces apoptosis and arrest cell cycle in ME-180 cells. The data implicate that the combinational therapy of p53 expression and cannabidiol mark the potent therapeutic platform for treatment of metastatic human cervical carcinoma.

From this study, CBD is found to be one of the apoptotic natural agents at IC_{50} of 3µg/ml. Although CBD showed to possess apoptotic properties, in this study it was more effective when used in

combination with TP53 gene expression which interestingly showed the desired results. Targeting TP53 as a sole therapy for treatment of cervical carcinoma halt cell growth and induces apoptosis in metastatic cervical carcinoma cells, however from this study it is certain that therapy is not as effective as when implemented concurrently with CBD compound. That is according to the data collected from the study, the p53-CBD cotreatment is elucidated to be the potential endogenous therapeutic strategy for treatment of cervical cancer especially the metastatic ME-180 cells.

5.6 Conclusions and recommendations.

The p53 gene therapy is now in the clinical trials for treating other carcinomas (Chen et al., 2014), thus the therapy in combination with other natural compound would develop the efficient cotreatment to eradicate cervical cancer without causing any harm to the patients. The results from the current study demonstrated that the p53-cannabidiol cotreatment does not only upregulate the TP53 expression in the ME-180 cells but also knockdown the negative regulator MDM2 of tumour suppressor p53 in the cervical cancer cells consequently arresting cell cycle to let the apoptotic cell death to result.

This data implicates that the p53-cannabidiol cotreatment is the ideal therapeutic tool with full potency to treat the metastatic human cervical cancer. Although the p53-cannabidiol cotreatment is considered the potential therapeutic biomarker for treating cervical cancer, the mechanisms in which this cotreatment implement to eradicate cancer is yet to be studied and explicated for fully understanding. Moreover, expression of other genes implemented in cell division and apoptosis need to be studied for further elucidation of the cotreatment as biomarker for cervical cancer therapy. The investigation still needs to be taken for the *in vivo* studies for solid validation of the cotreatment being the pursued tool in developing the anticancer therapeutic for metastatic cervical cancer.

CHAPTER 6: REFERENCES

Abbady, A.Q., Twair, A., Ali, B. and Murad, H. (2017). Characterization of Annexin V Fusion with the Superfolder GFP in Liposomes Binding and Apoptosis Detection. *Front. Physiol. 8*, 317. doi: 10.3389/fphys.2017.00317.

Amaral, J. D., Xavier, J. M., Steer, C. J., and Rodrigues, C. M. (2010). The role of p53 in apoptosis. *Discovery Medicine*, *9*(45), 145-152.

Anita, Sharma, H. P., Jain, P., and Amit, P. (2014). Apoptosis (Programmed Cell Death)-A Review. *World Journal of Pharmaceutical Research*, *3*(4), 1854-1872.

Arbyn, M., Castellsague, X., de Sanjose, S., Bruni, L., Saraiya, M., Bray, F., Ferlay, J. (2011). Worldwide burden of cervical cancer in 2008. Annals of Oncology 22: 2675–2686. Doi:10.1093/annonc/mdr015.

Bačević, K., Lossaint, G., Achour, T.N., Georget, V., Fisher D., Dulić, V. (2017). Cdk2 strengthens the intra-S checkpoint and counteracts cell cycle exit induced by DNA damage. *Scientific Reports,* 7, 13429. DOI:10.1038/s41598-017-12868-5.

Baig, S., Seevasant, I., Mohamad, J., Mukheem, A., Huri, H. Z., and Kamarul, T. (2016). Potential of apoptotic pathway-targeted cancer therapeutic research: Where do we stand? *Cell Death and Disease, 7*, e2058. doi:10.1038/cddis.2015.275

Baldi, A., De Luca, A., Esposito, V., Campioni, M., Spugnini, E.P., Citro, G. (2011). *Review article*: *Tumor Suppressors and Cell-Cycle Proteins in Lung Cancer.* Vol, 2011, 1-12.

Bank, S., Maiti, S., and Sinha, A. K. (2017). Apoptosis in Neurodegenerative Disease, Myocardial Infarction and in Cancer. *J Cell Sci Apo,* 1, 107.

Barnum, K. J., and O'Connell, J. M. (2014). Cell Cycle Regulation by Checkpoints. *Methods Mol Biol*. 2014; 1170: 29–40. doi:10.1007/978-1-4939-0888-2_2.

Bell, R., and Megeney, L.A. (2017). Evolution of caspase-mediated cell death and differentiation: twins separated at birth*. Cell Death and Differentiation*,*24*, 1359–1368.

Bergamaschi, M. M., Queiroz, R. H. C., Crippa, J. A. S., and Zuardi, A. W. (2011). Safety and side effects of Cannabidiol, a *Cannabis sativa* constituent. *Current Cancer Therapy, 6* (4).

Bernard, B., Fest, T., PreÂtet, J-L. and Mougin, C. (2001). Staurosporine-induced apoptosis of HPV positive and negative human cervical cancer cells from different points in the cell cycle. *Cell Death and Differentiation, 8*, 234-244.

Berridge, M.J. (2014). Cell Signalling Biology. doi:10.1042/csb0001009.

Besson, A., Dowdy, S. F. Roberts, J. M. (2008). CDK Inhibitors: Cell Cycle Regulators and Beyond. *Developmental Cell, 14* (2), 159–169. doi.org/10.1016/j.devcel.2008.01.013.

Bruni, L., Barrionuevo-Rosas, L., Albero, G., Serrano, B., Mena, M., Gómez, D., Muñoz, J., Bosch, F. X., de Sanjosé, S. (2017). ICO Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in South Africa. *Summary Report* 27 July 2017.

Chappell, W. H., Lehmann, B. D., Terrian, D. M., Abrams, S. L., Steelman, L. S., and McCubrey, J. A. (2012). p53 expression controls prostate cancer sensitivity to chemotherapy and the MDM2 inhibitor Nutlin-3. *Cell Cycle 11*(24), 4579–4588.

Chen, G., Zhang, S., He, X., Liu, S., Ma, C., and Zou, X. (2014). Clinical utility of recombinant adenoviral human p53 gene therapy: current perspectives. *Onco Targets and Therapy, 7*, 1901–1909.

Choene, M., and Motadi, L. (2016). Validation of the Antiproliferative Effects of Euphorbia tirucalli Extracts in Breast Cancer Cell Lines. *Molecular Biology, 50,* (1), doi: 10.1134/S0026893316010040

Colombo, N., Carinelli, S., Colombo, A., Marini, C., Rollo, D., and Sessa, C. (2012). Cervical cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, *23* (7) vii27-vii32. doi.org/10.1093/annonc/mds268.

Cragg, G.M., and Newman, D.J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology, 100*, 72–79.

Cupit-Link, M. C., Kirkland, J. L., Ness, K. K., *et al*. (2017). Biology of premature aging in survivors of cancer. ESMO Open, 2, e000250. doi: 10.1136/esmoopen-2017-000250.

Do, K., Doroshow, J. H., and Kummar, S. (2013). Wee1 kinase as a target for cancer therapy. *Cell Cycle, 12*(19): 3348-3353. doi.org/10.4161/cc.26062.

Dominguez-Brauer, C., Thu, K.L., Mason, J.M., Blaser, H., Bray, R. M., and Mak, T.W. (2015). Targeting mitosis in cancer: Emerging strategies. *Molecular Cell*, *60*, doi.org/10.1016/j.molcel.2015.11.006.

Duenas-Gonzalez, A., Serrano-Olvera, A., Cetina, L., Coronel, J. (2014). New molecular targets against cervical cancer. *International Journal of Women's Health*. (6) 1023-1031.

Duronio, R. J., Xiong, Y. (2013). Signaling Pathways that Control Cell Proliferation. *Cold Spring Harbor Perspectives in Biology, 5*(3), a008904. doi.org/10.1101/cshperspect.a008904

Ehtisham, M., Wani, F., Wani, I., Kaur, P., and Nissar, S. (2016). Polymerase Chain Reaction (PCR): Back to Basics. *Indian Journal of Contemporary Dentistry, 4*(2), 31-35. doi: 10.5958/2320-5962.2016.00030.9

Einsele-Scholz, S., Malmsheimer, S., Bertram, K., Stehle, D., Johänning, J., Manz, M., Daniel, P. T., Gillissen, B. F., Schulze-Osthoff, K., and Essmann, F. (2016). Bok is a genuine multi-BH-domain protein that triggers apoptosis in the absence of Bax and Bak. *Journal of Cell Science*,*129*, 2213-2223. doi:10.1242/jcs.181727

Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol. 35*(4): 495–516.

Fridman, J.S., and Lowe, S.W. (2003). Control of apoptosis by p53. *Oncogene. 22*, 9030–9040. Doi: 10.1038/sj.onc.1207116.

Gascoigne, K. E., and Taylor, S. S., (2009). How do anti-mitotic drugs kill cancer cells? *Journal of Cell Science 122*, 2579-2585. Doi:10.1242/jcs.039719

Gatti, R., Belletti, S., Orlandini, G., Bussolati, O., Dall'Asta, V., and Gazzola, G. C. (1998). Comparison of Annexin V and Calcein-AM as Early Vital Markers of Apoptosis in Adherent Cells by Confocal Laser Microscopy. *The Journal of Histochemistry Cytochemistry.* Volume *46*(8): 895–900.

Giarre, M., Caldeira, S., Malanchi, I., Ciccolini, F., Leao, M.J., Tommasino, M. (2001). Induction of pRb Degradation by the Human Papillomavirus Type 16 E7 Protein Is Essential to Efficiently Overlook $p16^{INK4a}$-Imposed G_1 Cell Cycle Arrest. *Journal of Virology, 75*(10): 4705-4712.

Gulati, K.H., Anand, M., Deshmukh, D.S. (2013). Squamous cell carcinoma of the uterine cervix extending to the corpus in superficial spreading manner and causing hematometra. 4(1): 63–64.

Gurtu, V., Kain, R. S., and Zhang, G. (1997). Fluorometric and Colorimetric Detection of Caspase Activity Associated with Apoptosis. *Analytical Biochemistry, 251*, 98–102. Article NO. AB972220.

Hengstermann, A., Linares, L. K., Ciechanover , A., Whitaker, N. J., and Scheffner, M. (2001). Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells. *PNAS, 98* (3), 1218–1223.

Hengstermann, A., Linares, K. L., Ciechanover, A., Whitaker, N. J., and Scheffner, M., (2001)l. Complete switch from Mdm2 to human papillomavirus E6-mediated degradation of p53 in cervical cancer cells. *PNAS, 98* (3): 1218-1223.

Henry, C. M., Hollville, E., and Martin, J. S., (2013). Measuring apoptosis by microscopy and flow cytometry. *Methods, 61* (2013) 90–97.

Hongmei, Z. (2012). Extrinsic and intrinsic apoptosis signal pathway review. *Apoptosis and Medicine, 1*, 3-22.

Hoste, G., Vossaert, K., Poppe, W.A.J. (2013). Clinical Role of HPV testing in primary and secondary cervical cancer screening. Volume 2013, 1-7. Article ID: 610373.

Hüttemann, M., Pecina, P., Rainbolt, M., Sanderson, T. J., Kagan, V. E., Samavati, L., Doan, J. W., and Lee, I. (2011). The multiple functions of cytochrome c and their regulation in life and death decisions of the mammalian cell: from respiration to apoptosis. *Mitochondrion*. *11*(3), 369–381. doi:10.1016/j.mito.2011.01.010.

Joshi, M., and Deshpande J.D. (2011). Polymerase Chain Reaction: Methods, principles and application. *International Journal of Biomedical Research, 1*(5), 81-97.

Jürgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., Reed, J. C. (1998). Bax directly induces release of cytochrome *c* from isolated mitochondria. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(9), 4997–5002.

Kabel, A. M., Adwas, A. A., Elkhoely, A. A., Abdel-Rahman, M. N., and Eissa, A. A. (2016). Apoptosis: Insights into Pathways and Role of p53, Bcl-2 and Sphingosine Kinases. *Journal of Cancer Research and Treatment, 4*, (4), 69-72. doi: 10.12691/jcrt-4-4-3.

Kalu, N. N., Mazumdar, T., Peng, S., and Shen, L., Sambandam, V., Rao, X., Xi, Y., Li, L., Qi, Y., Gleber-Netto, F. O., Patel, A., Wang, J., Frederick, M. J., Myers, J.N., Pickering, C. R., and Johnson, F. M. (2017). Genomic characterization of human papillomavirus-positive and -negative human squamous cell cancer cell lines. *Oncotarget, 8*, (49), pp: 86369-86383.

Kastan, M. B., and Bartek, J., (2004). Cell-cycle checkpoints and cancer. *Nature*: VOL 43218. www.nature.com/nature.

Kavya, S. R. (2015). PCR Technique with its Application. *Research and Reviews: Journal of Microbiology and Biotechnology, 4*, 1.

Kijima, M., and Mizut, R. (2019). Histone H1 quantity determines the efficiencies of apoptotic DNA fragmentation and chromatin condensation. *Biomedical research (Tokyo), 40* (1), 51–56.

Kiraz, Y., Adan, A., Yandim, K. M., and Baran, Y. (2016). Major apoptotic mechanisms and genes involved in apoptosis. *Tumor Biol, 37*:8471–8486. doi 10.1007/s13277-016-5035-9

Krämer, C. E. M., Wiechert, W., and Kohlheyer, D. (2016). Time-resolved, single-cell analysis of induced and programmed cell death via non-invasive propidium iodide and counterstain perfusion. *Scientific Reports, 6*, 32104. doi: 10.1038/srep32104.

Kumar, S. (2004). Measurement of Caspase Activity in Cells Undergoing Apoptosis. *Methods in Molecular Biology, 282,* 19-30.

Kumar, S., Jena, L., Galande, S., Daf, S., Mohod, K., Varma, A.K. (2014). Elucidating Molecular Interactions of Natural Inhibitors with HPV-16 E6 Oncoprotein through Docking Analysis. *Genomics Inform. 12*(2):64-70.

Kurien B.T., and Scofield R.H. (2015). Electrophoresis – Blotting Techniques. In: Reedijk, J. (Ed.) *Elsevier Reference Module in Chemistry, Molecular Sciences and Chemical Engineering. Waltham, MA: Elsevier*. 24-Apr-15 doi: 10.1016/B978-0-12-409547-2.11157-6.

LaVigne, A. W., Triedman, S.A., Randall, T. C., Trimble, E. I., Viswanathan, A. N., (2017). Cervical cancer in low- and middle-income countries: addressing barriers to radiotherapy delivery. *Gynecologic Oncology Reports* 22: 16–20.

Li, F., Jiang, T., Li, Q., and Ling, X. (2017). Review Article Camptothecin (CPT) and its derivatives are known to target topoisomerase I (Top1) as their mechanism of action: did we miss something in CPT analogue molecular targets for treating human disease such as cancer? *Am J Cancer Res, 7*(12), 2350-2394.

Liu, Y., Li, W., Moris-Natschke, S. L., et al. (2015). Perspectives of biologically active Camptothecin derivatives. *Med Res Rev, 35*(4), 753-789. doi: 10.1002/med.21342.

Lukhele, S., and Motadi, L. R. (2016). Cannabidiol rather than *Cannabis sativa* extracts inhibit cell growth and induce apoptosis in cervical cancer cells. *BMC Complementary and Alternative Medicine*16:335 doi 10.1186/s12906-016-1280-0.

Martinez, M. M., Reif, R. D., and Pappas, D. (2010). Detection of apoptosis: A review of conventional and novel techniques. *Analytical Methods, 2,* 996–1004. doi: 10.1039/c0ay00247j.

Martínez-Flores, K., Salazar-Anzures, T. A., Fernández-Torres, J., Pineda, C., Aguilar-González, C. A., López-Reyes, A. (2017). Western blot: a tool in the biomedical field. Vol. 6, No. 3 pp 128-137.

Matassov, D., Kagan, T., Leblanc, J., Sikorska, M., and Zakeri, Z. (2004) .Measurement of Apoptosis by DNA Fragmentation. *Methods in Molecular Biology, 282*. doi: 10.1385/1-59259-812-9:001.

Matheson, C. J., Backos, D. S., and Reigan, P. (2016). Targeting WEE1 kinase in cancer. *Trends in Pharmacological Sciences, 37*(10) 872-881. http://dx.doi.org/10.1016/j.tips.2016.06.006.

McAllister, S. D., Christian, R. T., Horowitz, Garcia, A., and Desprez, P. (2007). Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells. *Mol Cancer Ther*, *6*(11), 2921–7.

Merghoub, N., Btaouri, H. E., Benbacer, L., Gmouh, S., Trentesaux, C., Brassart , B., Attaleb, M., Madoulet, C., Wenner , T., Amzazi, S., Morjani, H., and Mzibri, M. E. (2017). Tomentosin Induces Telomere Shortening and Caspase-Dependant Apoptosis in Cervical Cancer Cells. *Journal of Cellular Biochemistry, 118*,1689–1698. DOI 10.1002/jcb.25826

Moela, P., and Motadi, L. R. (2016). RBBP6: a potential biomarker of apoptosis induction in human cervical cancer cell lines. *OncoTargets and Therapy*, *9*, 4721–4735. http://doi.org/10.2147/OTT.S100964.

Moela, P., Choene, M.S., Lesetja R. Motadi, L. R. (2014). Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitises breast cancer cells MCF7 to staurosporine and Camptothecin-induced cell death. *Immunobiology*, 219, 593–601. doi: 10.1016/j.imbio.2014.03.002.

Mohammed, S., Birhan, G., Admassu, B., Shite, A., and Yeneneh, H. (2015). Review on Polymerase Chain Reaction and its Diagnostic Merit Over Conventional Techniques in Animal Disease. *African Journal of Basic & Applied Sciences, 7* (5), 262-281. doi: 10.5829/idosi.ajbas.2015.7.5.95294.

Moodley, M. (2009). Cervical cancer in Southern Africa: The challenges. *SA Journal of Gynaecological Oncology*. 1(1)

Moudi, M., Go, R., Yien, C. Y. S., Nazre, M., (2013). Vinca Alkaloids. *International Journal of Preventive Medicine*, *4*(11), 1231–1235

Munger, K., and Howley, P. M. (2002). Human papillomavirus immortalization and transformation functions. *Virus Research, 89*(2), 213-28.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T.T., Liu, B., and Bao, J. K., (2012). Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Prolif., 45,* 487–49. doi: 10.1111/j.1365-2184.2012.00845. x

Palou, G., Palou, R., Guerra-Moreno, A., Duch, A., Travesa, A., and Quintana, D. G. (2010). Cyclin Regulation by the S Phase Checkpoint. *J. Biol. Chem,* 285: 26431-26440. doi: 10.1074/jbc.M110.138669

Peyressatre, M., Prével, C., Pellerano, M., and Morris , M. C. (2015). Targeting Cyclin-Dependent Kinases in Human Cancers: From Small Molecules to Peptide Inhibitors. *Cancers*, 7, 179-237. doi:10.3390/cancers7010179

Pfeffer, C.M., and Singh, A.T.K. (2017). Apoptosis: A Target for Anticancer Therapy. *International Journal of Molecular Sciences, 19,* 448. doi:10.3390/ijms19020448

Pisanti, S., Malfitano, A. M., Ciaglia, E., Lamberti, A., Ranieri, R., Cuomo, G., Abate, M., Faggiana, G., Proto, M. C., Fiore, D, Laezza, C., and Bifulco, M. (2017). Cannabidiol: State of art and new challenge for therapeutic challenges. *Pharmacology & Therapeutics 175*, 133–150, http://dx.doi.org/10.1016/j.pharmthera.2017.02.041

Potapova, T.A., Sivakumar, S., Flynna, J.N., Lib, R., and GorbskY, G.J. (2011). Mitotic progression becomes irreversible in prometaphase and collapses when Wee1 and Cdc25 are inhibited. *Molecular Biology of the Cell*, 22, 1191-1206

Prenek, L., Boldizsár, F., Kugyelka, R., Ugor, E., Berta, G., Németh, P., and Berki, T. (2017). The regulation of the mitochondrial apoptotic pathway by glucocorticoid receptor in collaboration with Bcl-2 family proteins in developing T cells. *Apoptosis 22*, 239–253. doi 10.1007/s10495-016-1320-8

Pu, X., Storr, S. J., Zhang, Y., Rakha, E. A., Green, A. R., Ellis, I. O., Martin, S. G. (2017). Caspase-3 and caspase-8 expression in breast cancer: caspase-3 is associated with survival. *Apoptosis 22*, 357–368. doi 10.1007/s10495-016-1323-5

Radogna, F., Dicato M., and Diederich, M. (2015). Cancer-type-specific crosstalk between autophagy, necroptosis and apoptosis as a pharmacological target. *Biochemical Pharmacology 94*,1–11.

Ramer, R., Heinemann, K., Merkord, J., et al. (2013). COX-2 and PPAR-γ Confer Cannabidiol-Induced Apoptosis of Human Lung Cancer Cells. *Molecular Cancer Therapeutics, 12*, 69-82. doi: 10.1158/1535-7163.MCT-12-0335.

Rassol, M., Malik, A., Manan, A., Arooj, M., Qazi, M.H., Kamal, M.A., Sheikh I.A., Gan, S.H., Asif, M., and Naseer, M.I. (2015). Roles of Natural Compounds from Medicinal Plants in Cancer Treatment: Structure and Mode of Action at Molecular Level. *Med Chem*, 11(7):618-28.

Riccardi, C., and Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature protocols, 1*(3) 1458-1461. doi:10.1038/nprot.2006.238

Santosh, S., Rajagopalan, M. D., Pallavi, B. A., Rudramurthy, G. R., Rajashekar, V., Sridhar, K. A., and Swamy, M. K. (2018). Anticancer Plants: Chemistry, Pharmacology, and Potential Applications. *Cancer Therapies: Current Scenario, Management, and Safety Aspects* 1, DOI: 10.1007/978-981-10-8548-2_21.

Satyanarayana, A., Hilton, M. B., and Kaldis, P. (2008). p21 Inhibits Cdk1 in the Absence of Cdk2 to Maintain the G1/S Phase DNA Damage Checkpoint. *Molecular Biology of the Cell*, 19, 65–77.

Scheffner, M., Munger, K., Byrne, J.C., Howley, P.M. (1991). The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Journal of Medical Sciences*. 88: 5523-5527.

Schuler, M., and Green. D. R. (2001). Mechanisms of p53-dependent apoptosis. *Biochemical Society Transactions*. *29* (6).

Segurado, M., and Tercero, J.A. (2009). The S-phase checkpoint: targeting the replication fork. *Biol. Cell*, *101*(11), 617–627. doi:10.1042/BC20090053.

Sen, G. S., Mohanty, M., Hossain, D. S., Bhattacharyya, S., *et al*. (2011). Curcumin Enhances the Efficacy of Chemotherapy by tailoring p65NFκB-p300 Cross-talk in Favor of p53-p300 in Breast Cancer. *Journal of biological chemistry*. *286*(49): 42232–42247. doi 10.1074/jbc.M111.262295.

Shrivastava, A., Kuzontkoski, P. M., Groopman, J. E., and Prasad, A. (2011). Cannabidiol Induces Programmed Cell Death in Breast Cancer Cells by Coordinating the Cross-talk between Apoptosis and Autophagy. *Molecular Cancer Therapeutics, 10*(7), 1161–72. doi: 10.1158/1535-7163.MCT-10-1100

Śledziński, P., Zeyland, J., Słomski, R., & Nowak, A. (2018). The current state and future perspectives of cannabinoids in cancer biology. *Cancer Medicine, 7*(3), 765–775. doi: 10.1002/cam4.1312

Stanton, R. A., Gernert, K. M., Nettles, J.H., Aneja, R. (2011). Drugs That Target Dynamic Microtubules: A New Molecular Perspective. *International Journal of Preventive Medicine*, 31(3): 443–481.

Stellwagen, N. C. (2009). Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. *Electrophoresis, 30,* S188–S19. doi 10.1002/elps.200900052.

Sullivan, K.D., Galbraith, M. D., Andrysik, Z., and Espinosa, J. M. (2017). Mechanisms of transcriptional regulation by p53. *Cell Death and Differentiation advance*, doi:10.1038/cdd.2017.174

Takayama, S., Reed, J. C., and Homma, S. (2003). Heat-shock proteins as regulators of apoptosis. Oncogene *22*, 9041–9047. doi: 10.1038/sj.onc.1207114.

Tamiru Y, Abebe N and Kebede A. (2017). Review on mechanisms of regulating apoptosis in animal cells. World Journal of Biomedicine and Pharmaceutical Sciences 2: 33-38. https://doi.org/10.5281/zenodo.837832.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., Kaur, H. (2011). Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Sciencia*,1(1).

Valones, M.A.A., Guimarães, R. L., Brandão, L. A. C., de Souza, P. R. E., de Albuquerque, A., Carvalho, T., and Crovela, S. (2009). Principles and applications of polymerase chain reaction in medical diagnostic fields: A review. *Brazilian Journal of Microbiology, 40*, 1-11.

van den Heuvel, S. (2005). Cell-cycle regulation. WormBook, ed. *The C. elegans Research Community, WormBook,* doi/10.1895/wormbook.1.28.1, http://www.wormbook.org.

Velasco, G., Sánchez, C., and Guzmán M. (2016). Anticancer mechanisms of cannabinoids. *Current Oncology , 23*(2), 23-32. DOI: http://dx.doi.org/10.3747/co.23.3080.

Venditto, V. J., and Simanek, E. E. (2010). Cancer Therapies Utilizing the Camptothecin: A Review of the in Vivo Literature. *Molecular pharmaceutics, 7*(2), 307–349.

Visconti, R., Monica, R. D., and Grieco, D. (2016). Cell cycle checkpoint in cancer: a therapeutically targetable double-edged sword. *Journal of Experimental & Clinical Cancer Research, 35*:153. doi 10.1186/s13046-016-0433-9.

Vishnoi, K., Mahata, S., Tyagi, A., Pandey, A, Verma, G., Jadli, M., Singh, Singh, S. M. and Bharti, A. C. (2016). Cross-talk between Human Papillomavirus Oncoproteins and Hedgehog Signaling Synergistically Promotes Stemness in Cervical Cancer Cells. *Scientific Reports, 6*, 34377. doi: 10.1038/srep34377.

Wang, C., and Youle, R. J. (2009). The role of mitochondria in apoptosis. *Annu Rev Genet*, *43*, 95–118. doi:10.1146/annurev-genet-102108-134850.

Wang, X., Simpson, E. R., and Brow, K.A. (2015). p53: Protection against Tumor Growth beyond Effects on Cell Cycle and Apoptosis. *Cancer Res 75*, 5001-5007.

Wang, Z., and Sun, Y. (2010). Targeting p53 for novel anticancer therapy. *Translational oncology, 3*(1), pp. 1–12.

Weber, T.S., Jaehnert, I., Schichor, C., Or-Guil, M., and Carneiro, J. (2014). Quantifying the Length and Variance of the Eukaryotic Cell Cycle Phases by a Stochastic Model and Dual Nucleoside Pulse Labelling. *PLoS Comput Biol, 10*(7): e1003616. doi:10.1371/journal.pcbi.1003616.

Widowati, W., Mozef, T., Risdian, C., Yellianty, Y. (2013). Anticancer and free radical scavenging potency of *Catharanthus roseus, Dendrophthoe petandra, Piper betle* and *Curcuma mangga* extracts in breast cancer cell lines. *Oxid Antioxid Med Sci*, *2*(2):137-142

Wong, R. S. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental Clinical Cancer Research: CR*, *30*(1), 87. http://doi.org/10.1186/1756-9966-30-87.

Xiao, J., Zhou, J., Fu, M., Liang, L., Deng, Q., Liu, X., and Liu, F. (2017). Efficacy of recombinant human adenovirus-p53 combined with chemotherapy for locally advanced cervical cancer: A clinical trial. *Oncology letters, 13,* 3676-3680. doi: 10.3892/o1.2017.5901.

Xu, W., Mi, Y., He, P., He, S., and Niu, L. (2017). γ-Tocotrienol Inhibits Proliferation and Induces Apoptosis via the Mitochondrial Pathway in Human Cervical Cancer HeLa Cells. *Molecules, 22*, 1299. doi:10.3390/molecules22081299.

Yoshida, K. and Miki, Y. (2010), The cell death machinery governed by the p53 tumor suppressor in response to DNA damage. *Cancer Science, 101*: 831–835. doi:10.1111/j.1349-7006.2009.01488. x.

Zhu, H., Yang, K., Xie, Y., Lin, Y., Mao, Q., and Xie, L. (2013). Silencing of mutant p53 by siRNA induces cell cycle arrest and apoptosis in human bladder cancer cells. *World Journal of Surgical Oncology, 11*:22.

Zhuang, J., Ma, W., Lago, C. U., Hwang, P. M. (2012). Metabolic regulation of oxygen and redox homeostasis by p53: lessons from evolutionary biology? *Free Radical Biology Medicine*, *53*(6), 1279–1285. doi.org/10.1016/j.freeradbiomed.2012.07.026.

Zlatic, N. M., and Stankovic, M. (2015). Medicinal plants in the treatment of cancer. *Communications in plant science, 5*(3-4), 35-43.

ANNEXURES

ANNEXURE A: BUFFER AND REAGENTS PREPARATIONS

A1. DMEM growth medium

450ml	DMEM
45ml	FBS
5ml	Pen/strep

A2. 1X PBS

250ml	Autoclaved distilled water
1	Pellet of PBS

A3. 0.1% DEPC treated water

500µl	100% DEPC treated water.
500ml	Autoclaved distilled water.

A4. 10X TBE buffer

108g	Tris
55g	Boric acid
9.3g	EDTA

Add to 1L of autoclaved water.

ANNEXURE B

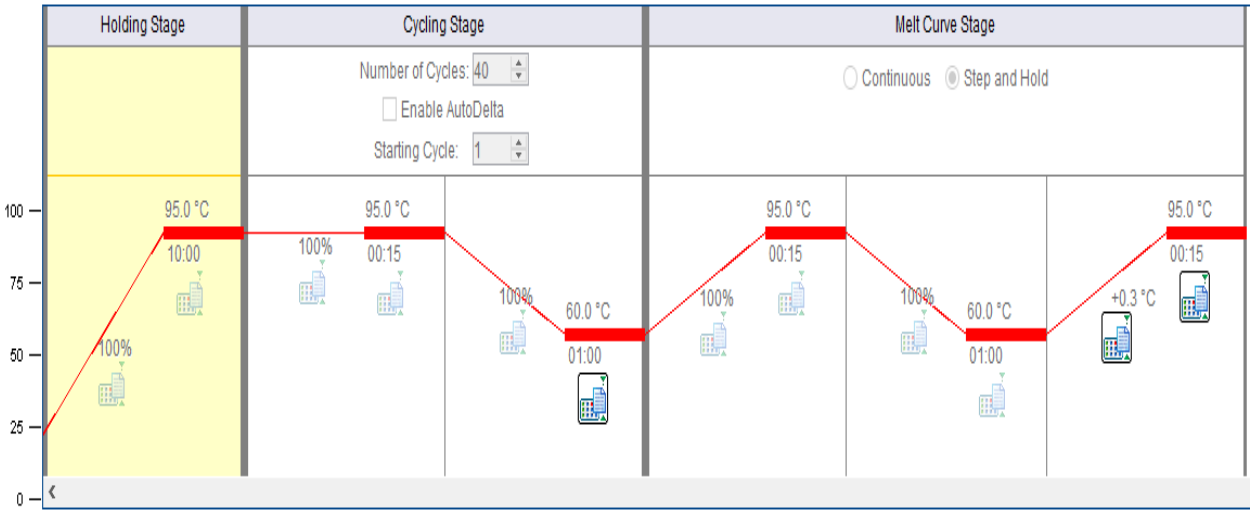

Figure B.1 Detailed conditions used to run the real time PCR assay.

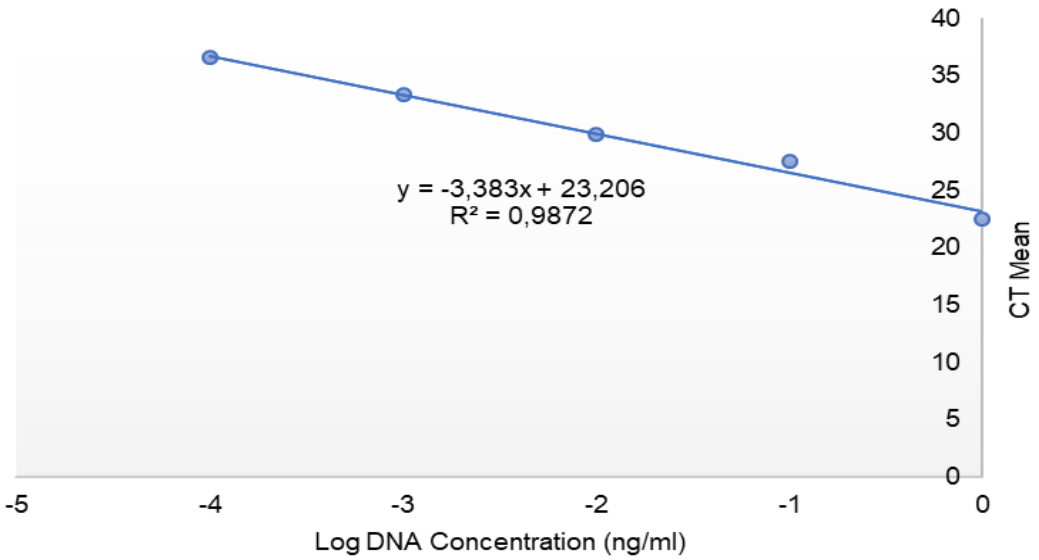

Figure B2. The standard curve generated for determination of DNA concentration.

The unknown concentrations of genes for particular treated cells where calculated using the equation from the standard curve as follows:

$$y = mx + b, \quad where\ y = CT\ value,\ m = slope\ of\ the\ regression\ line,\ x = unknown.$$

Hence,

$$Unknown\ DNA\ concentrattion = 10^{x}$$

Equation for amplification efficiency:

$E = 10^{\frac{-1}{slope}}$, where E is efficiency, and slope is the slope of the regression line represented by the "m" on the equation $y = mx + b$

$$E = 10^{\frac{-1}{-3,382}} = 1,976$$

Percentage amplification efficiency was calculated as follows:

$$\%E = (E - 1)\ X\ 100\%$$

$$\%E = (1,976 - 1)X\ 100\% = 97,6\%$$