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Crude extracts isolated from *Cannabis sativa* plant inhibit growth and induce apoptosis in cervical cancer cells

Sindiswa Thandeka Lukhele

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Masters in Science.

December 2015

DECLARATION

I declare **"Crude extracts isolated from *Cannabis sativa* inhibit growth and induce apoptosis in cervical cancer cells''** to be my own unaided work. It is being submitted for Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination at any other University.

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Sindiswa Thandeka Lukhele

Signature…….....  .....

.....08.....December.....2015.



"When others believe you are capable

of great things, they give you a chance

to unlock your greatness"

Author unknown

RESEARCH OUTPUT

Poster presentation

Sindiswa T. Lukhele, Mpho S. Choene, and Lesetja R. Motadi. Expressional analysis of p53 and its related genes following treatment of MCF-7 breast cancer cells with *Euphorbia mauritanica*. SABMB conference, Worcester, Cape Town, 6-9 July 2014.

Sindiswa T. Lukhele, Mpho S. Choene, and Lesetja R. Motadi. Crude extracts isolated from *Cannabis sativa* inhibit cell growth and induce apoptosis in cervical cancer cells. EMBO conference, Braamfontein, Johannesburg, 6-11 July 2015.

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ABSTRACT

Cervical cancer remains a global health related issue among females of Sub-Saharan Africa, with over half a million new cases reported each year. Different therapeutic regimens have been suggested in various regions of Africa, however, over a quarter of a million women die of cervical cancer, annually. This makes it the most lethal cancer amongst black women in this area, and makes it important to search for new effective therapeutic drugs through screening of medicinal plant extracts used by many in Sub-Saharan Africa as potential anti-cervical cancer agents.

The aim of this study was to evaluate the anti-proliferative effects of *Cannabis sativa* extracts and its isolate, cannabidiol on cervical cancer cell lines HeLa, SiHa, and ME-180. To achieve our aim, phytochemical screening, MTT assay, cell growth analysis, flow cytometry, morphology analysis, Western blot, caspase 3/7 assay, and ATP measurement assay were conducted were conducted. Results obtained indicate that both plant extracts induced cell death at an IC_{50} of 50 – 100µg/ml and the Inhibition of cell growth was cell line dependent. Flow cytometry confirmed that, with or without cell cycle arrest, the type of induced cell death was apoptosis. *Cannabis sativa* extracts led to the up-regulation of apoptosis proteins (p53, Bax, caspase-3, and caspase-9) and the down regulation of anti-apoptosis proteins (Bcl-2 and RBBP6), signalling the execution of apoptosis. Apoptosis induction was further confirmed by morphological changes, an increase in Caspase 3/7 and a decrease in the ATP levels.

In conclusion, this data implies *Cannabis sativa crude* extracts has the potential to inhibit growth and induce apoptosis in cervical cancer cell lines, which may be due to the presence of cannabidiol.

Key words: Apoptosis, cervical cancer cells, cannabidiol, and *Cannabis sativa* extracts

DEDICATION

I dedicate this dissertation to my mother, Martha Lukhele and my late brother, Dennis Karabo Kgagara.



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All the honour and glory goes unto thee Lord Almighty for making things possible from the beginning till the end of this study.

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LIST OF ABBREVIATIONS

ASMR	Age Standardized Mortality Rates
ASIR	Age Standardized Incidence Rate
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid assay
Bcl-2	B-cell lymphoma 2
CIN	Cervical intraepithelial neoplasia
DD	Death Domain
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E6AP	E6 Associated Protein
FACS	Fluorescence activated cell sorting
FADD	Fas Associated protein with Death Domain
FBS	Fetal bovine serum
FITC	Fluorescein isothiocynate
HRP	Horse radish peroxidase
HPV	Human papillomavirus
P53	Phosphoprotein 53
PBS	Phosphate buffered saline
Rb1	Retinoblastoma 1
RBBP6	Retinoblastoma binding protein 6

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UNITS

h\hrs	hour\hours
kDa	kilodaltons
Min	minute
µM	micromolar
nM	nanomolar
nm	nanometer

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CHAPTER ONE

LITERAURE REVIEW

1.1. **Cervical cancer**

Cervical cancer develops in the cervix, between the uterus and the vagina. It is one of the top five cancers that affect women worldwide following lung, breast, and colorectal cancer (GLOBOCAN, 2012). The two main types of cervical cancer are squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma affects the flat cells lining the inner layer of the cervix while adenocarcinoma targets glandular cells lining the outside of cervix (Green *et al*., 2003).

1.1.1. **Epidemiology**

Cervical cancer is the fourth most commonly diagnosed cancer in women around the world (GLOBOCAN, 2012). An estimated 528 000 women were diagnosed with cervical cancer in 2012 (GLOBOCAN, 2012). A high burden of cervical cancer is most notable among women residing in rural areas of developing countries than developed countries (Francis *et al*., 2010; Arybn *et al*., 2011). The reported variation in the global burden may be due to remarkable geographical differences in the distribution of socio-economic factors and the rate of HPV prevalence (Arbyn *et al*., 2011). Over 455 000 new cases of cervical cancer (86%) were reported in developing countries when compared to 83 000 new cases (15%) of the developed countries (GLOBOCAN, 2012). Incidence rates vary greatly with each country worldwide, ranging from 4.5 per 100 000 women in Western Asia to 34.8 per 100 000 women in Sub-Saharan Africa (GLOBOCAN, 2012). The incidence rates in Sub-Saharan Africa, Melanesia, , the Caribbean, Latin America, and South Central and South East Asia exceed 20 per 100 000 women (Jemal *et al*., 2010; GLOBOCAN, 2012). The lowest incidence rates were observed in countries such as North America (6.6 per 100 000) and Australia/ New Zealand (4.5 per 100 000) (Arybn *et al*., 2011 and GLOBOCAN, 2012).

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Vaccinating young girls before predisposal to HPV and the use of Pap smear to detect cell changes in the cervix are some of the prevention strategies implemented to reduce the risk of cervical cancer (Denney *et al*., 2013).However; these strategies cannot eliminate the burden of cervical cancer in poor countries, where women who have already been exposed to the disease often present advanced stages (Francis *et al*., 2010; Arybn *et al*., 2011; Bray *et al*., 2013). As a result, cervical cancer remains the fourth most common cause of death amongst women with almost 266 000 women reported only in 2012 (GLOBOCAN, 2012). High mortality rates were observed in rural regions of Sub-Saharan Africa with approximately 22.5 per 100 000 women were reported dead in 2012 as compared to North America with 2.5 per 100 000 (GLOBOCAN, 2012). Lack of skilled individuals with expertise and knowledge on the signs and symptoms of cervical cancer and its preventative measures is one of the factors that contribute immensely to the death rate in developing countries (Arybn *et al*., 2011; Bray *et al*., 2013; Kumar and Bhasker, 2014). The lowest mortality were recorded in western Asia, Europe, Australia and New Zealand with less than 2 per 100 000 women (GLOBOCAN, 2012).

Regionally, cervical cancer is one of the biggest health issues in South Africa. Among Southern African countries, South Africa ranks third in the number of diagnosed cases of cervical cancer with 31.7 per 100 000 women following Swaziland (53.1 per 100 000) and Lesotho (38.4 per 100 000) as illustrated by **Figure 1.1**(GLOBOCAN, 2012). It is the second most effective cancer following breast cancer, with an annual diagnosis of 7735 new cases and the highest incidence rates were recorded among females aged 15-44 years (26.6 per 100 000) (GLOBOCAN, 2012). Black African women are at the highest risk of developing cervical cancer because most of them do not know of the disease and have never been screened for the presence of cervical lesions (Moodley, 2009). In a recent study conducted by Denny *et al* (2014), it was observed that almost 184 out of 300 South African black women exhibited the highest invasive cervical cancer cases in comparison to white women (4/300 cases) and Asian women (1/300 cases). The substantial increase of cervical cancer figures may be due to failure of women to avail themselves for screening, to follow up after diagnosis, and deficiency of cervical cancer treatment regimen and which according to Moodley

(2009), has resulted in high death rates among women residing in the rural regions of South Africa. Over 4248 deaths occur annually in South Africa, making cervical cancer the leading cause of death.

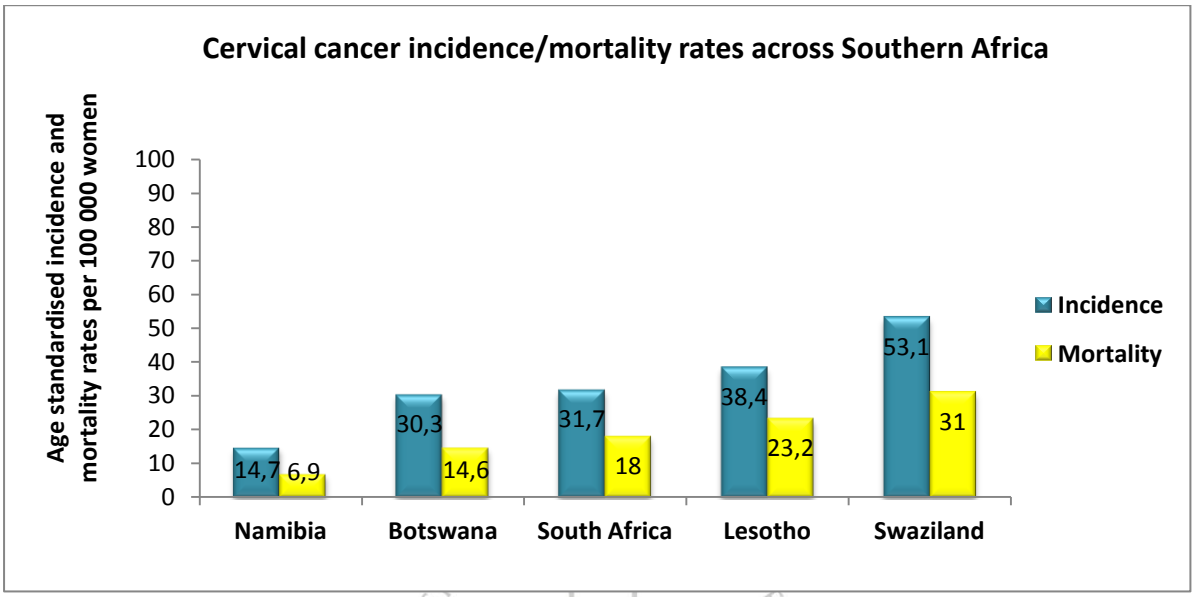

Figure 1.1: Cervical cancer age-standardised incidence and mortality rates (ASIR/MR) among Southern African countries.

Cervical cancer survival rates differ across each region around the world. Prevalence of HPV plays a fundamental role in the observed survival rates. Low- and middle-income countries have the lowest survival rates (Bray *et al*., 2013). In Sub-Saharan Africa, HPV prevalence rate accounted for almost 24% followed by Eastern Europe (21%), and Latin America (16%) (Forman *et al*., 2012; Bray *et al*., 2013). The initiative taken by upper- and high-income countries such as Eastern Europe to vaccinate sexually active girls above the age of 15 and also create awareness on cervical cancer has seen the reduction of a number deaths and improvement of survival rates over the years (Bray *et al*., 2013; Forman *et al*., 2012). Therefore, early detection and diagnosis via screening for the presence of HPV remains a cornerstone for the prevention of cervical malignancies and improvement of survival rates.

1.1.2. **Cervical cancer and the burden of HPV**

Human papillomavirus is the main causative agent of cervical cancer. This is evident in the number of cervical cancer cases (~ 99%) found to be associated with the virus

(Tota *et al*., 2011; Ibrahim *et al*., 2013; Pang and Theirry, 2012 and Bray *et al*., 2013). HPV is a non-enveloped and icosahedral structured virus made up of at least 8000 bases and consists of an episomal double stranded DNA (Burd, 2003). HPV is acquired through direct skin to skin contact with an infected individual and it also has the ability to infect other anatomical sites including oral mucosa, vulvar, anus, and the cervix (Burd, 2003; Pang and Theirry, 2012). This virus is responsible for diseases such as condyloma acuminata (genital warts), skin associated tumours, squamous cell carcinoma, oral and neck cancer (Pang and Theirry, 2012). An estimated 75% of sexually active individuals are at risk of being infected with HPV, making it the most common sexually transmitted viral infection (Arybn *et al*., 2011).

Currently, over 100 HPV genotypes of varying virulence have been identified with only 30 genotypes classified as sexually transmittable (Horvath *et al*., 2010). Half of these thirty genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70) have been suggested to be high-risk oncogenic subtypes and are most likely responsible for the development of precancerous lesions predisposing to cervical neoplasia (Pang and Theirry, 2012; Burd, 2003). Some of the high-risk genotypes were found in squamous intraepithelial lesions (Burd, 2003). HPV-16 and -18 are two of the most frequently detected high risk genotypes in pre-cancerous lesions that advance to high intraepithelial neoplasia and eventually to carcinoma (Burd, 2003; zur Housen, 2000; Horvath *et al*., 2010).This is evident in a series of studies wherein persistent infection with high risk subtype 16, 18, 31and 45 were observed to be strongly associated to the development of cervical intraepithelial neoplasia grade I-III and invasive cervical cancer (zur Housen, 2000; Burd, 2003). HPV 16 accounted for almost 50% of the overall cervical cancer cases followed by HPV18 with only 10% (Pang and Thierry, 2012; Ibrahim *et al*., 2013; Tota *et al*., 2011; Burd, 2003). In South Africa, HPV 16 and 18 are the most predominant genotypes detected in cervical cancer cases. The low risk genotypes (6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81) are frequently detected in genital warts (Burd, 2003). HPV subtype 6 and 11 are responsible for the development of benign lesions that seldom progress to cervical neoplasia (Burd, 2003; Horvath *et al*., 2010).

1.1.3. **Pathogenesis of HPV**

HPV infection is opportunistic, and it only gains entry to the basal cells (keratinocytes) of the squamous epithelium through mild abrasion or trauma to the skin (Horvath *et al*., 2010). HPV entry is modulated by the interaction of HPV receptors to alpha integrin receptors of the host or through attachment with stabilizing proteoglycan molecules present on the surface of the host cell (Burd, 2003; zur Housen, 2000; Tota *et al*., 2011 and Ibrahim *et al*., 2013). Other host receptors such as laminin receptors have also been implicated to facilitate the binding and entry of HPV into the host cell, however, not much has been reported on the mechanism of entry using these receptors (Horvath *et al*., 2010). The replicative life cycle of HPV is dependent on the differentiative state of the host keratinocytes (Münger *et al*., 2001; Burd, 2003; Horvath *et al*., 2010). Firstly, HPV encapsidates and exposes its viral genome wherein it infects undifferentiated keratinocytes and establishes itself episomally within these cells (Burd, 2003, Stanely, 2012). The virus uses the host's cellular chromosome to replicate from low copy number to high copy number (Dueñas-Gonzălez *et al*., 2005). The daughter cells migrate away from the basal layer as the host cells progress to form an epithelium layer (Münger *et al*., 2001; Burd, 2003; Di Domenico *et al*., 2012). This leads to the production and release of mature virions to seek new host cells (Di Domenico *et al*., 2012). Integration of HPV viral genome encodes for early proteins (E1, E2, E4, E5, E6, and E7) and late proteins (L1 and L2) (Münger *et al*., 2001; Burd, 2003; zur Housen, 200). The expression of these proteins is dependent on the differentiation stage of the host during the virus life cycle (Münger *et al*., 2001). E1 and E2 modulate the replication and early transcription of the viral genome in the cells. According to review by Côté-Martin *et al* (2008) E1 has similar enzymatic activity to that of a helicase protein, which helps unwind HPV DNA strands during amplification of the viral genome (Côté-Martin *et al*., 2008). It arranges itself in a double hexamer manner to help disassemble the strands of DNA (Côté-Martin *et al*., 2008; Münger *et al*., 2001). E2, a sequence-dependent DNA binding protein, recruits and binds to E1 protein following recognition of a specific sequence in the virus origin of replication (Côté-Martin *et al*., 2008). It also acts as a transcription factor of the viral genome (Côté-Martin *et al*., 2008). Over-expression of

E2 has been implicated to repress E6/7 expression, thus acting as an apoptosis promoting protein (Côté-Martin *et al*., 2008). However, the disruption of E2 caused by E6/7 over-expression leads to the alteration of programmed cell death via degradation of two important suppressor proteins p53 and pRb, and inactivation other targets genes (Côté-Martin *et al*., 2008). This in turn activates aberrant proliferation and immortalization of cervical cancer cells (Côté-Martin *et al*., 2008).

E6 inactivates the function of cellular protein p53 and E7 inhibits Rb1 protein function therefore initiating proliferation of cervical cancer cells and thus maintaining their state of malignancy (Münger *et al*., 2001). E6 interacts with E6-associated protein (E6AP) through its substrate recognition domain located on the N-terminal end to form a complex that allows binding of E6 (Dueñas-Gonzălez *et al*., 2005). E6AP is an E3 ubiquitin ligase and it helps transfer ubiquitin monomers that aid in the degradation of target genes such as p53 (Dueñas-Gonzălez *et al*., 2005). Following binding, E6 interacts with p53 through the E6- binding site present on the core domain of p53, thus promoting p53 degradation via the ubiquitin-26S proteosomal pathway (Dueñas-Gonzălez *et al*., 2005). E7 binds and disrupts the E2F-Rb complex, as a result releasing an active E2F (Dueñas-Gonzălez *et al*., 2005). An active E2F stimulates replication of DNA and promotes cell division by activating factors and target genes that trigger cell cycle progression (Dueñas-Gonzălez *et al*., 2005). Deregulation of both p53 and Rb1 proteins by HPV E6/7 contributes to the development of tumors in the cervix.

1.1.4. **Risk factors**

Development of cervical cancer cannot be attributable to infection with HPV only. HPV infection often clears off within a year or two after predisposal, with or without treatment and depending on the severity of the lesions (Burd, 2003; zur Housen, 2000). High grade lesions have a high progression rate and are more severe than low grade lesions (Burd, 2003). Persistent infection with high-risk oncogenic HPV leads to the occurrence of precancerous lesions predisposing to cervical neoplasia, however, it may take years for cervical cancer to develop (zur Housen, 2000; Burd, 2003). Therefore, certain risk factors act synergistically or additively with HPV, contributing

to the development and progression of precancerous lesions to cervical carcinoma (Kumar and Bhasker, 2014). These factors do not necessarily cause cancer, but they only increase the risk of developing malignant tumours.

The rates of cervical cancer estimates correlate to the increasing number of sexual partners, and younger age. Sexually active women of reproductive age (15-45 years) in developing countries present most cases (75%) of the disease and are at high risk of developing cervical cancer (Arbyn *et al*., 2011; Soumuya and Arun, 2011; WHO, 2014). A lower number of cases have been reported among women of menopausal age (≥50) (Somuya and Arun, 2011). Sexual behaviour determines the exposure to HPV. Teenagers and young women that engage in early age sexual intercourse with multiple sexual partners, without protection and with a history of sexual transmitted diseases such as HIV are highly susceptible to HPV infections (Moodley, 2009). According to Moodley (2009) women infected with HIV are five times more prone to infections with high-risk HPV when compared to non-infected women and this increases the chance of developing cervical cancer precancerous lesions by forty times.

Long term use of estrogen containing drugs, oral contraceptives, and hormone replacement therapeutic drugs are among the most important reproductive factors that lead to cervical cancer predisposition. The risk of developing cervical cancer correlates with long term use of these drugs. In a review by Chung *et al* (2010), it is implicated that increased levels of estrogen elevate the rate of proliferation and differentiation of cells lining the cervix in HPV-infected transgenic mice (Chung *et al*., 2010). It is evident that increased levels of estrogen, in cooperation with HPV, promote the progression of precancerous lesions in the cervix.Women that smoke are at high risk of developing cervical cancer and this is because of the carcinogenic tobacco by-products that are absorbed and carried in the bloodstream. In most cases of the smokers, the carcinogenic content of a cigarette has been found in mucus lining the cervix and it is speculated that it causes DNA damage in the cells (Fonseca-Moutinho, 2011). This also increases the progression rate of precancerous lesions in an individual that is already infected with HPV because smoking compromises the immune system making it less effective in fighting infections (Fonseca-Moutinho,

2011). Early age pregnancy has also been implicated to play a fundamental role in the development of cervical cancer. During pregnancy, cells lining the reproductive tract undergo changes and these changes could result in DNA damage (Soumuya and Arun, 2011). Other cofactors including a family history of cervical cancer and the occurrence of mutations on apoptosis promoting genes contribute to cervical malignancies (Soumuya and Arun, 2011). P53 and Rb1 protein are two of the most widely studied tumor suppressor genes that play an important role in preserving genomic stability and regulating the cell cycle. Occurrence of mutations in both genes leads to the development of cervical tumours. P53 has been found to be the most commonly mutated gene in almost 50% of all human cancers thus promoting growth of aberrant cells (Hainaut and Hollstein, 2000). Primary breast carcinomas including triple negative breast cancers present almost 18- 20% cases of p53 mutations (Hainaut and Hollstein, 2000).

1.2 **P53**

P53, also known as *Tp53*, is regarded as the guardian of the genome based on its inhibitory role to conserve genomic stability and also prevent the occurrence of mutations on the genome (Hinds and Weinberg, 1994; Sager, 1992). It is located on the seventeenth chromosome position 13.1 (Sager, 1992). P53 is made up of 393 amino acid subunits and consists of domains that facilitate the transactivation of downstream target genes, recognize specific DNA sequences (core domain), tetramerization of the protein, and a domain that recognizes damaged DNA as a result of the occurrence of single stranded DNA breaks or misalignment of base pairs (Hinds and Weinberg, 1994).

1.2.1 **Functions of p53**

P53 acts as a tumor suppressor. In normal cells, p53 expression levels are low (Hinds and Weinberg, 1994). However, exposure to a damaged DNA triggers the increase p53 protein levels leading to growth arrest until DNA is repaired or induction of apoptosis (Freeman, Wu, and Levine., 1999). P53 levels are tightly regulated by the presence of MDM_2 in an autoregulatory feedback loop (Hinds and Weinberg, 1994;

Freeman, Wu, and Levine., 1999). Briefly, the increase of p53 protein levels after DNA damage transcriptionally induces Mdm2 expression in the cells leading to its protein blocking p53 activities while facilitating p53 degradation (Freeman, Wu, and Levine., 1999). Therefore, Mdm2 functions as a negative regulator of p53 while p53 positively regulates Mdm2 (Freeman, Wu, and Levine., 1999). Exposure to DNA damage and other various stress signals including radiation light, chemotherapeutic agents, epigenetic factors activate protein kinases ATM and ATR including Chk1/2 to trigger and phosphorylate p53 at the ser15, thr 18 or ser20 residues thus disrupting the p53-Mdm2 complex (Freeman, Wu, and Levine., 1999). Increased levels of p53 triggers and activates pro-apoptotic proteins and inhibits anti-apoptotic proteins thus executing cell death.

P53 encodes for a protein that controls a network of signalling pathways essential for growth regulation and cell survival including transactivation of a multitude of pathways, cell cycle arrest, senescence, DNA repair, and induction of cell death by triggering apoptosis (Freeman, Wu, and Levine., 1999). Prior to the disruption of p53-Mdm2 complex, p53 levels increase in the cells leading to transactivation of downstream effector proteins involved in the cell cycle ($p21^{Waf1}$, GADD45, 14-3-3σ), DNA repair (p53R2) and apoptosis(Bax, Noxa, PUMA, PIG3, p53AIP1) to either arrest the cell cycle or induce apoptosis in case of irreparable DNA damage (Freeman, Wu, and Levine., 1999).

1.2.2 **The role of p53 in carcinogenesis**

Hinds and Weinberg (1994) described tumour suppressor p53, as a gene whose loss of function leads to development of tumour malignancies. P53 serves a regulatory purpose to negate activated cancer-promoting oncogenic products thus inhibiting their activity (Sager, 1992). However, the occurrence of mutations, activation of proto-oncogenic genes, and abnormal over expression of p53 contribute to tumorigenesis (Sager, 1992; Hinds and Weinberg, 1994). A defective or mutant p53 could allow aberrant cells to proliferate therefore resulting in cancer (Hinds and Weinberg, 1994). In virus associated cancers such as cervical cancer, p53 degradation is facilitated by an increased expression and interaction of oncogenic product E6 to p53 (Sager, 1992).

Upon the intergration of HPV in the basal cells, p53 is blocked from performing its tumour suppressor function following an increased expression of E6 (**Figure 1.2**) (Ryan *et al*., 2001). As illustrated by figure 1.2 (arrows highlighted in red), cellular protein E6AP recruits and binds to E6 (Ryan *et al*., 2001). E6 changes conformation and allows the binding of p53 therefore facilitating its ubiquitination and degradation (Ryan *et al*., 2001). Upon binding of E6 to p53, E6AP adds ubiquitin monomers to facilitate ubiquitylation and degradation of p53 via the ubiquitin-dependent/ 26-S-proteosomal pathway (). There are other pathways and their protein products implicated to contribute the dysregulation of p53 and carcinogenesis. These include the over expression of ubiquitin ligase protein such as RBBP6 which causes a reduced expression of p53. The status of the cell is compromised as a result of p53 being unnecessarily degraded thus causing aberrant cell growth.

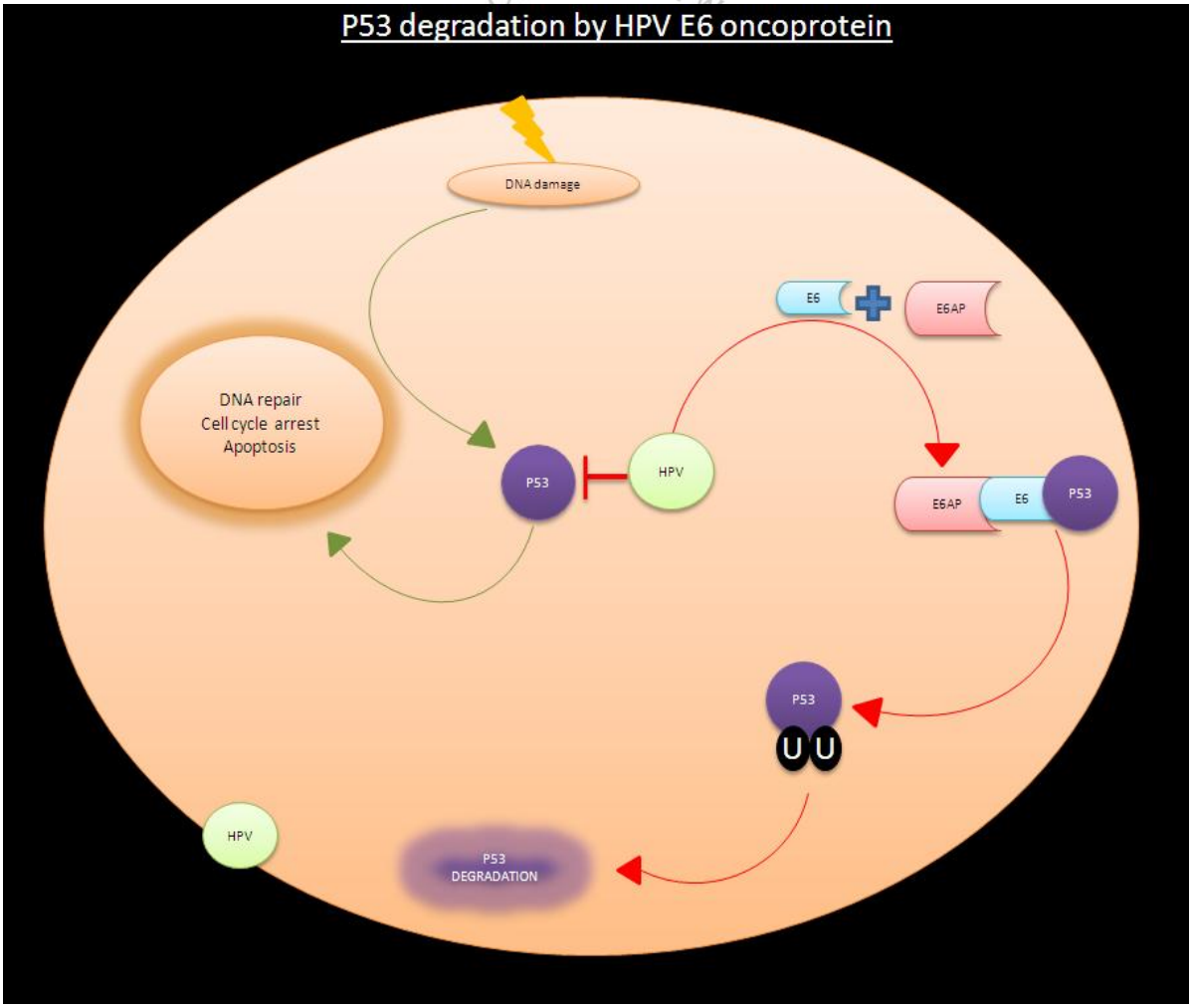

Figure 1.2: A schematic diagram illustrating the degradation of p53 protein by HPV E6 protein. Upon exposure to DNA damage, p53 becomes activated and leads to the induction of cell cycle arrest or apoptosis. E6AP and E6 recruit and bind to p53 therefore promoting its degradation via the ubiquitin-dependent pathway

1.3 **Retinoblastoma binding protein 6 (RBBP6)**

Retinoblastoma binding protein 6, also known as p53-associated cellular protein-testes (PACT), proliferation protein-related protein (P2P-R/PP-RP) and retinoblastoma binding Q protein 1(RBQ-1), is a 250kDa multi-domain protein implicated to contribute to tumor development (Chibi *et al*., 2008; Li *et al*., 2007; Motadi *et al*., 2010). It is localized on chromosome 16p12.2 and consists of 18 exons wherein at exon 16 it is spliced to make transcript variants of 1.1 kb, 6.0 kb and 6.1 kb (Pugh *et al*., 2006). All three transcripts encode for three different protein isoforms 1, 2, and 3 respectively (Motadi *et al*., 2010; Moela *et al*., 2013; Pugh *et al*., 2006). RBBP6 is made up several domains including the N-terminal ubiquitin-like domain, cysteine-rich Ring finger domain, and a Domain With No Name (DWNN) making it a multifunctional protein (Mbita, 2012 thesis; Chibi *et al*., 2008). Isoform 1 and 2 comprise of an additional zinc domain while isoform 3 consists of a Domain With No Name (DWNN) (Mbita, 2012 thesis). RBBP6 is over-expressed in oesophageal cancer (Pugh *et al*., 2006). *In vitro* studies conducted by Motadi *et al*., 2010; Moela *et al*., 2013; Ledwaba and Dlamini, 2005; and Chen *et al*., 2013, have also confirmed the expression of the 250 kDa protein product on various cell lines including A549, MCF-7, HeLa, and HT116 cells, which makes it a therapeutic target for the development of cancer therapy. RBBP6 is involved in various roles including ubiquitination, translation, transcription and mRNA processing (Pugh *et al*., 2006).

1.3.1 **Role of RBBP6 in cancer**

RBBP6 and its isoforms (PACT, RBQ1, and P2P-R) are part of the most widely studied proteins implicated to contribute to carcinogenesis (Pugh *et al*., 2006). According to Moitto *et al* (2014), RBBP6 plays a major role in maintaining genomic stability and preventing DNA damage. RBBP6 ubiqutinates and destabilizes ZBTB3,

transcriptional repressor. ZBTB3 negatively regulates MCM10 levels, which according to Moitto *et al* (2014) plays a vital role as replication factor of chromatin. Deregulation of RBBP6 during this pathway contributes to genomic instability. And this phenomenon is well documented in a study by Moitto *et al* (2014) wherein knockdown of RBBP6 in HeLa, HCT 116, p53 -/-HCT116, U2OS , and a Rb-depleted U2OS cells led to an increase in the levels of phospho-H2AX, phosphorylated ATM and phosphorylated Chk2. HeLa cells were used as a control (Moitto *et al*., 2014). This effect was also recorded in a normal lung fibroblast (MRC-5) wherein depletion of RBBP6 led to what Moitto *et al* (2014) described as spontaneous appearance of DNA damage. This clearly indicates that RBBP6 is an important gene to maintain stability of the DNA.

Not only is RBBP6 limited to conserving genomic stability, it has also been implicated to regulate the cell cycle and apoptosis (Li *et al*., 2007). During apoptosis, RBBP6 helps maintain the levels of p53 following response to DNA damage. RBBP6 mediates the assembly of p53-Mdm2 complex subjecting p53 to ubiquitination and 26S proteosomal degradation (Vassilev *et al*., 2012). According to Mbita *et al* (2012), knock down of RBBP6 isoform 3 resulted in the induction of a reduced cell cycle arrest at G_2/M phase using propidium iodide, which demonstrates that RBBP6 may be a regulator of the cell cycle. The mechanism of cell cycle regulation is still unclear. Therefore, gene studies could help elucidate the mechanism.

1.4 **The cell cycle**

Execution of the cell cycle is for normal cells to divide and produce identical daughter cells in the process eliminating aberrant cells. Cells are required to undergo division to multiply and equate the number of eliminated cells and maintain homeostasis in the body. The cell cycle consists of two main events called interphase and the mitotic phase (Schwartz and Shah, 2005). Both events are involved in fulfilling the main functions of the cell cycle, which is to synthesize vast amounts of chromosomal DNA and equally segregate the genomic material between two daughter cells (Caldon *et al*., 2006). The interphase involves long growth periods and it is subdivided into three distinct phases including G_1, S-phase and G_2 phase to ensure that cells are ready for

the next phase (**Figure 1.3**) (Caldon *et al*., 2006; Schwartz and Shah, 2005). In the first gap (G_1), cells actively grow and divide to until they are ready to progress to the S-phase (Schwartz and Shah, 2005). Not only is growth and division promoted in the first gap, however, cells are also monitored to ensure only healthy cells progress to the next phase (Kastan and Bartek 2004). In the S-phase, newly formed cells commit to DNA replication, to manufacture copies of its own DNA into two identical daughter cells (Kastan and Bartek 2004). The genetic material and its accessory proteins of the mother cells are equally divided among the two daughter cells before committing to the second gap, G_2 (Kastan and Bartek 2004). Following entry in the G2 phase, cells continue to grow slowly as it prepares for cell division (Kastan and Bartek, 2004; Schwartz and Shah, 2005). The second gap also ensures that only non-aberrant cells progress to the M-phase (Kastan and Bartek, 2004). And therefore, it can be concluded that G1/2 phase both act as a checkpoint factors of the cell cycle, ensuring only normal cells are duplicated in the cell cycle (Kastan and Bartek, 2004). In the M-phase, mitosis and cytokinesis are executed, wherein the nucleus and cytoplasm is divided to make two daughter cells of identical genetic material and its end products (Chen *et al*., 2011; Huertas *et al*., 2008; Kastan and Bartek, 2004). Most of the cells enter a non dividing state called G_0 phase, an arrest or quiescent phase, wherein unnecessary proliferation of cells is inhibited. Detection of abnormal cell growth or damaged DNA in the cell cycle leads to the induction of cell cycle arrest until DNA is repaired or apoptosis is induced (Chen *et al*., 2011; Huertas *et al*., 2008).

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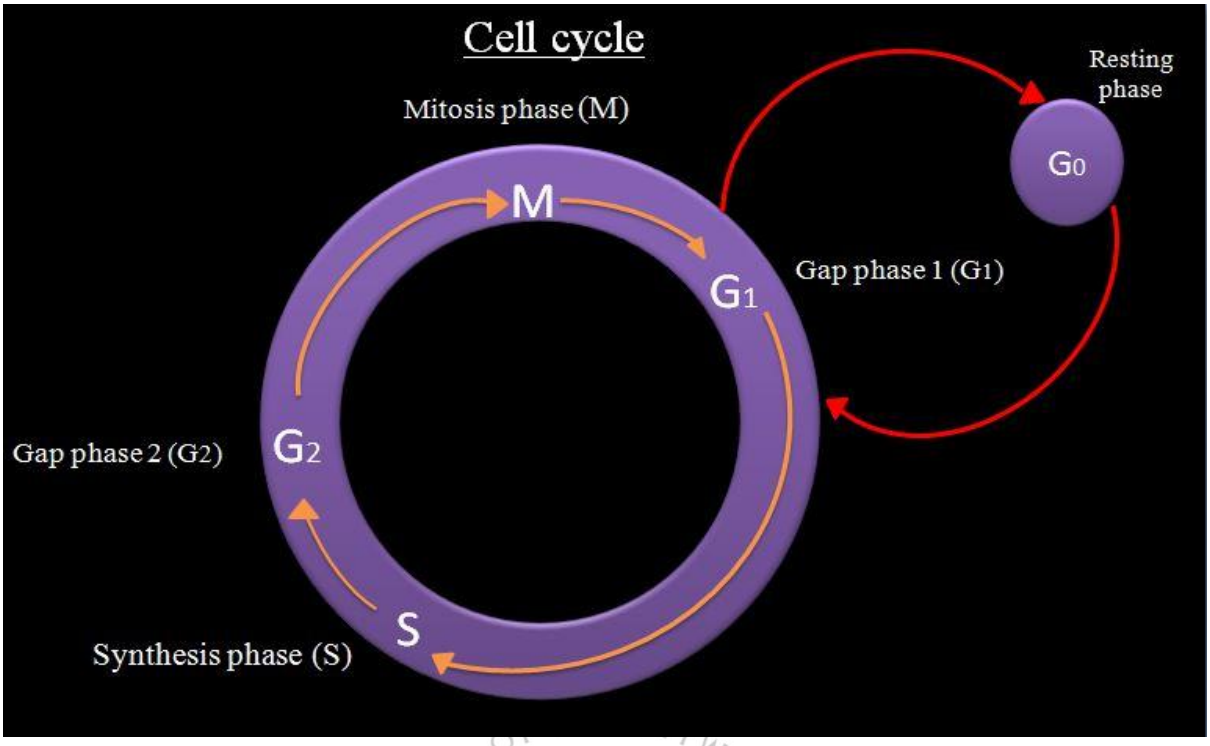

Figure 1.3: A schematic diagram of a cell cycle made up of four phases. The resting phase promotes cell growth. Cells enter gap phase 1 to undergo cell division until they are ready to replicate their DNA in the S-phase. Following DNA synthesis, cells continue to grow and divide in the G2 phase before entering the mitosis phase, wherein, DNA is equally segregated amongst two daughter cells.

The cell cycle is regulated by a number of genes including the cyclins, cyclin dependent kinases (CDKs), cyclin inhibitors and other genes such as p53 and a retinoblastoma gene, pRb (Hunter and Pines, 1994; Morgan, 2007). Cyclin inhibitors, (KIN/CIP (p21 and p27) and INK4 (p15, p16, p18, and p19)), p53 and pRb screen and prevent abnormal DNA proliferation from progressing to the next phase through the induction of cell cycle arrest until DNA is repaired or by triggering apoptosis (Morgan, 2007). Loss of function of these regulatory genes leads to uncontrolled cell proliferation and contributes to tumorigenesis. Under normal conditions, cyclins A, B, D, and E activate specific CDKs for smooth progression of the cell cycle through constant degradation by p53(Chen *et al*., 2011; Huertas *et al*., 2008; Morgan, 2007). Over expression of cyclins has been implicated to cause cancer (Huertas *et al*., 2008). *In vivo* studies indicate a subsequent increase in the expression of cyclins in aberrant cells which may be as a result of oncoproteins E6/E7 (Chakrabarti and Krishna, 2003).

In low and high-grade precursor lesions including carcinoma in situ and invasive cancer, it was found that the expression of oncoproteins E6/E7 led to the up-regulation of cyclins and their expression played a remarkable role in tumorigenesis (Ryan *et al*., 2007). This is as a result of disrupted complexes required for the degradation of cyclins (Morgan, 2007). A defect in cyclin inhibitors and CDKs has also been linked to cause cancer. In cervical carcinoma, progression of the cell cycle is triggered by activation of oncogenic proteins and repression of cyclin inhibitors and CDKs, which is modulated by the interaction of the viral proteins with those of the host cell (Ryan *et al*., 2001). It is suggested that HPV oncogenes alter the expression of cyclin inhibitors via E6-p53 interaction, therefore inhibiting the degradation of tumor suppressor, p21. In low and high grade CIN lesions it has been noted that binding of E7 to pRb family proteins disrupts the E2F-pRb complex causing over expression of cyclins A or E (Chakrabarti and Krishna, 2003).

1.5 **Apoptosis and cervical cancer**

Previously, carcinogenesis was regarded as a result of mitotic disorders. Recent studies have however proven that carcinogenesis may also be as a result of failure of apoptosis through its regulatory genes and their protein products (Wong, 2011). Apoptosis is a form of programmed cell death that is required by the immune system to maintain homeostasis in the human body (Chipuk *et al*., 2004). It plays a fundamental role in early cellular development, immune system, and tissue and cell differentiation in multicellular organisms (Chipuk *et al*., 2004; Wu *et al*., 2001). This form of programmed cell death is characterized by morphological, pathological, and biochemical changes including the condensation of chromatin, convolution of nuclear and cellular outlines, nuclear fragmentation, formation of apoptotic blebs within the plasma membrane, cell shrinkage, and leakage of organelles in the cytoplasm (Wu *et al*., 2001; Chipuk *et al*., 2004). Programmed cell death is regulated by a number of genes which facilitate cellular responses such cell cycle arrest, cell proliferation, senescence, DNA repair and genomic stability (Igney and Krammer, 2002; Magal *et al*., 2005). Induction of apoptosis occurs through the exposure of cells to various stimuli including ultraviolet light, hypoxia, DNA damage, activation of death

receptors, and chemotherapeutic agents (Igney and Krammer, 2002; Zangemeister-Wittke and Simon, 2001). Deregulation of apoptosis has alsobeen linked to the development of various disorders such as Alzheimer's disease, autoimmune diseases, and neoplastic disorders (Magal *et al*., 2005). Too little apoptosis contributes to cancer, autoimmune and certain chronic inflammatory diseases while too much apoptosis results in stroke-induced neuronal damage and neurodegenerative disorders (Zangemeister-Wittke and Simon, 2001),

Two apoptosis pathways are initiated upon exposure to a stress-inducing agent, the mitochondrial pathway and the death receptor pathway (Zangemeister-Wittke and Simon, 2001; Li-weber, 2013; Magal *et al*., 2005). The mitochondrial pathway, also known as the intrinsic pathway, is regulated by the expression of pro- and anti-apoptotic bcl-2 family proteins in which members are either antagonists or agonists of apoptosis (Li-weber, 2013). In response to intracellular stress such as DNA damage, various multidomain anti-apoptotic bcl-2 family proteins are inhibited (Magal *et al*., 2005). In turn, pro-apoptotic genes are activated and inserted into the outer membrane of the mitochondria therefore forming lipid pores that are lethal to proteins providing support and rigidity to the outer membrane (Li-weber, 2013). Permeabilization of the outer membrane facilitates the release of cytochrome c into the cytoplasm and binds to Apaf-1, an apoptosis-protease-activating factor 1, in the outer membrane space of the mitochondrion forming a complex (Chipuk *et al*., 2004; Fulda and Debatin, 2006). This complex binds to dATP resulting in an apoptosome complex which activates caspases-9 (Wong, 2011; Cárdenas-García and González-Pérez, 2013). The active form of caspase 9 activates and cleaves caspases 3 via a protein cascade which leads to the induction of apoptosis (Magal *et al*., 2005).

The extrinsic pathway occurs via death receptors located on the surface of the cells. Receptors such as fas (CD95), tumor necrosis factor- alpha (TNF-α), TNF receptor (TNFR1) and nerve growth factors (NGF) mediate programmed cell death in cancerous cells (Igney and Krammer, 2002). Fas receptor employs the use of DD (death domain), DED (death effector domain), and Fas-associated death domain (FADD) adaptor protein to facilitate the binding of procaspase-8 and -10 (Wong,

2011). Following interaction of the ligand FasL to Fas, the adaptor protein is recruited and binds to the death domain of Fas (Igney and Krammer, 2002; Wong, 2002). As a result, a ligand-receptor-adaptor protein complex known as a death inducing signalling complex (DISC) is formed (Wong, 2011; Hoetelmans *et al*., 2000). DISC promotes the binding of procaspase-8 thus activating it into caspase 8 (Wong, 2011; Igney and Krammer, 2002; Hoetelmans *et al*., 2000). Active procaspase-8 and -10 act as initiators of apoptosis by further activating and cleaving caspase-9 and other executioner caspases (Ibrahim *et al*., 2013; Hoetelmans *et al*., 2000).

Figure one point four (**Figure 1.4**) elucidates the apoptosis mechanism in the presence of cannabinoids, the active ingredient of *Cannabis sativa* plant. In response to a chemotherapeutic stimulus (Δ^9-THC, CBN, and CBD), apoptosis has been observed to be triggered via the extrinsic or the intrinsic pathways (Bla´zquez *et al*., 2000). Delta-9-tetrahydrocannabinol, Δ^9-THC, binds to the CB2 receptors on the surface of the cell, triggering ceramide syntheisis (Go´mez del Pulgar, 2002). Ceramide accumulates when cells are treated with apoptosis inducing agents including ionizing radiation and chemotherapeutic drugs and it further triggers effector proteins that lead to Bcl-2 inhibition (Go´mez del Pulgar, 2002; Bla´zquez *et al*., 2000; Hoetelmans *et al*., 2000). In the process, bax is activated and it porates the mitochondria causing the release of cytochrome c (Hoetelmans *et al*., 2000). On the other hand, Δ^9-THC, CBN, and CBD have been implicated to bind to the Fas ligand causing the activation of caspase 8 (Jia *et al*., 2006). Caspase 8 triggers and activates caspase 3, which further activates the caspase cascade leading to apoptosis (McKallip *et al*., 2006; Jia *et al*., 2006).

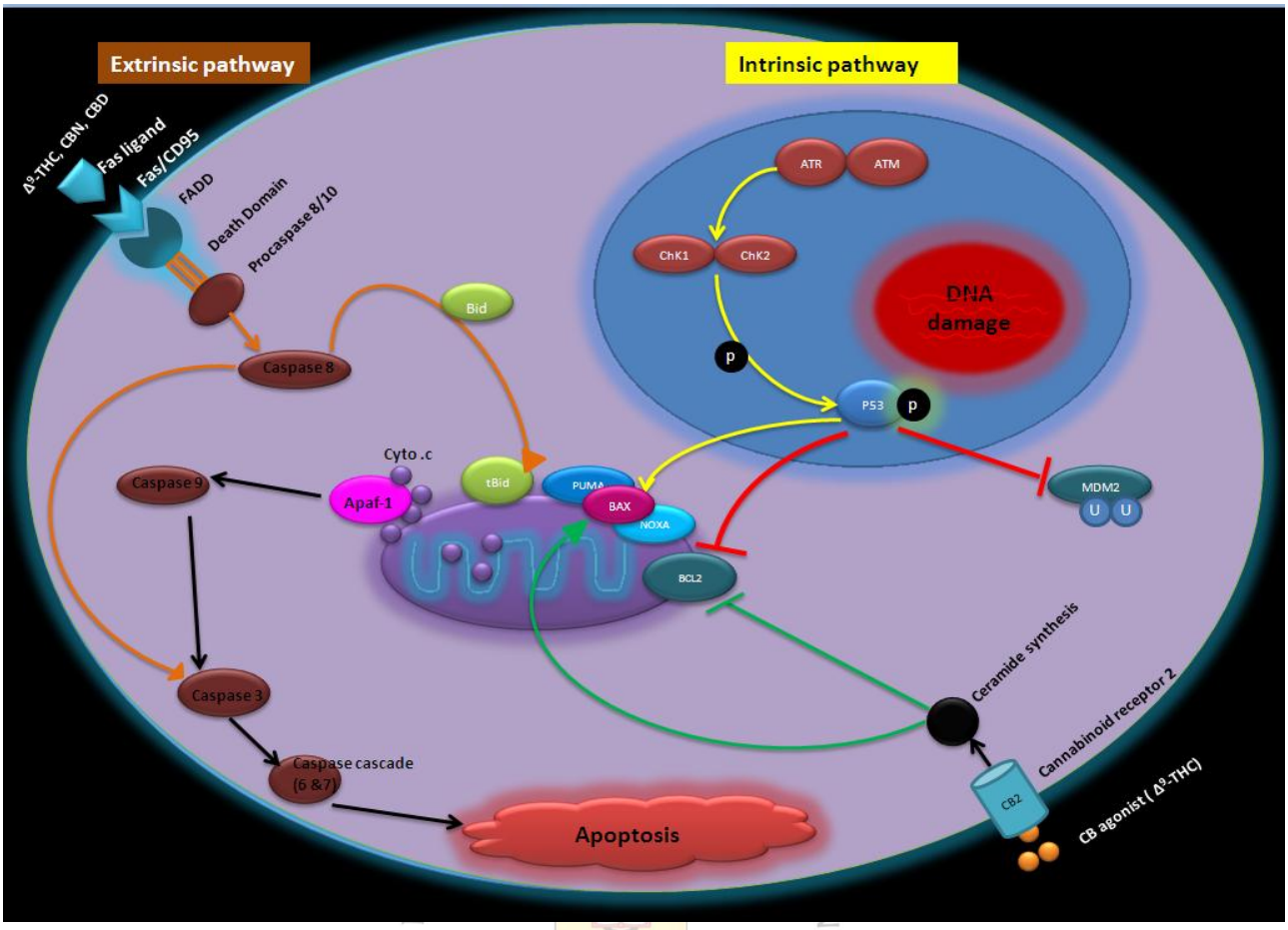

Figure 1.4: Apoptosis induction following exposure to the major constituents of *Cannabis sativa* plant. Cannabinoids initiate apoptosis via the extrinsic pathway or through the CB1 receptors. Cannabinoids bind to the fas receptor and activate procaspase 8/10 to caspase-8.

The dysfunction of apoptosis leads to the development of malignancies including cervical neoplasia. A study conducted by Magal *et al*, (2005), clearly indicates that HPV oncoproteins have the ability to interfere with apoptosis, therefore leading to cervical cancer. Oncopotein E6 and E7 are capable of causing an imbalance in the proliferation of normal uterine tissue cells and apoptosis through inactivation of tumour suppressor genes Rb1 and p53 (zurHousen, 2000). A defect in apoptosis may also be as a result of reduced caspase function, and impairment of signalling mechanism in death receptor, and the imbalance between pro-apoptotic and anti-apoptotic Bcl_2 family proteins (Wong, 2011).

1.5.1. **Caspases**

Apoptosis induction occurs as a result of the activation and cleavage of caspases. Caspases are cysteinyl aspartate-specific proteases that naturally occur as inactive zymogens (Chang and Yang, 2000; Lavrik *et al*., 2005). They are made of N-terminal domain followed by a 20 kDa subunit, p20, and a small subunit of about 10 kDa, p10 (Chang and Yang, 2000). Almost 14 caspases have been identified in mammalian tissue and they are divided into three major groups based on the structure of the prodomain and its function (Lavrik *et al*., 2005; Chang and Yang, 2000). Initiator caspases and inflammatory caspases are made up of a large prodomain while effector caspases have a short prodomain of less than 30 amino acid residues (Chang and Yang, 2000). Initiator caspases (caspase-9, -8, and -10) activate and cleave effector caspases in response to a death signal (Lavrik *et al*., 2005). Effector caspases (caspases-3, -6, and -7) further activate and cleave caspase cascade to execute cell death (Chang and Yang, 2000).

Caspases are activated via proteolytic events at a specific aspartic residue located within the prodomain and the two subunits (Chang and Yang, 2000). Activation of caspases depends on the apoptosis pathway. Procaspase-8 and -10 are triggered through the death receptors including CD95, TRAIL, fas-APO1, and DR3 or DR6 (Chang and Yang, 2000; Lavrik *et al*., 2005). These receptors belong to the TNF family and consist of the Death Domain in their intracellular domains (Chang and Yang, 2000). Binding of a ligand to a death receptor, results in the formation of a death inducing signalling complex, DISC (Chang and Yang, 2000). The DISC contains isoforms of procaspase 8, FADD/MORT1, FLICE inhibitory proteins and procaspase 10 (Lavrik *et al*., 2005). The death domain of the receptor interacts with the death domain of FADD, while DED of FADD interacts with N-terminal tandem of DEDs of procaspase 8 and -10 leading to their activation (Lavrik *et al*., 2005). Procaspase 10 is activated at the DISC thus forming an active heterotetramer, however, the exact mechanism of whether procaspase 10 is activated in the presence of a mature caspase 8 remains unknown (Lavrik *et al*., 2005). Activation of the effector casapses differs between the death receptor and mitochondria mediated pathways (Lavrik *et al*., 2005; Chang and Yang, 2000). In response to a death signal, initiator caspases are activated and cleaved resulting in a

conformational change, which enables interaction of the adaptor protein, Apaf-1 (Chang and Yang, 2000). As a result, an apoptosome is formed (Lavrik *et al*., 2005). The effector proteins in turn execute apoptosis by cleaving cellular proteins following the aspartic residues (Korsmeyer *et al*., 2000).

1.6 **Traditional medicine**

Natural plants have been used for centuries as traditional medicine to treat diverse human ailments. Various ethnic groups living in rural geographical areas still depend on indigenous medicinal plants as a primary source of health care (Street and Prinsloo, 2012 and Rao *et al*., 2008). It is reported that almost 70-80% of the world's population rely on medicinal plants to combat diseases and reccuring infections (Mamedov, 2012; Street and Prinsloo, 2012). Only a minority (20%) of the population in developing regions are dependent on orthodox medicine for health care (Street and Prinsloo, 2012). In countries such as China, India, South Africa, South America, and East Asia, it is a common practice to use traditional medicine to treat infections (Mathabe *et al*., 2006). Medicinal plants and herbs remain a cheap and affordable option compared to commercialized synthetic products (Mthembu *et al*., 2012; Rao *et al*., 2008). In over-populated countries with increased levels of poverty, modern medicine is inaccessible and costly. Therefore, the use of traditional medicine is strongly encouraged and individuals opt for cheap and easily available natural plant-based products (Mthembu *et al*., 2012). Strong cultural influences and traditional beliefs also play a huge role in people often seeking out help from traditional healers as their preferred choice for treating diseases (Mthembu *et al*., 2012). Medicinal plants formed part of a healing ritual often practiced by different African, Asian and Native American societies (Mamedov, 2012). People living in rural areas of South Africa still believe in the healing abilities of plants (Street and Prinsloo, 2012). In a study by Street and Prinsloo (2012), it is suggested that almost 80% of the South African population is dependent on traditional medicine for health reasons and often consult traditional herbalist for medicinal plants.

Currently, plant medicines play a pivotal role in drug discovery. Screening of various medicinal plants has led to isolation of biologically active components that are used as

potent drugs (Newman and Cragg, 2003). Analysis of prescribed modern medicine indicates the presence of one or more plant derivatives in them (Mamedov, 2012). To date, at least 119 chemical constituents extracted from 90 plants are of use in one or more countries and form part of their primary health care systems (Mamedov, 2012).There are many examples where plants have been used for medicinal purposes, including plants such as *Digitalis purpurea, Artemisia annua, Catharanthus roseus, Rauvolfia serpentine and Taxus brevifolia* (Itokawa *et al*., 2008 and Cragg and Newman, 2005). Today, some of these plants or drugs derived there from, are used treat various chronic, infectious and psychological diseases. Madagascar periwinkle is involved in the treatment of childhood leukemia and Hodgkin's disease (Mamedov, 2012). In India, *Rauvolfia serpentine* forms part of the Aryuverdic medicine used in cases of insomnia, and artemisinin treats malaria (Mamedov, 2012; Pezzuto, 1997 and Huang *et al*., 2010). Further research on indigenous plants could lead to discovery of new novel drugs for different type of cancers including cervical cancer.

1.7 **Medicinal plants as anticancer agents**

Medicinal plants play an important role in preventing and treating cancer. Over the past five decades, medicinal plants have gained recognition as possible future medicines for cancer due to their healing abilities. In a review by Mamedov (2012), it is suggested that almost 3000 plants have been screened and used as anticancer agents. Over 60% of the prescribed industrialized drugs are derived from plant, marine, animal and microbial sources (Cragg and Newman, 2005) and almost 25% of the total drugs yielded are from higher plants (Mamedov, 2012). In the United States of America alone, over 121 drugs prescribed are derivatives of natural products (Newman and Cragg, 2007).

Medicinal plants are known to contain active ingredients and antioxidants with complex structures that work synergistically during the healing process. It has been suggested that these metabolites either directly or indirectly impact on human cells, limiting the spread of diseases in the body (Mamedov, 2012; Rao *et al*., 2008; Gurib-Fakim, 2006). Currently, some medicinal plants are undergoing clinical trials as drugs that can be used in the treatment of different cancers. This is aimed at reducing

adverse effects demonstrated by orthodox medicine and for this purpose, various active compounds derived from plants are being assessed for their efficacy and tolerability against cancer (Cragg and Newman, 2005). Pharmacologists, chemists, and ethno-botanists assist in identifying plants of medicinal use and further isolate their chemical compounds for development of new anticancer drugs and their analogues, with the aid of assays, extraction, and isolation techniques (Pezzuto, 1997).

Evaluation of medicinal plants has seen the dawn of many anticancer agents. Over 40% of anticancer agents available in the market today are either derived from natural plant products or are mimics of natural products (Newman and Cragg, 2007). A total of 19 plant-derived drugs were approved by the Food and Drug Administration, of which 7 of the 19 are natural plants (Mamedov, 2012). Plant derived drugs that are currently in clinical use for the treatment of cancer include camptothecins, taxanes, vinca alkaloids, and epipodophyllotoxins (Newman and Cragg, 2007). These drugs also serve as starting materials or models for the production of their synthetic analogues (Newman and Cragg, 2007; Mamedov, 2012). Eleven of the total 19 drugs are semi-synthetic and at least two drugs contain one or more of the active ingredients found in plants (Newman and Cragg, 2007; Mamedov, 2012). Preclinical synthetic drugs flavopiridol and combrestatins are some of the semi-synthetic drugs (Rahman, 2008). Camptothecin, a known cell death inducer, was also tested for its antiproliferative effects against cervical cancer.

1.7.1 **Camptothecin**

Camptothecin was first isolated from a bark and stem of a Chinese tree, *Camptotheca acuminata*, in 1966 by Wall *et al* (1966). It is a cytotoxic alkaloid-containing agent which targets and inhibits the activity of DNA topoisomerase I enzyme (topo I) (Nobili *et al*., 2009). Nuclear enzyme topo I covalently bind and relax the strands of a supercoiled DNA through a reversible transesterification reaction. A cleavage complex causes single stranded breaks in a double helix of the DNA (Nobili *et al*., 2009). Once torsional stress is relieved, the enzyme rejoins the broken strands and disassociates from the double strands, allowing the DNA to easily undergo translation, transcription, and recombination (Nobili *et al*., 2009; Garcia-Carbonero and Supko,

2002). Camptothecin binds and stabilizes the DNA topo I cleavage complex, inhibiting its religation step (Nobili *et al*., 2009). This causes the accumulation of single stranded breaks of the DNA (Nobili *et al*., 2009). It has been widely used in a number of *in vivo* studies against a series of cell lines and has however been proven to be effective as an individual agent or in combination with other agents. This is evident in a study conducted by Moela *et al* (2014) wherein treatment of MCF-7 breast cancer cells with camptothecin alone and in combination siRNA significantly increased cell death and reduced cell growth through the down-regulation of RBBP6, proliferative gene, and up-regulation of p53.

Two camptothecin analougues, topotecan and irinotecan, have been approved by Federal Drug Administration (FDA) and are used in cancer therapy for their antitumor properties (Garcia-Carbonero and Supko, 2002). Topotecan is administered in patients suffering from ovarian carcinoma, small-cell lung cancer while irinotecan has been proven to be successful for patients with advanced colorectal cancer (Garcia-Carbonero and Supko, 2002). To date, several synthetic camptothecin analogues including lurtotecan, exatecan, mesylate, karenitecan and gimatecan are undergoing clinical evaluation for various human cancers and are currently in phase I-II of clinical trials (Nobili *et al*., 2009; Garcia-Carbonero and Supko, 2002). It is therefore imperative to screen plants of different species to determine the effect they have on cancerous cells (Rahman, 2008).

1.8 ***Cannabis sativa***

Cannabis sativa is a dioecious plant that belongs to the Cannabaceae family and it originates from Central and Eastern Asia (Turner *et al*., 1975; Flemming *et al*., 2007). It is widely distributed in countries including Morocco, South Africa, United States of America, Brazil, India, and parts of Europe (Turner *et al*., 1975; Happyana *et al*., 2013). *Cannabis sativa* is a narrow-leafed plant (**Figure 1.5**) that grows annually in tropical and warm regions around the world and it has been used for decades as part of a herbal remedy to treat internal sores, cough, ear infections, and flu (Fankhauser, 2002). Different ethnic groups with various cultural influences use *Cannabis sativa* for reasons such as smoking, preparing concoctions to treat diseases, and for cultural

purposes (Lozano, 2001). According to Turner *et al* (1975), it is composed chemical constituents including cannabinoids, nitrogenous compounds, flavanoid glycosides, steroids, terpenes, hydrocarbons, non-cannabinoid phenols, vitamins, amino acids, proteins, sugars and other related compounds.



Figure 1.5: *Cannabis sativa* plant species. The image of *Cannabis sativa* plant was captured at Elukwatini in Mpumalanga province.

Cannabinoids are a family of naturally occurring compounds highly abundant in *Cannabis sativa* plant (Caffarel *et al*., 2012; Alexander *et al*., 2009; Safaraz *et al*., 2008; Happyana *et al*., 2013). Screening of *Cannabis sativa* has led to isolation of at least 66 types of cannabinoid compounds (Alexander *et al*., 2009; Yamaori *et al*., 2010; Happyana *et al*., 2013). Furthermore, cannabinoids can be classified into endocannabinoids such as anandamide and synthetic cannabinoids (Safaraz *et al*., 2008; Alexander *et al*., 2009). Endocannaboids are produced endogenously and often target cannabinoid receptors located on the surface of the cell, while synthetic cannabinoids are analogs of either endocannabinoids or plant derived cannabinoids

(Caffarel *et al*., 2012). These compounds are almost structurally similar or possess identical pharmacological activities and offer various potential applications including the ability to inhibit cell growth, proliferation and inflammation (Safaraz *et al*., 2008). One such compound is cannabidiol, which is among the top three most widely studied compound, following delta-9-tetrahydrocannabinol (Δ^9-THC) (Safaraz *et al*., 2008; Alexander *et al*., 2009).

1.8.1 **Cannabidiol**

Cannabidiol is the second most abundant compound found in *Cannabis sativa* plant, following delta-9-tetrahydrocannabinol (Δ^9-THC) (Yamouri *et al*., 2010; Happyana *et al*., 2013). It has been observed to be effective against a variety of disorders including neurodegerative disorders, autoimmune diseases, and cancer (Shrivastava *et al*., 2011; Solinas *et al*., 2012). In a research study conducted by Shrivastava *et al* (2011), it is suggested that CBD inhibits cell proliferation and induces apoptosis in a series of human breast cancer cell lines including MCF-10A, MDA-MB-231, MCF-7, SK-BR-3, and ZR-7-1. Another study conducted on PC-3 prostate cancer cell line demonstrated the ability of Δ^9-THC, CBN, and CBD to induce apoptosis via down-regulation of AKT protein (Sharma *et al*., 2014). CBD has also been implicated to play an inhibitory role against angiogenesis which involves the formation of new blood vessels from pre-existing ones (Solinas *et al*., 2012). This is evident in a study by Solinas *et al* (2012), wherein CBD was observed to inhibit migration of an invasive cell line, human umbilical vein endothelial cell (HUVEC). Therefore, in this study, cannabidiol was validated for its anti-cervical properties.

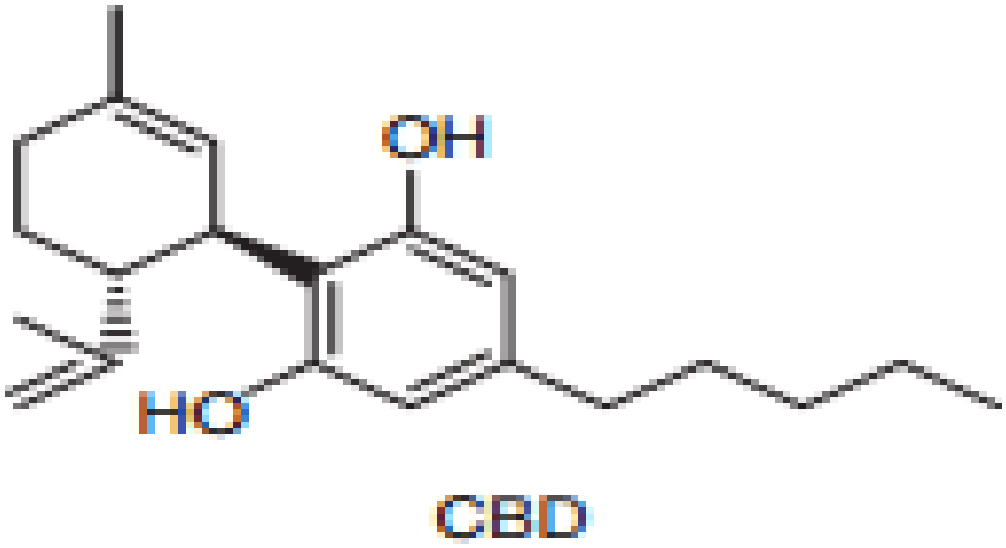

Figure 1.6: The chemical structure of cannabidiol (CBD), the second major cannabinoid constituent isolated from Cannabis sativa plant extracts (Adapted from Yamouri *et al*., 2010).

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RATIONALE AND OBJECTIVES

It is hypothesized that *Cannabis sativa* and its psychoactive substances have been used to alleviate pain and nausea in cancer and HIV patients, to allow the body to fight the disease. Therefore it is of utmost importance to explore this medicinal plant and its secondary metabolites for its anti-cervical cancer properties. Therefore, the aim of this study was to evaluate for the cytotoxic and anti-growth properties of *Cannabis sativa* and its isolate, cannabidiol, after treatment of cervical cancer cell lines and to check for any apoptosis induction in cervical cancer.

Objectives:

- To screen for the presence of secondary metabolites present in *Cannabis sativa.*
- To identify and separate compounds found in *Cannabis sativa*
- To evaluate for the cytotoxic effect of *Cannabis sativa* and cannabidiol in cervical cancer cells.
- To monitor the effect of *Cannabis sativa* and cannabidiol on ATP levels.
- To monitor cell growth in real time.
- To analyse the effect of *Cannabis sativa* and cannabidiol on the cell cycle.
- To analyse the mode of cell death induced.
- To analyse the morphology of the cells
- To evaluate protein expression of proapototic genes p53, and bax and antiapoptotic genes bcl-2, and RBBP6 before and after treatment with plant extract.
- To monitor the effect of *Cannabis sativa* and cannabidiol on caspase3/7 expression.

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CHAPTER TWO

METHODS AND MATERIALS

1. Introduction

This chapter serves to outline principles of each molecular technique and their procedures, to elucidate the mechanism of *Cannabis sativa* crude extracts following treatment of cervical cancer cells.

2.1 **Materials**

2.1.1. **Preparation of plant extracts**

Fresh leaves stem and roots of *Cannabis sativa* were collected in Elukwatini located in Mpumalanga, South Africa. *Cannabis sativa* was identified by a Forensic specialist in Silverton, lab number: 201213/2009, Pretoria The voucher specimen number CAS: 239/02/2009 of *Cannabis sativa* was deposited at Booysens Police station, Johannesburg. The plant material was washed with distilled water to remove dirt and contaminants. This was followed by drying the plant material in an oven at room temperature (25ºC). Dried material was ground to fine powder using a blender at low speed to avoid denaturing heat sensitive compounds within *Cannabis sativa* plant. Crude extracts were prepared separately using *n*-hexane and *n*-butanol. Ten grams (10 g) of fine powder was dissolved in a 100 ml of hexane and another 10 g was dissolved in butanol. Ground material was allowed to soak in the solvents for 4 days, to allow efficient isolation of metabolites. Following soaking, crude extracts were filtered using a syringe and microfilter of 0.22 µM. Filtered extracts were allowed to dry in a fume hood to evaporate the solvent. This was then followed by the addition of 4% DMSO to dissolve dried filtered extracts also to minimise contamination. Desired concentrations (50, 100, and 150 µg/ml) were prepared to treat the cells and the remaining crude extracts were stored at -20ºC.

2.1.2. Phytochemical screening of *Cannabis sativa* extracts

Cannabis sativa plant extracts were screened for the presence of secondary metabolites such as flavonoids, glycosides, phenols, phytosterols, triterpenoids, terpenes, tannins, steroids, and saponins, using standard procedures (Appendix) by Kalaisezhiyen and Sasikumar (2012) and Tiwari *et al* (2011).

2.1.3. **Cell culturing**

Two tumourigeneric cell lines (SiHa and HeLa) and a metastatic cell line (ME-180) were purchased from ATCC. SiHa is implicated to be caused by HPV 16, HeLa by HPV 18 while ME-180 is caused by HPV 39. These cell lines were chosen because they account for almost 70% of the total burden of cervical cancer worldwide. HPV 16 and 18 are two of the most common cause cervical cancer in South Africa and contribute immensely to the number of cases diagnosed and the mortality rates observed annually.

Cell culture media was prepared using Dulbecco's modified eagle's media (DMEM) (Highveld biological) supplemeted with 10% fetal bovine serum (FBS) (Highveld biological,) and 1% penicillin and streptomycin antibiotic (pen/strep) (Sigma,). Cells were initiated from a frozen vial wiped with 70% ethanol to reduce the risk of contamination and thawed in a laminah flow hood for less than a minute. SiHa and HeLa cells were added in 10 ml of media. To grow ME-180 cells, 50% of supplemented DMEM was mixed with 50% nutrient media (). Cells were incubated at 37°C with 5% carbon dioxide (CO_2) until they are 70% confluent.

2.1.4. **Cell detachment**

At 70% confluence, cells were detached using a 1X citric saline buffer (**APPENDIX A**) following cell detachment procedure (**APPENDIX 1B**). Cells were split at a ratio of 1:2 with 9 ml of media added into each flask. The flasks were further incubated at 37°C with 5% carbon dioxide (CO_2) until they become 70% confluent.

2.1.5. **Cell counting**

Old media was discarded and the cells were washed twice with 2 ml with PBS. A 2 ml of citric saline buffer was added to the cells for detachment. The flask was incubated

at 37°C with 5% carbon dioxide (CO_2) until cells were completely detached. Ten millilitres of DMEM was added to the flask to deactivate cell detachment. Cells were counted using Neubar Hemocytometer slide and an inverted microscope using the formula below:

$$\text{Number of cells/ml} = \frac{average\ number\ of\ cells}{4} \times 10^4$$

10^4 represent the dilution factor.

The counted cells were used during MTT assay and the xCELLigence assay.

2.2. **Methods**

2.2.1 **MTT assay**

MTT assay is a colometric method that is used to assess cell proliferation, viability, and cytotoxicity following treatment of cells with a toxic substance such as *Cannabis sativa*.. This method involves the use of a yellow MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), a water soluble tetrazolium salt, which is converted into insoluble formazan crystals. Only viable cells with an active metabolism can uptake the dye. This event occurs in the mitochondria wherein the active mitochondrial succinate dehyrogenase enzyme cleaves the tetrazolium ring and reduces the MTT reagent into purple crystals. These insoluble purple crystals are dissolved with a suitable solvent such as isopropanol or DMSO to measure the absorbance spectophotometrically at a specific wavelength. In this study, MTT assay was used to evaluate the cytotoxic effect of Cannabis sativa following treatment of SiHa, HeLa, and ME-180 cells and also account for IC_{50} which represents the half maximal concentration that induces 50% cell death.

Ninety microlitres of media only was added in some of the wells to serve as a blank. Five thousand cells in a 90 µl of DMEM were seeded in the remaining wells of a 96-well plate and incubated overnight at 37°C, to allow cells attachment. Following attachment, cells were treated with different concentrations (0, 50, 100, and 150

µg/ml) of *Cannabis sativa* hexane and butanol extracts and further incubated at 37°C for a period of 24 hrs. Treatment of cells with 0.3 µM camptothecin were included as a positive control while cells treated with 0.1% DMSO were included as a vehicle control. After 24 hrs, 10 µl of MTT reagent 5 mg/ml was added into each well except the blank followed by a 4 hr incubation period. Ninety microlitres of DMSO were added into each well after 4hrs, to dissolve the formazan crystals in each well and the absorbance was measured using a spectrophotometer plate reader (Thermoscientific, USA) at a wavelength of 570 nm.. The following formula was used to determine the IC50:

% Cell viability = (Abs treated - Abs blank / Abs untreated - Abs blank) x 100

Abs represents the absorbance readings

This procedure was conducted in duplicates and the best IC_{50} was further assessed during cell growth analysis, cell cycle analysis, apoptosis detection, and western blot.

2.2.2. **Mitochondrial assay (ATP detection)**

To assess the effect of treatment on the ATP levels in cervical cancer cells, an ATP detection assay was conducted using a mitochondrial assay kit (Promega) and a GLOMAX instrument. Mitochondrial assay is a molecular technique that is used not only to assess mitochondrial functioning before and after treatment with drug. This assay uses ATP and membrane integrity as biomarkers to assess dysfunction of the mitochondria following cytotoxic events.

Twenty five microlitres of $1x10^4$ cells per well were plated in a white 96-well luminometer plate overnight. Cells were treated with 25µl of IC_{50} concentrations of *Cannabis sativa* crude extracts and cannabidiol dissolved in a glucose free media supplemented with 10mM galactose. The plate was incubated at 37° in a humidified and CO2-supplemented incubator for a period of 24 hrs. Fifty microlitres of ATP detection reagent was added to each well and the plate further incubated for 30 min. Luminiscence was measured using GLOMAX (Promega, USA). The assay was

conducted in duplicates and ATP levels were reported as a mean of Relative Light Units (RLU). The following formula was used to calculate the ATP levels in RLU:

RLU =Luminiscence (sample) - Luminiscence (blank)

2.2.3. **Cell growth analysis**

To monitor cell growth, xCELLigence system was used. The xCELLigence system is a continuous method that involves the use of the real time cell analysis (RTCA) software, RTCA DP machine and 16-well E-plate to monitor cell proliferation, invasion, migration, viability, and cytotoxicity. The E-plates are integrated with microelectronic cell sensor arrays that detect cell attachment, size, density and morphology. During cell growth, the sensor arrays give off a signal in the form of electrical impedance that is displayed as cell index (CI). The cell index accounts for the relative change in the status of cell. In this study, the xCELLigence system was used to analyze cell growth before and after treatment with IC50 concentration of *Cannabis sativa* crude extracts.

A titration assay was performed for each cell line to determine the optimal cell density required for this experiment. Briefly, 100 µl of media only was added in each well incubated for 30 minutes (min) to equilibrate the plate before recording the background readings. After recording the background readings, a 100 µl of cells was added into each well and incubated for five days at 37˚C under 5% CO_2. A titration curve was constructed for each cell line. For the experiment, a 100µl of cells were seeded for each cell line according to their optimal response into a 16 well E-plate and incubated at 37˚C under 5% CO_2 for 30 min to allow cells to attach to the bottom of the plate. The E-plate was placed on the xCelligence machine (Roche) and incubated for 24 hrs, to monitor cell growth. This was then followed by addition of IC_{50} concentration of *Cannabis sativa* crude extracts and further incubated for a period of 24hrs. Camptothecin and DMSO were included as controls for comparative purposes. Cell index readings were recorded at equal intervals.

2.2.4. **Cell cycle analysis**

Cell cycle analysis is an important method used to determine cell cycle status and to distinguish between populations of cells in different stages of the interphase. This method uses a DNA- specific fluorescent dye and flow cytometry to measure the relative amount of DNA present in the cells. In this study propidium iodide (PI) was used to stain the cells. Viable cells with an intact plasma membrane cannot uptake the dye. Propidium iodide can only intercalate into the DNA of fixed and permibilised cells with a compromised plasma membrane or cells in the late stage of apoptosis. The intensity of stained cells correlates with the amount of DNA within the cells. HeLa, SiHa, and ME-180 cells were stained with PI and analysed using flow cytometry.

Cells were harvested with 2 ml of citric saline buffer. Ten millilitres of media was added to the cells to inactivate citric saline buffer and the cell suspension was centrifuged at 1500 rpm for 10 min. The supernatant media was discarded and the pellet was resuspended twice in 1 ml PBS. Cell suspension was transferred to a 1.5 ml eppendorf tube and centrifuged at 5000 rpm for 2-5 min wherein PBS was discarded. To permeabilize and fix the cells, 700 µl of pre-chilled 100% absolute ethanol stored at -20º C was added to the cells and the tube was inverted a few times to ensure proper mixing. The cells were then stored at -20º C for 30 min to allow efficient permeabilisation and fixing of the cells. After 30 min, cells were centrifuged at 5000 rpm for 5 min to remove ethanol. The pellet was washed twice with PBS and centrifuged at 5000 rpm to remove PBS. Five hundred microlitres of FxCycle^{TM}PI/RNase Staining solution (Life technologies, USA) and was vortexed for 30 seconds (sec). The cells were analysed with FACSCalibur (BD Biosciences, USA).

2.2.5. **Apoptosis assay**

Apoptosis assay allows the detection of cells undergoing apoptosis using a fluorescent dye and flow cytometry instrument for analysis. In this study, an Annexin-V dye conjugated with FITC and PI was used to distinguish live cells from apoptotic and necrotic cells following treatment of SiHa, HeLa, and ME-180 cells with IC_{50} *Cannabis sativa* crude extracts. Viable cells do not uptake both dyes as a result of an intact plasma membrane and necrotic cells can only uptake propidium iodide (PI), as result of cells bursting leading to an exposure of DNA content. However, apoptotic

cells uptake both dyes as a result of treatment compromising the plasma membrane, thus leading to an exposure of the phosphotidylserine (PS) residues. Annexin-V binds to the PS residues while PI further intercalates into the DNA and binds to the nucleic acids.

Cells were harvested using citric saline buffer and 10 ml of media was added to the cells to deactivate cell detachment. Media was discarded after centrifuging at 1000 rpm and cells were twice with PBS to remove any residual media. Flow cytometry was performed using Biolegend FITC Annexin V Apoptosis Detection kit with PI, following manufacturer's protocol with slight modifications. Briefly, cells were washed twice with 100 µl of cold Biolegend's cell staining buffer followed by resuspension in 100 µl of Annexin V binding buffer. A 100 µl of cell suspension was transferred into 15 ml test tube and 5 µl of FITC Annexin V was added to it. Ten microlitres of PI was added into the untreated and treated cell suspension. The cells were gently vortexed and incubated at room temperature (25º C) in the dark for 15 min. After 15 min, 400 µl of Annexin V binding buffer was added to the cells. The stained cells were analysed using FACSCalibur (BD Biosciences, USA).

2.2.6 **Morphological analysis**

To confirm the induction of apoptosis following treatment of cells with $IC_{50,}$ Fluorescence confocal microscopy was conducted. This was done in order to assess for any morphological changes that might occur during treatment with *Cannabis sativa* extracts.

Five hundred microlitre of 1x 10^4 cells was added onto a 6-well plate containing coverslips. The plate was incubated overnight to allow the cells to attach. Following attachment, media was removed and cells were washed twice with PBS, prior to incubation with IC_{50} of Cannabis sativa extracts for 24hrs. After 24hrs, media was removed and cells were washed twice with PBS. Four percent (4%) was added into each well and the plate incubated for 20 min at room temperature, to allow efficient fixation of cells. cells were washed twice with PBS and once with 0.1% BSA wash

buffer. Cells were stained with DAPI and Annexin V/FITC for 5 min and visualized using BX-63 Olympus microscope (Germany).

2.2.7. **Western blotting**

The media was carefully removed from a flask containing confluent cells and washed twice with 2 ml of cold PBS, to remove any residual media. An amount of 200 µl of RIPA buffer (Thermo scientific, USA) was added into the cells and the flask was placed on ice for approximately 5 min, to ensure that there is efficient lysis of the cells. After cell lysis, the adherent cells were scraped from the surface of the flask. The lysate was transferred to an eppendorf tube and centrifuged at 14000 g for 20minutes to get a pellet.

2.2.7.1. **Protein quantification**

The supernatant was aliquoted and the protein content in the lysate was measured using BCA protein assay (Thermo scientific pierce, USA) according to the manufacturers' protocol. Briefly, protein concentration was determined and reported with reference to standards of bovine serum albumin (BSA). One vial of BSA was serially diluted with distilled water into an eppendorf tube in a working rage of 0-2000 µg/ml in a volume of duplicates. A serial dilution of an unknown sample of protein extracted from SiHa, HeLa, and ME-180 cell line of both the untreated and treated with *Cannabis sativa* and cannabidiol were prepared for measurement. Equal amounts of unknown sample of protein lysates and BSA standards were loaded onto a 96-well plate. Two hundred microlitres of working reagent (WR) containing a 50:1, BCA reagent A: B was added to each well and mixed thoroughly on a plate shaker for 30 seconds, then covered and incubated at 37°C for 30 minutes. The plate was cooled to room temperature the absorbance at or near 560 nm was read using a microplate reader (Thermo scientific, USA). The absorbance of the standards versus their concentration was plotted determine the concentration of the unknown protein sample.

2.2.7.2. **Protein separation**

Aliquots of the lysates were boiled for 10min at 70°C in a laemmlie sample buffer (**APPENDIX B**). Equal amounts of treated and untreated protein (30µg of protein) were separated on a 12% SDS-PAGE and transferred to a nitrocellulose membrane overnight at 4°C using transfer buffer (25 mM Tris-Hcl, 192 nM glycine and 20% methanol [v/v]). The membranes were incubated for 10 min in a Ponceau S staining solution to confirm protein transfer. The membranes were washed with three times PBS-Tween buffer which was followed by blocking for 1h with 5% non-fat dried milk dissolved in PBS-Tween. Membranes were further washed with PBS-Tween, to remove milk residues that my affect binding of an antibody. The membrane was incubated in a primary antibody (anti-human p53 (1:750), Bcl-2(1:750), Bax (1:500), RBBP6 (1: 1000), caspase 9 (1:750), caspase 3 (1:750) and Beta actin (β-actin) (1:500)) overnight followed by washing three times with PBS-Tween for 10 minutes per wash. A secondary antibody, (Santa cruz, USA), dissolved in milk containing PBS-Tween was added to the membrane and incubated at room temperature for 2 hrs. A goat anti-mouse horseradish peroxidise-conjugated horse IgG at a dilution of 1:5000 (Santa cruz, USA) was used as a secondary antibody. The membranes were developed using chemiluminescence detection kit and imaged using a Chemidoc MP from Biorad. Densitometry analysis was performed using Totallab quantification software to measure the relative expression of p53, Bax and Bcl-2 protein. Beta actin was used as a reference protein, to normalize the expression of p53, Bax, RBBP6, Bcl-2, caspase 9 and -3 proteins.

2.2.8. **Capsase 3/7 assay**

Caspase 3/7 assay is a qualitative method that determines the activity of caspase 3 and 7 in the cells. It uses a caspase substrate that is conjugated with an enzyme luciferin. Caspase substrate lyses and enters the cell to interact with caspase 3/7, which causes the cleavage and activation of luciferin enzyme, resulting emmsion of light, measured in relative light units (RLU) by using a luminometer.

A hundred microlitres of $1x10^4$ cells were plated overnight on a 96-well luminometer plate and allowed to attach overnight. The next day, cells were treated with 0.3 µM camptothecin and the IC_{50} concentrations of *Cannabis sativa* crude extracts and

further incubated for a period of 24 hrs. Caspase-Glo 3/7 assay was performed according to manufacturer's protocol (Promega, USA). Briefly, following treatment, media was replaced with caspase glo 3/7 reagent mixed with a substrate at a ratio of 1:1 v/v of DMEM: Caspase-glo 3/7 reagent and was incubated for 2 hrs at 37ºC in 5% CO_2. Luminescence was quantified using GLOMAX from Promega (USA).The assay was conducted in duplicates and caspase 3/7 activity was reported as a mean of Relative Light Units (RLU). The following formula was used to calculate caspase 3/7 activity in RLU:

RLU =Luminiscence (sample) - Luminiscence (blank)

2.2.9 **Data analysis (Statistics)**

Experiments were performed in duplicates. Statistical analysis of the graphical data was expressed as the mean standard deviation. The p-value was analysed for significance in comparison to the untreated using Students t-Test ($p < 0.05$).

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CHAPTER THREE

RESULTS

3. **Introduction**

The aim of this study was to screen and evaluate the cytotoxic effect of *Cannabis sativa* extracts in cervical cancer cells. Most *in vivo* and *in vitro* studies have reported results based on the screening of chemical constituents isolated from *Cannabis sativa* against cancer and other disorders. However, studies conducted on *Cannabis sativa* being used as a crude extract in cervical cancer and the underlying mechanism of cell death induced by the extracts have not been well documented so far. This chapter of results serves to report on the ability of *Cannabis sativa* crude extracts to inhibit growth and induce cell death during treatment of cervical cancer cells and also account for the mode of cell death induced.

3.1 ***Phytochemical screening of Cannabis sativa extracts shows the presence of secondary metabolites.***

In order to evaluate for the presence of secondary metabolites, which have been shown to have anti-neoplastic effects, biochemical assays were conducted. Based on these biochemical assays, the presence of flavonoids, glycosides, phenols, quinones, steroids, saponins, tannins, triterpenes, and triterpenoids were detected.

Table 3.1: Phytochemical screening of secondary metabolites from *Cannabis sativa* crude extracts.

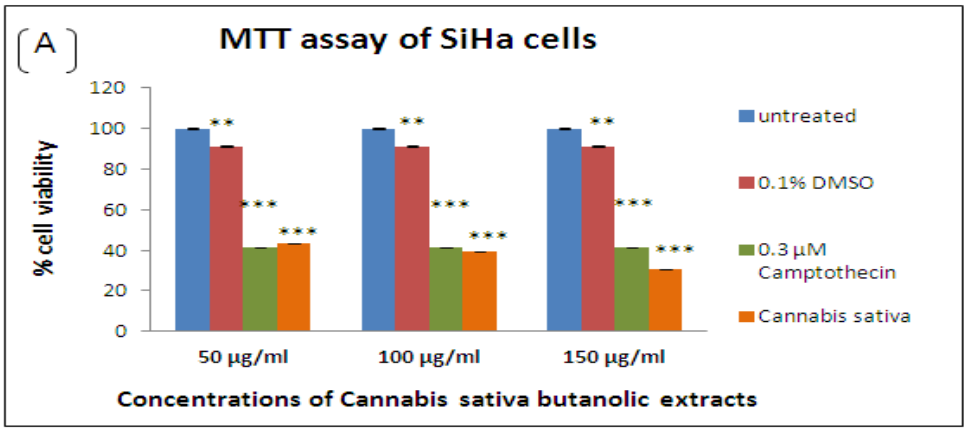
Secondary metabolites	Butanol extracts	Hexane extracts
Flavonoids	+	++
Glycosides	++	++
Phenols	++	++
Quinones	++	+

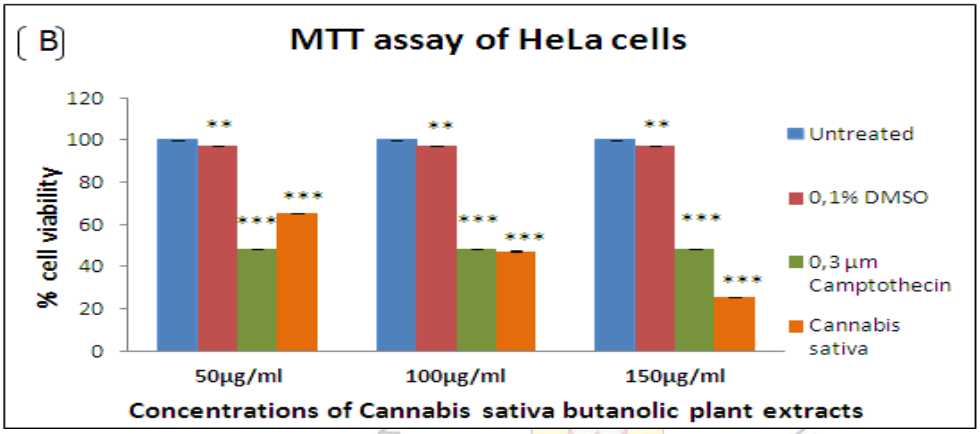
Steroids	++	+
Saponins	-	++
Tannins	++	++
Triterpenes	++	+
Triterpenoids	++	++

3.2 *The cytotoxic effect of Cannabis sativa extracts*

To evaluate the cytotoxic effect of *Cannabis sativa* crude extracts on cervical cancer cells and also account for IC_{50}, MTT assay was conducted. The IC_{50} represents the half maximal inhibitory concentration that causes 50% cell death. Briefly, cells were incubated with various concentrations of *Cannabis sativa* crude extracts for a period of 24 hrs. A known cell death inducing agent, camptothecin, was used as a positive control. Camptothecin significantly reduced cell viability of SiHa (40.36%), HeLa (47.19 %), and ME-180 cells (32.25%), respectively. In comparison to the positive control and the untreated, *Cannabis sativa* crude extracts reduced cell proliferation and increased cell death in all three cell lines in a dose dependent manner. As shown in **Figure 3.1** and **Figure 3.2**, the cytotoxic activities of each plant extract were different for each cell line.

After treatment of SiHa cells with butanol and hexane extract, the IC_{50} was found to be at the lowest concentration of 50 µg/ml. Fifty microgram per millitre of butanol extract reduced viability of SiHa cells to 56.6% while hexane reduced it to 48.4%. It can also be noted that a similar trend (reduction of cell viability at the same IC_{50} concentration) was observed during treatment of HeLa cells, however at a higher concentration. **Figure 3.1B** and **Figure 3.2E** shows that at an IC_{50} of 100µg/ml, the viability of HeLa cells significantly ($p<0.001$) reduced to 52.7% and 54%, respectively. On the other hand, treatment of ME-180 cells with *Cannabis sativa* butanolic extract exhibited an IC_{50} of a 100µg/ml, reducing viability to 48.6% (**Figure 3.1C**). Hexane extracts reduced viability ME-180 cells to 54% at an IC_{50} of 50µg/ml (**Figure 3.2F**).





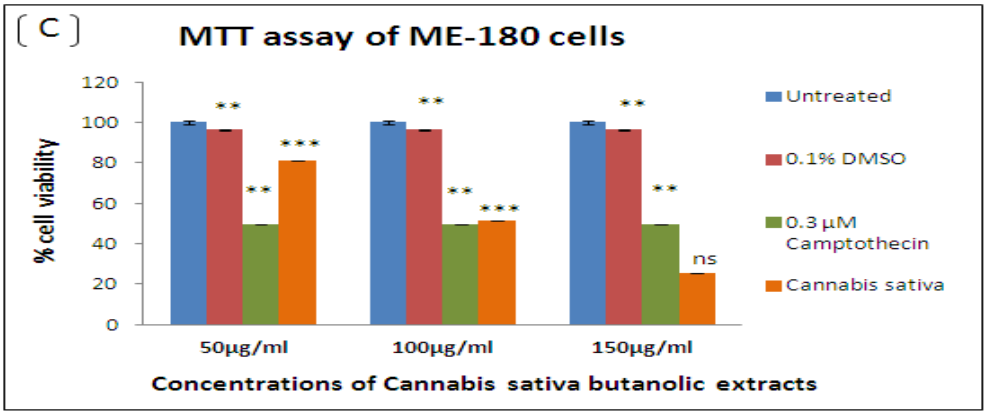
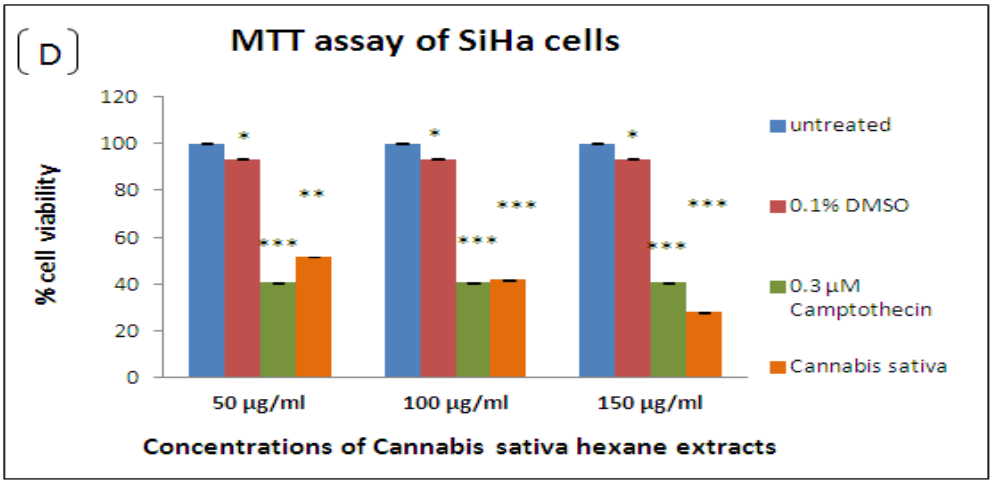
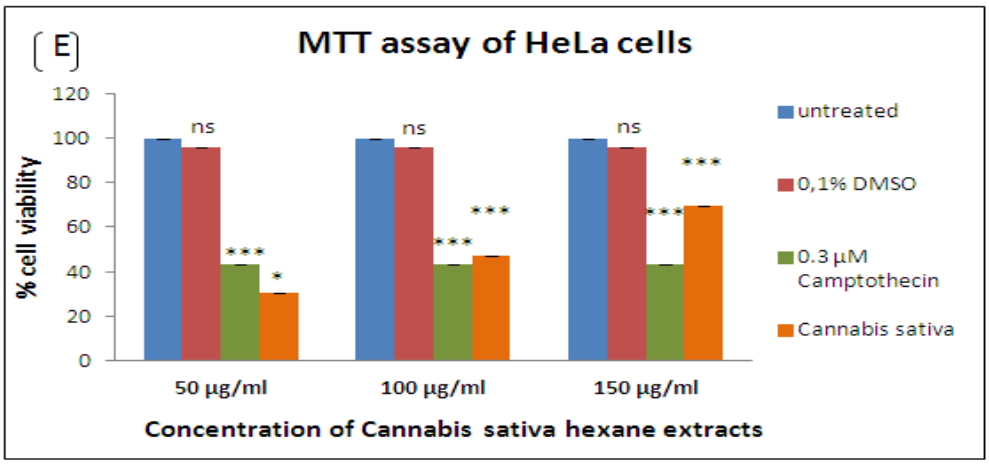


Figure 3.1: Cytotoxic effects of *Cannabis sativa* butanolic extract on cervical cancer cell lines. SiHa cells (**A**), HeLa cells (**B**), and ME-180 cells (**C**) were treated with various concentrations (50, 100, and 150 µg/ml) of *Cannabis sativa* butanolic extract for a period of 24 hrs. DMSO, camptothecin were included as controls. Data was expressed as the mean ± SD and ***$p<0.001$, **$p<0.01$ and ^{ns} $p>0.05$ representing the

level of significance in comparison to the untreated.





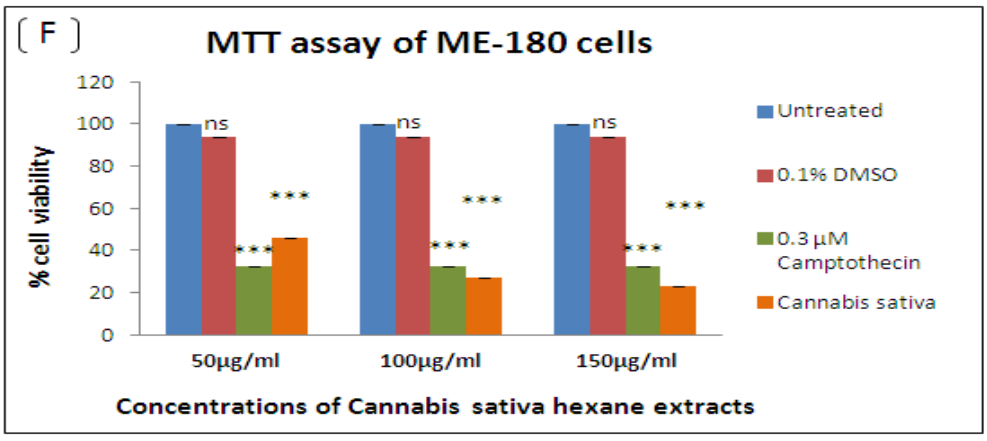


Figure 3.2: Cytotoxic effects of *Cannabis sativa* hexane extract on cervical cancer cell lines**.** SiHa cells (**D**), HeLa cells (**E**), and ME-180 cells (**F**) were treated with various concentrations (50, 100, and 150 µg/ml) of *Cannabis sativa* hexane extract for a period of 24 hrs. DMSO, camptothecin were included as controls. Data are

expressed as the mean ± SD and ***p<0.001, **p<0.01, *p<0.05, and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

DMSO was included as a vehicle control, to check whether the observed cell death was due to the plant extract or DMSO. Exposure of cells to DMSO had little effect on the cells (4-11%), which led to the conclusion that the plant extracts were responsible for the observed cytotoxic activities. In summary, the reduction of cell viability by the plant extract was observed to be cell line and concentration dependent.

3.3 *Effect of Cannabis sativa crude extracts on the ATP levels.*

Since Adenosine 5'-triphosphate acts as a biomarker for cell proliferation and cell death, an ATP assay was conducted. This was done in order to determine whether *Cannabis sativa* depletes ATP levels in cervical cancer cells. SiHa, HeLa, and ME-180 cells were treated at various time points, at 2 and 24 hrs. ATP levels were first detected after 2hrs.

Figure 3.3 shows that *Cannabis sativa* was able to reduce the levels of ATP in a cell line dependent manner. At 2 hrs, there was a significant difference (p<0.01) between the ATP levels of *Cannabis sativa* treated and untreated SiHa cells. At an IC_{50}= 50µg/ml, the ATP levels of both butanol and hexane treated cells were significantly reduced by 31% (from 4719589 RLU to 3221245) and 22.5% (4719589 to 3655730 RLU) when compared to untreated cells. HeLa cells displayed the highest reduction of ATP levels during treatment with butanol and hexane extract (**Figure 3.3B**). At a concentration of 100µg/ml, butanol and hexane extracts significantly reduced ATP levels by 74% (from 627621 to 164208 RLU) and 78% (from 627621 to 133693 RLU). However, this was not the case with treatment of ME-180 cells. At an IC_{50}=100µg/ml, there was not much difference between the untreated and butanol treated cells (from 803350 to 780363 RLU) as shown in **Figure 3.3C**. On the other hand, the IC_{50}=50µg/ml of hexane extract increased the levels of ATP in ME-180 cells (from 803350 to 888201 RLU). An increase in the ATP levels has been implicated to represent cell proliferation. The activity of *Cannabis sativa* extracts was similar to that of camptothecin, wherein treatment of all three cell lines either caused ATP levels to

decrease or increase during 2 hr incubation period (**Figure 3.3A, B, C**). Prolonged incubation periods (24 hrs) with camptothecin and the IC_{50} of *Cannabis sativa* extracts, resulted in a decrease of ATP levels in all three cell lines. At 24 hrs, camptothecin caused the reduction of ATP in SiHa (from 4868049 to 3255189 RLU), HeLa (from 601694 to 90525 RLU), and ME-180 cells (from 893657 to 782063 RLU). Treatment of SiHa and HeLa cells with IC_{50} of *Cannabis sativa* butanol and hexane extracts for a period of 24 hrs resulted in a severe depletion of ATP levels than camptothecin. At 24 hrs, the IC_{50} of butanol extract significantly reduced the levels of ATP in SiHa, HeLa, and ME-180 cells by ~42% (from 4868049 to 2822675 RLU), 80% (from 601694 to 117564 RLU), and 14% (from 893657 to 762302 RLU) when compared to the untreated cells. On the other hand, IC_{50} of hexane extracts was more or less effective than butanol in reducing ATP levels in SiHa (from 4868049 to 3278271), HeLa (from 601 694 to 90525), and ME-180 cells (from from 893657 to 680437). A series of studies have suggested that the depletion of ATP levels is a determinant of cell death by apoptosis or necrosis. Therefore, we can conclude that depletion of ATP levels may be caused by induction of cell death and that it may be cell-line dependent.

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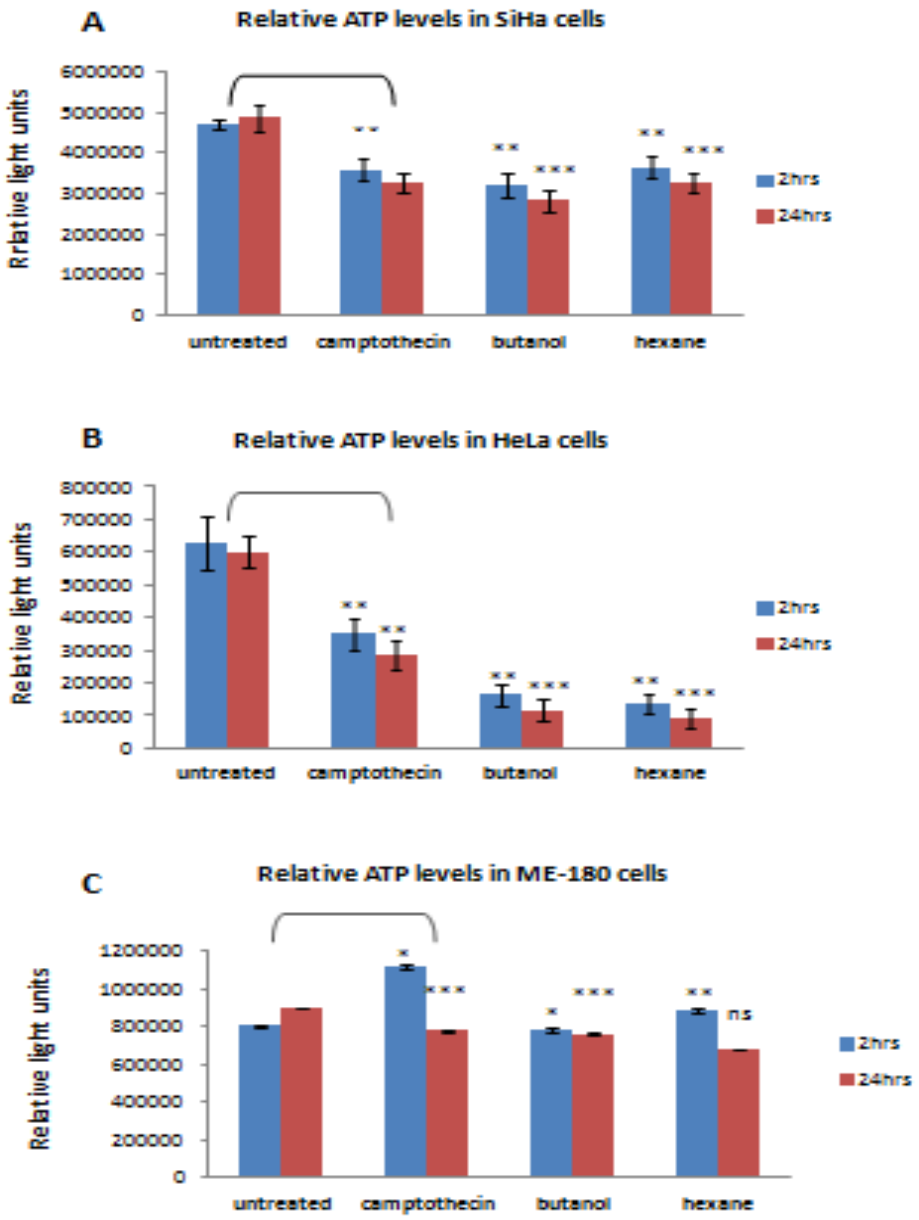


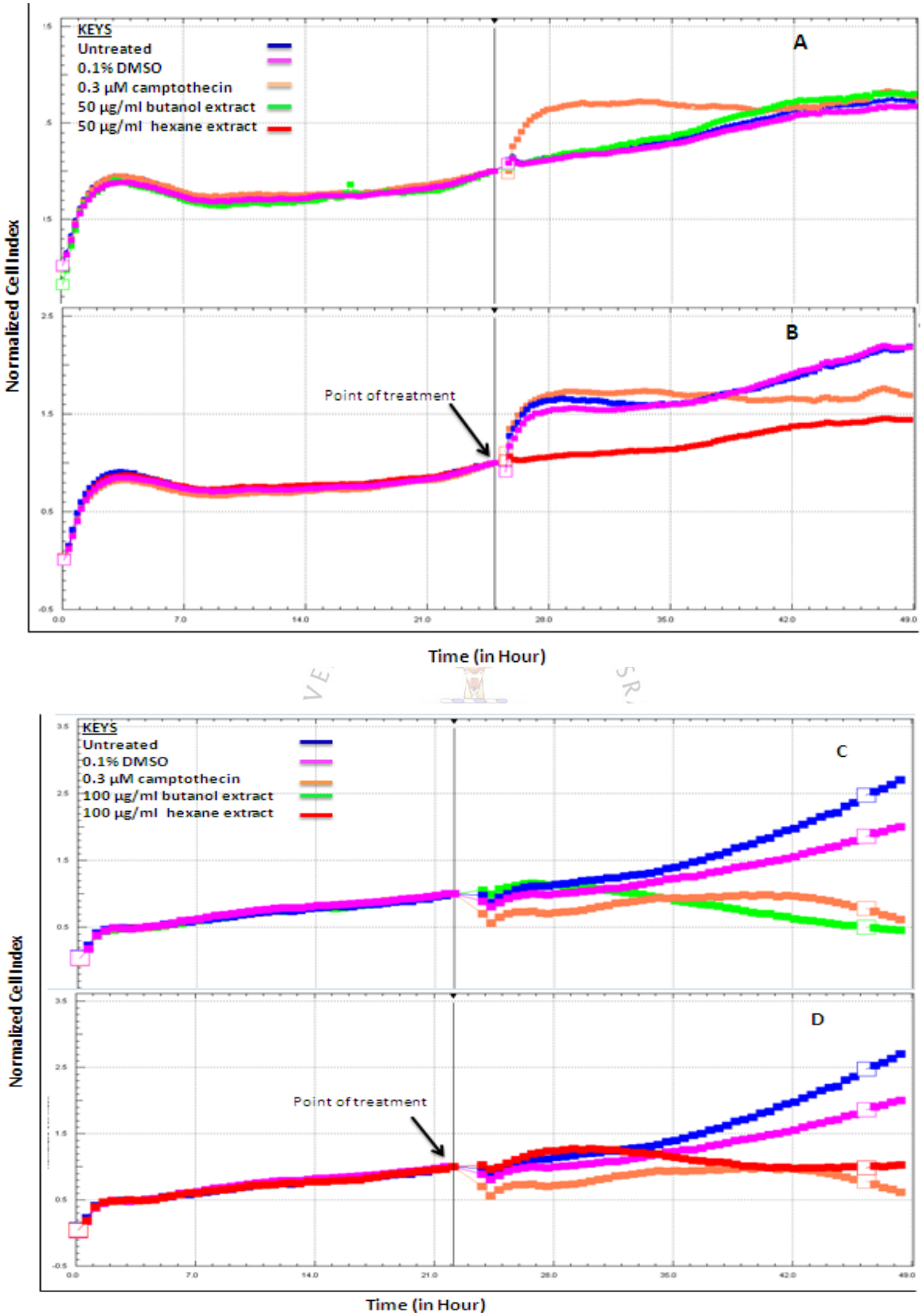
Figure 3.3: Representative bar graphs of changes in the ATP levels following treatment of cervical cancer cells with *Cannabis sativa* extracts. Cells were treated with IC_{50} of butanol and hexane extract for a period of 24 hrs. Untreated and camptothecin were included as controls for omparative purposes. Data represented as mean ± standard deviation with *** $p<0.001$, **$p<0.01$, *$p<0.05$, and ^{ns} $p>0.05$ representing the level of significance in comparison to the untreated.

3.4 ***Effect of Cannabis sativa extracts on cell growth of cervical cancer cells.***

The IC_{50} concentrations obtained during MTT assay were tested for their ability to alter cell viability in real time. An impedance based system was employed to validate the effect of the *Cannabis sativa* extracts on cervical cancer cell lines SiHa, HeLa, and ME-180. Firstly, the optimal response of each cell line was determined by seeding different cell densities, ranging between 625 and 20 000 cells per well. As shown on in **APPENDIX C**, the optimal response for SiHa and HeLa was obtained at 10 000 cells while ME-180 cells was at 20 000. Cells were further seeded E-plate according to their respective optimal densities and treated with IC_{50} for a period of 22-24 hrs, depending on their doubling time. Continuous changes in the impedance were measured and displayed as cell index (CI).

Analysis of cervical cancer cells with xCELLigence displayed a normal growth pattern before treatment. Treatment of HeLa and ME-180 cells with 0.3µM camptothecin reduced the impedance as shown in **Figure 3.4C, D, E, and F**. This means that the cells might have been detaching, thus reducing the cell index. However, this was not the case with SiHa cells. There was a rapid increase in the relative impedance for a period of 4 hrs after treatment with camptothecin followed by stabilization of the growth curve throughout the period of treatment. In such a case, camptothecin might have been inducing cytostasis, preventing the cells from growing and multiplying.

Cannabis sativa extracts inhibited growth in a cell type and concentration dependent manner. SiHa cells continuously grew after treatment with 50µg/ml butanol and hexane extracts as shown on **Figure 3.4** (**A and B**).On the other hand, exposure of HeLa and ME-180 cells to a 100µg/ml of butanol and 50µg/ml extract altered the growth curve and reduced the cell index, signalling of cell growth inhibition (**Figure 3.4C, D, E, and F**).In HeLa cells, IC_{50}= 100µg/ml of butanol extract reduced cell growth. HeLa cells managed to recover after treatment with 50µg/ml of hexane extract as observed in **Figure 3.4D**.



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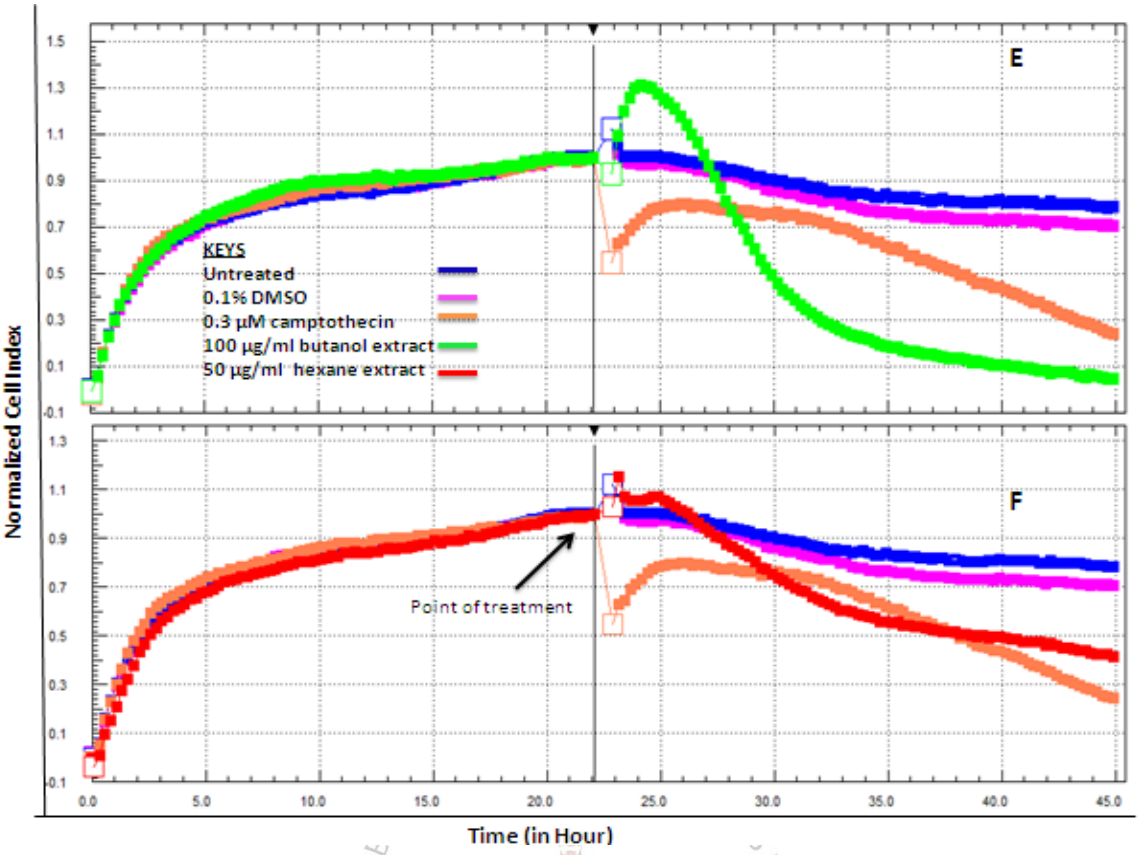

Figure 3.4: xCELLigence analysis of the cell growth pattern after treatment of SiHa (A and B), HeLa (C and D), and ME-180 (E and F) cells with *Cannabis sativa* extracts. Cells were seeded for a period of 24 hrs, followed by treatment with IC_{50} concentration of B) and D) hexane extracts represented by a curve highlighted in while A) and C) butanol extracts is represented by a growth curve highlighted in green. Untreated, DMSO, and camptothecin curves highlighted in blue, magenta and choral were included as controls for comparative purposes. Media only was used as a baseline cell index.

Treatment of cells with 0.1% DMSO did not alter the relative impedance (**Figure 3.4**). Cells continuously proliferated clearly indicating that DMSO does not affect cell viability. The crude plant extracts might be responsible for the reported activity.

3.5 ***Effect of Cannabis sativa crude extracts on cell cycle progression.***

Flow cytometry was conducted to evaluate the effect of *Cannabis sativa* extracts on cell cycle progression of SiHa, HeLa, and ME-180 cells. Cells were treated with IC_{50} concentration of each crude extract for a period of 24 hrs and stained with PI. The

number of cells detected in a specific phase is proportional to the relative amount of DNA. Relative DNA of untreated and treated cells was represented in all three phases including sub-G_0, initially known as the cell death phase. As shown in **Figure 3.5E**, no cell cycle arrest was exhibited following treatment of SiHa cells with camptothecin and *Cannabis sativa* crude extracts. Flow cytometry showed that in the presence of *Cannabis sativa* crude extracts and camptothecin, SiHa cells exhibited a significant increase ($p< 0.001$) in sub-G_0 population with a decrease in G0/G1, S, and G2/M phase in a dose dependent manner (**Figure 3.5**). At an IC_{50} concentration of 50µg/ml, butanol extracts increased the percentage of cells in the sub-G_0 phase from 4.2% to 20.1%, while decreasing the G_0/G_1 (from 64.0% to 48.7%), S-phase (from 9.3% to 6.5%), and G_2/M (from 18.5% to 17%) population as shown in (**Figure 3.5C**) and the bar graph. A similar event (accumulation of cells in the sub-G_0 phase) was observed after treatment of SiHa cells with hexane extracts at an IC_{50} concentration of 50µg/ml. Following treatment, most of the SiHa cells were accumulated at sub-G0 phase (39.1%) when compared to untreated cells (4.2%), with a simultaneous decrease in G_0/G_1 (from 64.0% to 30.4%), S (from 9.3% to 6.5%), and G_2/M (from 18.5% to 13.8%) population (**Figure 3.5D**). This could mean that, *Cannabis sativa* leads to activation of cell death without cell cycle arrest.

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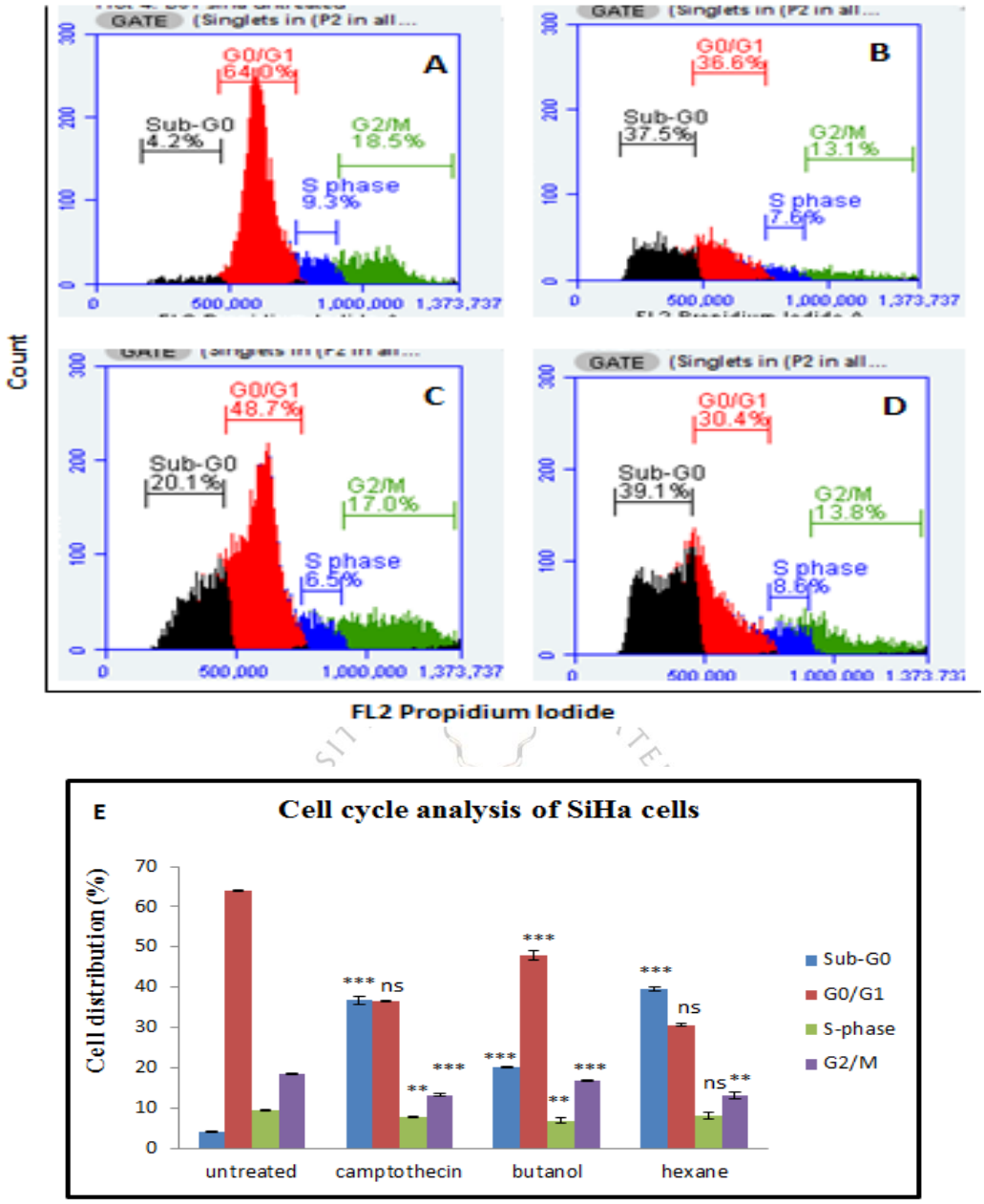
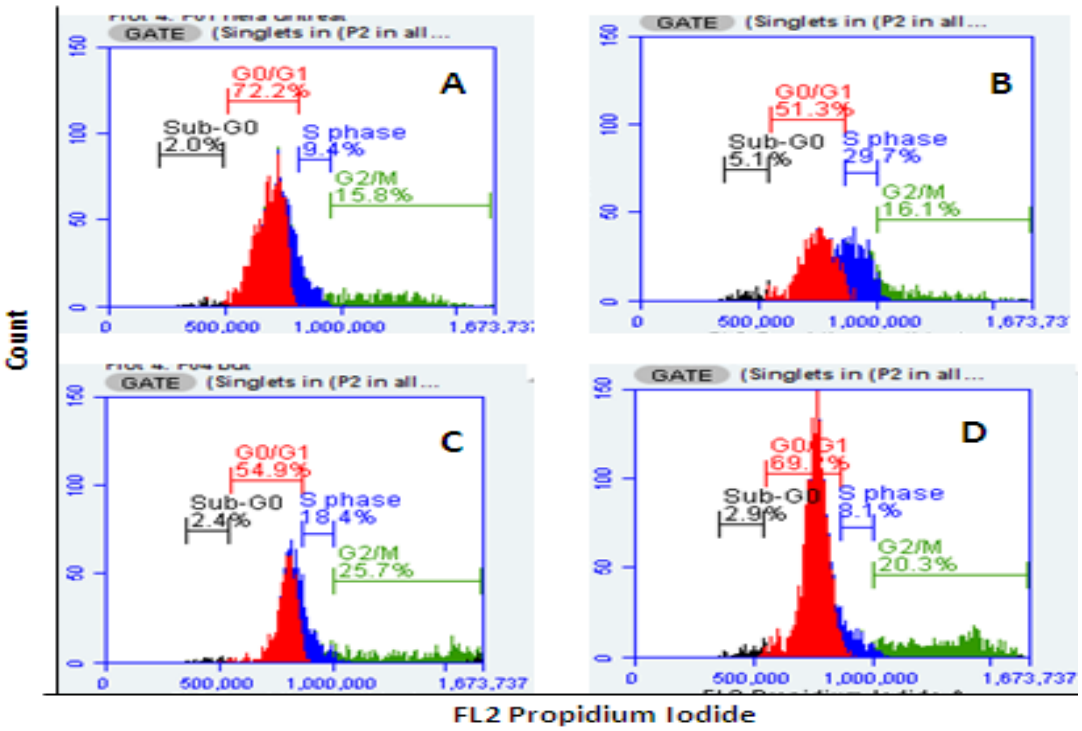


Figure 3.5: Representative histograms and bar graph of the SiHa cell cycle before and after treatment with camptothecin and *Cannabis sativa* crude extracts. Cells were harvested and treated with camptothecin and the IC_{50} concentrations of *Cannabis sativa* extracts. **A** is untreated cells, **B** is cells treated with camptothecin, **C** is cells treated with butanol extract, and **D** is cells treated with hexane extract. Data represented as mean ± standard deviation with *** $p<0.001$ and **$p<0.01$, and ^{ns} $p>0.05$ representing the level of significance in comparison to the untreated.

A different mechanism was observed upon treatment of HeLa cells with camptothecin and *Cannabis sativa* crude extracts. No cell cycle arrest was observed, however, a slight or no cell death was induced after treatment of HeLa cells. Camptothecin significantly increased the population of HeLa cells in the S-phase from 9.4% to 29.7% and in the G2/M phase, from 15.8% to 16.1% simultaneously reducing G_0/G_1 population from 72.2% to 51.3% (**Figure 3.6B**). A slight increase in the sub-G_0 population was also observed (from 2.0% to 5.1%). Similar results were observed after treatment of HeLa cells with IC_{50} concentrations. At 100µg/ml, butanol extracts reduced G_0/G_1 population to 54.9% while the S-phase and G_2/M significantly increased to 18.4% and 25.7%. This clearly indicates proliferation of the cell cycle. Treatment of HeLa cells with a 100µg/ml of hexane extracts led to an increase in the G_2/M phase (20.3%) and a decrease in the S-phase (8.1%) in comparison to the G_2/M (15.8%) and S-phase (9.4%) of untreated cells, signalling the blockage of mitosis induction (**Figure 3.6A** and **B**). Cell death (sub-G_0) slightly increased by a difference of 0.9%.



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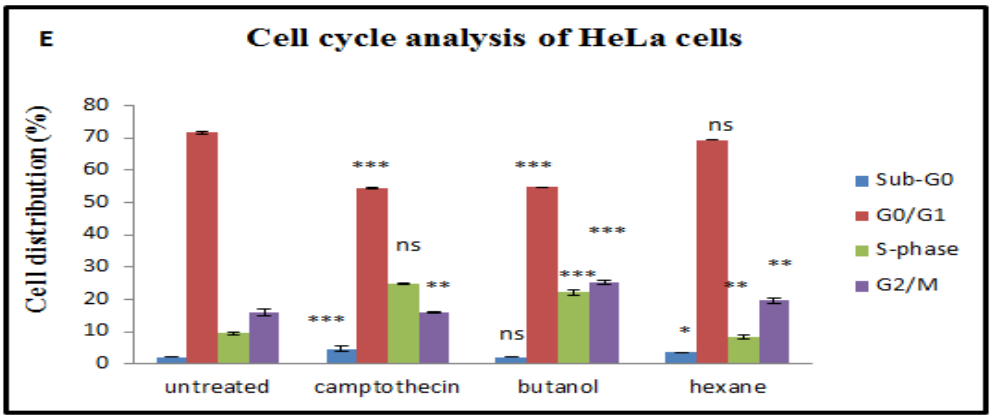

Figure 3.6: Representative histograms and bar graph of the HeLa cell cycle before and after treatment with camptothecin and *Cannabis sativa* crude extracts. Cells were harvested and treated with camptothecin and the IC_{50} concentrations of *Cannabis sativa* extracts. **A** is untreated cells, **B** is cells treated with camptothecin, **C** is cells treated with butanol extract, and **D** is cells treated with hexane extract. Data represented as mean ± standard deviation with *** $p<0.001$, **$p<0.01$, *$p<0.05$, and ^{ns} $p>0.05$ representing the level of significance in comparison to the untreated.

On the other hand, in ME-180 cells, there was a significant increase in the sub-G_0 population following treatment with all extracts including camptothecin (**Figure 3.7A and B**), signalling the activation of cell death. Treatment of ME-180 cells with 0.3 µM of camptothecin led to a significant increase ($p < 0.001$) in sub-G_0 population (from 1.8% to 15.9%) simultaneously decreasing the G2/M population from 24.0% to 17.8%. Cells continually progressed to the S-phase (from 5.2% to 13.0%) as shown on (**Figure 3.7E**). In comparison to the untreated, the hexane extract failed to inhibit cell cycle progression in ME-180 cells (**Figure 3.7B**). Although most of the cells were accumulated in the G_0/G_1 phase, however, an increase in the S-phase population following treatment with hexane (5.2% to 14.6%) clearly indicates proliferation of the cell cycle. A shift in the population of cells towards the sub-G0 phase was indicative of cell death induction without cell cycle arrest. Treatment of ME-180 cells with 100 µg/ml of butanol extract led to an increase in the G2/M population (27.5%) when compared to the untreated (24%), signalling the blockage of mitosis induction and the

inhibition of cell cycle progression. Figure 3.6A and B suggests the induction of cell death as well with 5.1%.

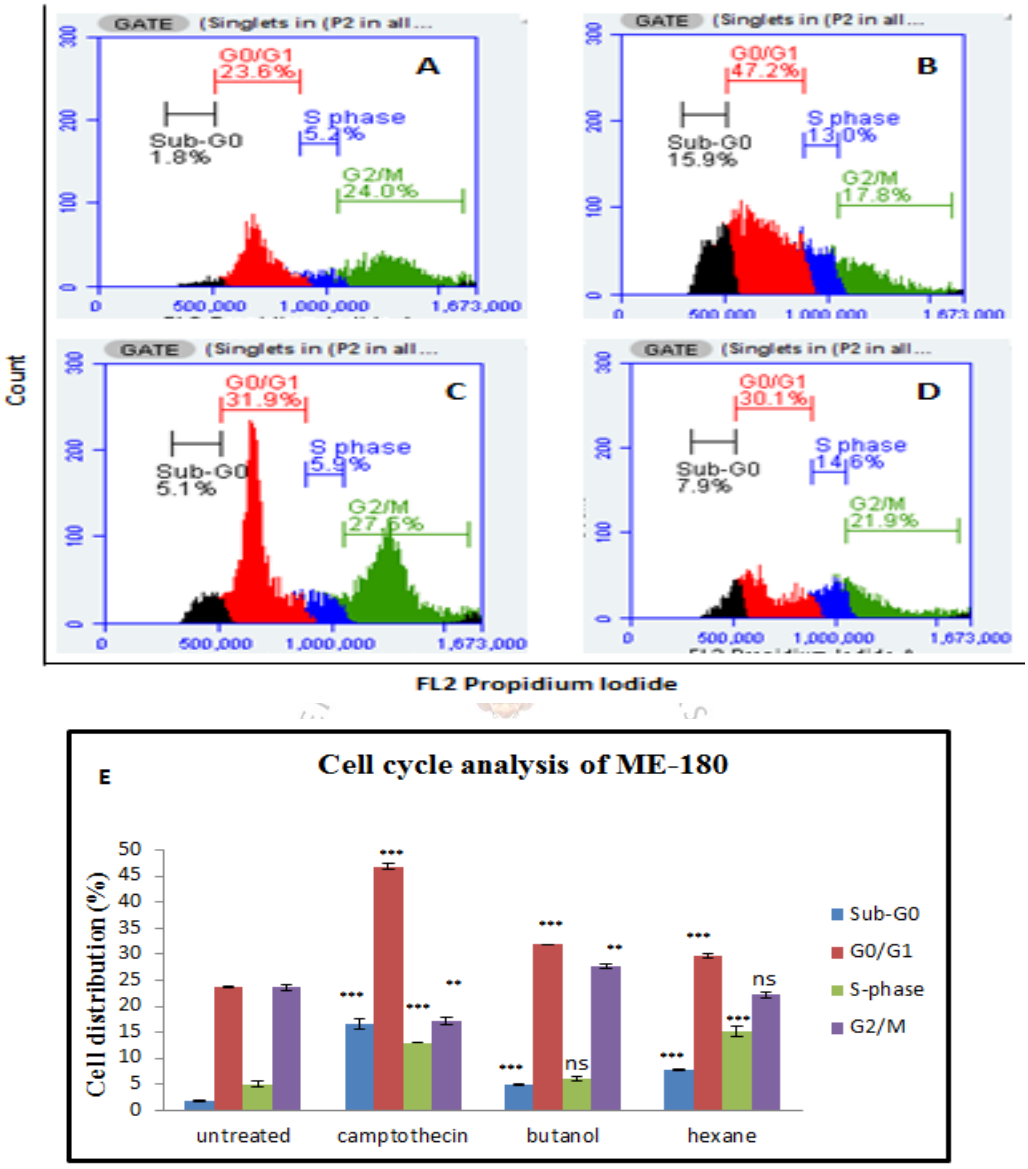

Figure 3.7: Representative histograms and bar graph of the ME-180 cell cycle before and after treatment with camptothecin and *Cannabis sativa* crude extracts. Cells were harvested and treated with camptothecin and the IC_{50} concentrations of *Cannabis sativa* extracts. **A** is untreated cells, **B** is cells treated with camptothecin, **C** is cells treated with butanol extract, and **D** is cells treated with hexane extract. Data represented as mean ± standard deviation with ***p<0.001, **p<0.01 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

3.6 ***Cannabis sativa crude extracts induce apoptosis in cervical cancer cells***.

To evaluate the type of induced cell death observed during cell cycle analysis, an apoptosis detection assay was conducted. This assay involves the use of an apoptosis detection kit and a flow cytometry instrument, to check whether the anti-proliferative effect of *Cannabis sativa* crude extracts was due to apoptosis or necrosis. Cells were treated with IC_{50} concentrations of *Cannabis sativa* crude extracts for a period of 24 hrs and were further stained with Annexin-V/propidium iodide (PI) dyes to help distinguish live cells from apoptotic and necrotic cells. Camptothecin was included as a positive control on the basis of its ability to induce apoptosis.

Before treatment, most of the untreated cells were accumulated in the live quandrant, with SiHa, HeLa, and ME-180 cells accounting for 91.9%, 92.7%, and 91%. Little apoptosis (2-5%) and necrosis (2-6%) was observed in all three cell lines as shown in **Figure 3.8A, Figure 3.9E,** and **Figure 3.10I**. After treatment with 0.3µM camptothecin and the IC_{50} concentrations of *Cannabis sativa* extracts for a period of 24 hrs, a shift in the population of cells towards the early (Q1-LR) and late (Q1-UR) quandrant was observed, signalling the induction of apoptosis. All treatments induced early apoptosis in SiHa and HeLa lines, which clearly indicates that the extracts have the ability to induce early onset of cell death (**Figure 3.8B** and **Figure 3.9F**). Flow cytometry revealed a significant increase in SiHa cells undergoing apoptosis during treatment with butanol (from 2% to 28.5%) and hexane (from 2% to 17.2%) when compared to camptothecin with 30.4%. necrosis was reduced in all treatment from 6.1% to 1.1%, 1,3%, and 3,6 % of butanol, camptothecin, and hexane extract.

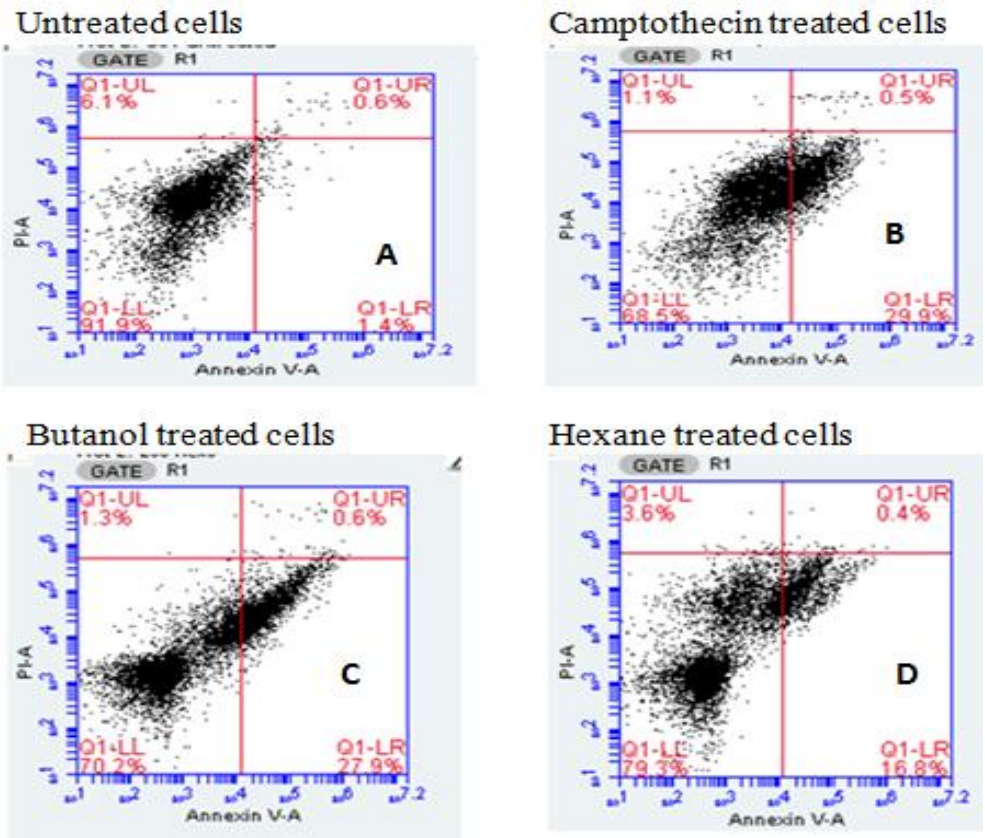

Figure 3.8: Apoptosis assessment following treatment of SiHa cells with IC_{50} concentrations of *Cannabis sativa* crude extracts. Dot plots illustrating the percentage of live, apoptotic, and necrotic cells, in comparison to the untreated cells and cells treated with camptothecin. Q1-LL represents live cells, Q1-LR is early apoptosis, Q1-UR is late apoptosis and Q1-UL is necrosis.

In HeLa cells, treatment with 0.3µM of camptothecin and the IC_{50} concentrations of *Cannabis sativa* extracts led to a significant increase ($p<0.001$) in the number of cells undergoing apoptosis (**Figure 3.9F, G, and H**). Following treatment with butanol extract, live cells were reduced to 59.0% while apoptotic cells increased to 31.9% when compared to untreated cells with 92.7% of live cells and 4.5% of apoptotic cells. On the other hand, hexane extract exhibited a slight increase in apoptosis induction, accounting for 15.3% of apoptosis and 80.3% of live cells (**Figure 3.11B**). Butanol extracts induced apoptosis (31.9%) similar to that of camptothecin (31.3%) with only a difference of 0.6%.

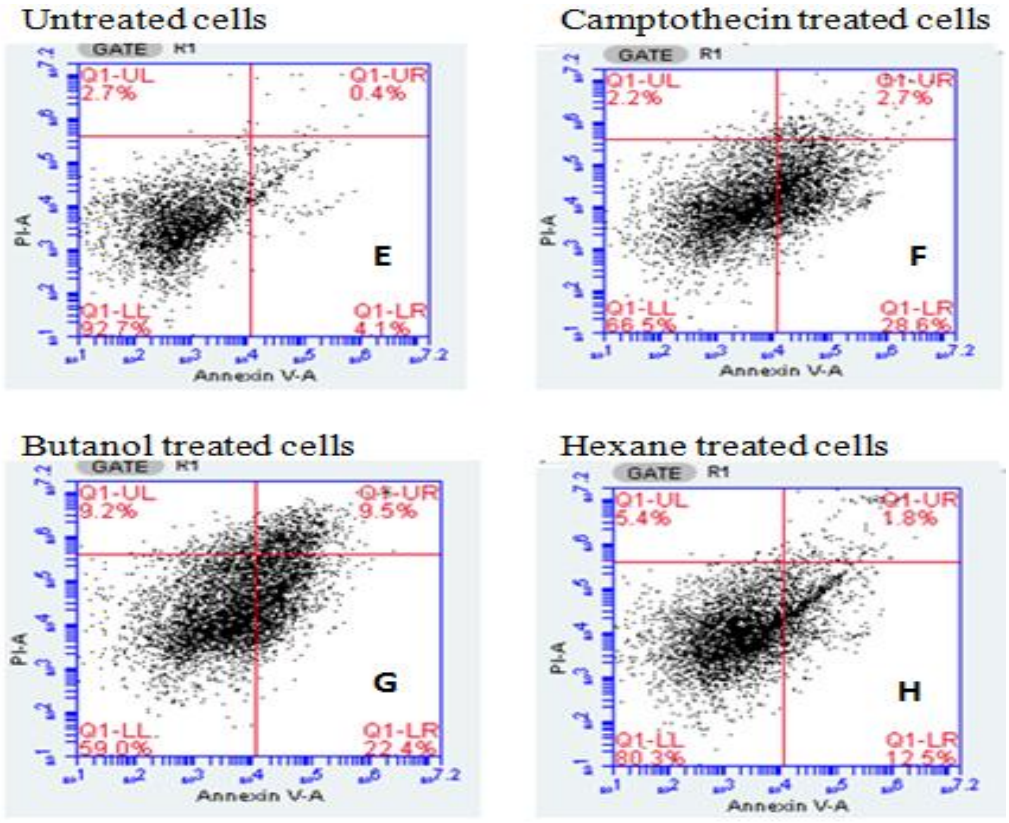

Figure 3.9: Apoptosis assessment following treatment of HeLa cells with IC_{50} concentrations of Cannabis sativa crude extracts. Dot plots illustrating the percentage of live, apoptotic, and necrotic cells, in comparison to the untreated cells and cells treated with camptothecin. Q1-LL represents live cells, Q1-LR is early apoptosis, Q1-UR is late apoptosis and Q1-UL is necrosis.

Apoptosis induction in ME-180 was higher than in SiHa and HeLa cells in all three treatments. Following treatment with 0.3µM of camptothecin, apoptosis induction increased significantly ($p<0.001$) to 45%, simultaneously reducing the percentage of live cells to 49.9% (**Figure 3.10J** and **Figure 3.11C**). A similar event was observed following treatment of ME-180 cells with butanol extract. Cells undergoing apoptosis accounted for 44.8% while live cells reduced to 44.7%. Butanol led to an increase in the percentage of necrotic cells with almost 10.5% when compared to camptothecin (5.1%). Both camptothecin and butanol had similar activity as they induced late apoptosis (25.3% and 29.4) than early (19.7% and 15.4%). However this was not the case with hexane. The hexane extract induced 43.2% of apoptosis with most of the

cells undergoing early apoptosis (28.9%) than late apoptosis (14.3%), simultaneously reducing live cells to 55.4% and necrotic cells to 1.3%. This suggests that, the hexane extract is responsible for an early event of apoptosis induction in ME-180 cells.

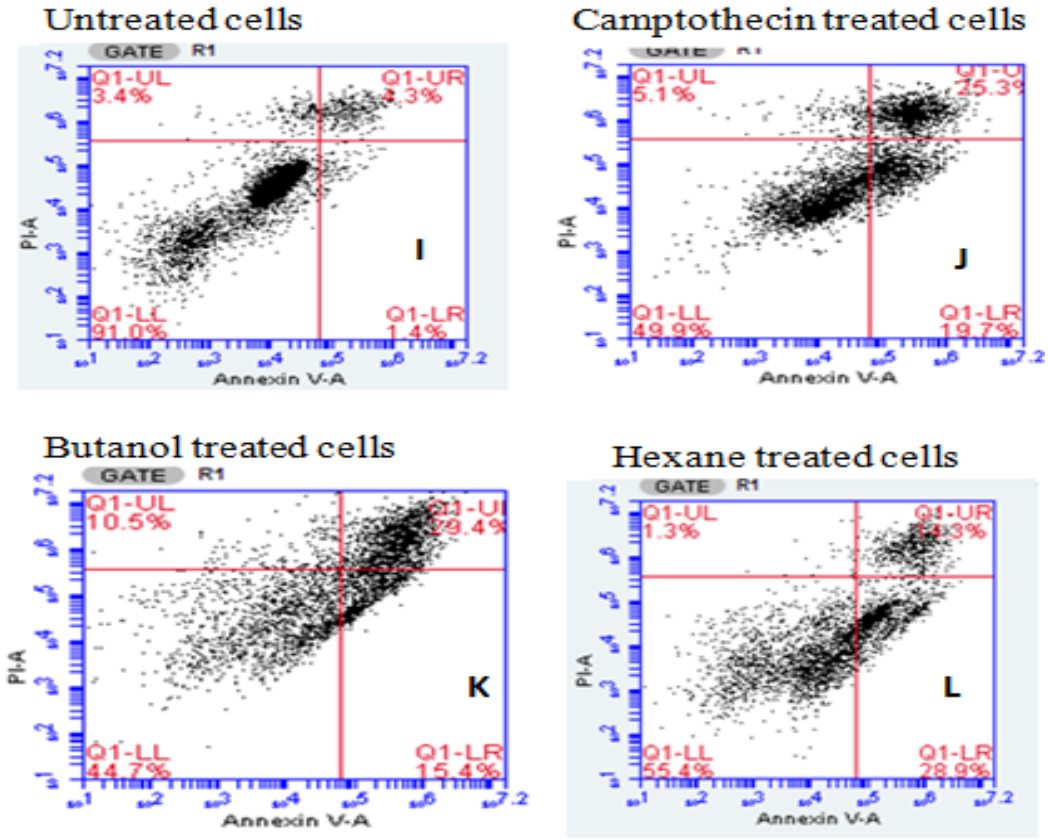

Figure 3.10: Apoptosis assessment following treatment of ME-180 cells with IC_{50} concentrations of *Cannabis sativa* crude extracts. Dot plots illustrating the percentage of live, apoptotic, and necrotic cells, in comparison to the untreated cells and cells treated with camptothecin. Q1-LL represents live cells, Q1-LR is early apoptosis, Q1-UR is late apoptosis and Q1-UL is necrosis.

Interesting to note was that, the activity of the crude extracts increases with severity of each cancer. And also the induction of apoptosis by butanol in all three cell lines was comparable to that camptothecin.

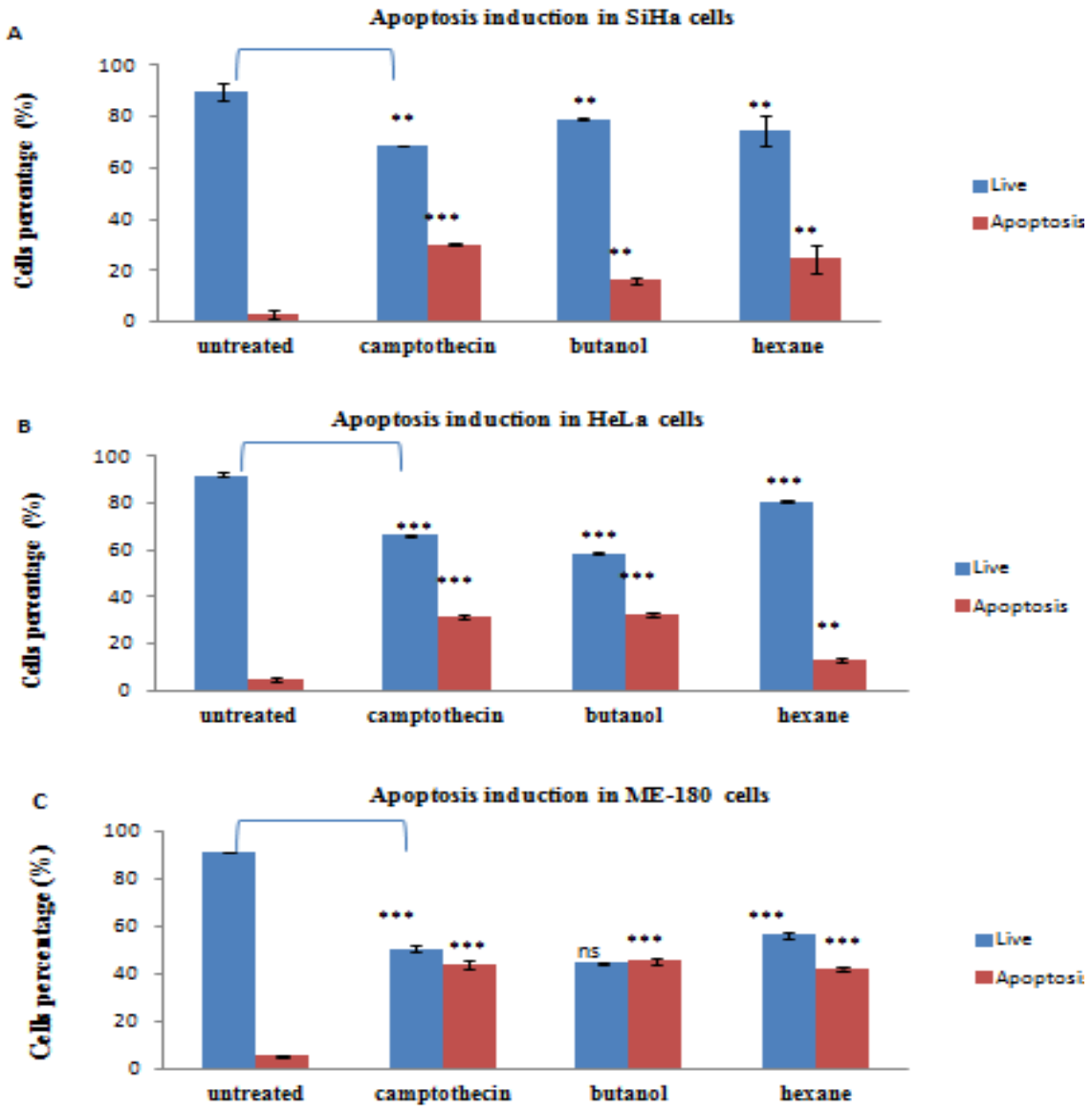

Figure 3.11: Representative bar graphs of apoptosis induction in cervical cancer cell lines. Cells were treated with IC50 of *Cannabis sativa* extracts for a period of 24 hrs and further stained with Annexin-V/PI. Data represented as mean ± standard deviation with ***p<0.001, **p<0.01 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

3.7 ***Effect of Cannabis sativa extracts on the morphology of SiHa cells.***

To determine whether *Cannabis sativa* changes the morphology of SiHa cells, a fluorescence confocal microscopy was conducted. DAPI and Annexin V/FITC were

used to stain the cells and to enable visualization. DAPI stains the nucleus of both live and dead cells while Annexin V binds to the phosphotidylserine (PS) residues exposed during early and late apoptosis.

Treatment of SiHa cells with IC_{50} of both butanol and hexane extracts, further confirmed the occurrence of apoptosis when compared to untreated. As shown in **Figure 3.12**, untreated did not uptake Annexin V dye (green fluorescence), which may be due to the fact that the cells were viable and with an intact cell membrane. Following treatment with IC_{50} of *Cannabis sativa* extracts resulted in an increase of Annexin V positive cells, signalling the induction of apoptosis. Another feature that is a representative of cell death is the change in morphology. Exposure of SiHa cells to camptothecin caused a change in morphology, from round to shapeless, when compared to untreated cells. Similar results (change in morphology) were obtained during exposure of cells to the IC_{50} of butanol extract. On the other hand, the hexane extract led to a reduction of cell size. This further proves that during cell growth analysis, SiHa cells were undergoing cell death while attached to the surface of the 16-well E-plate

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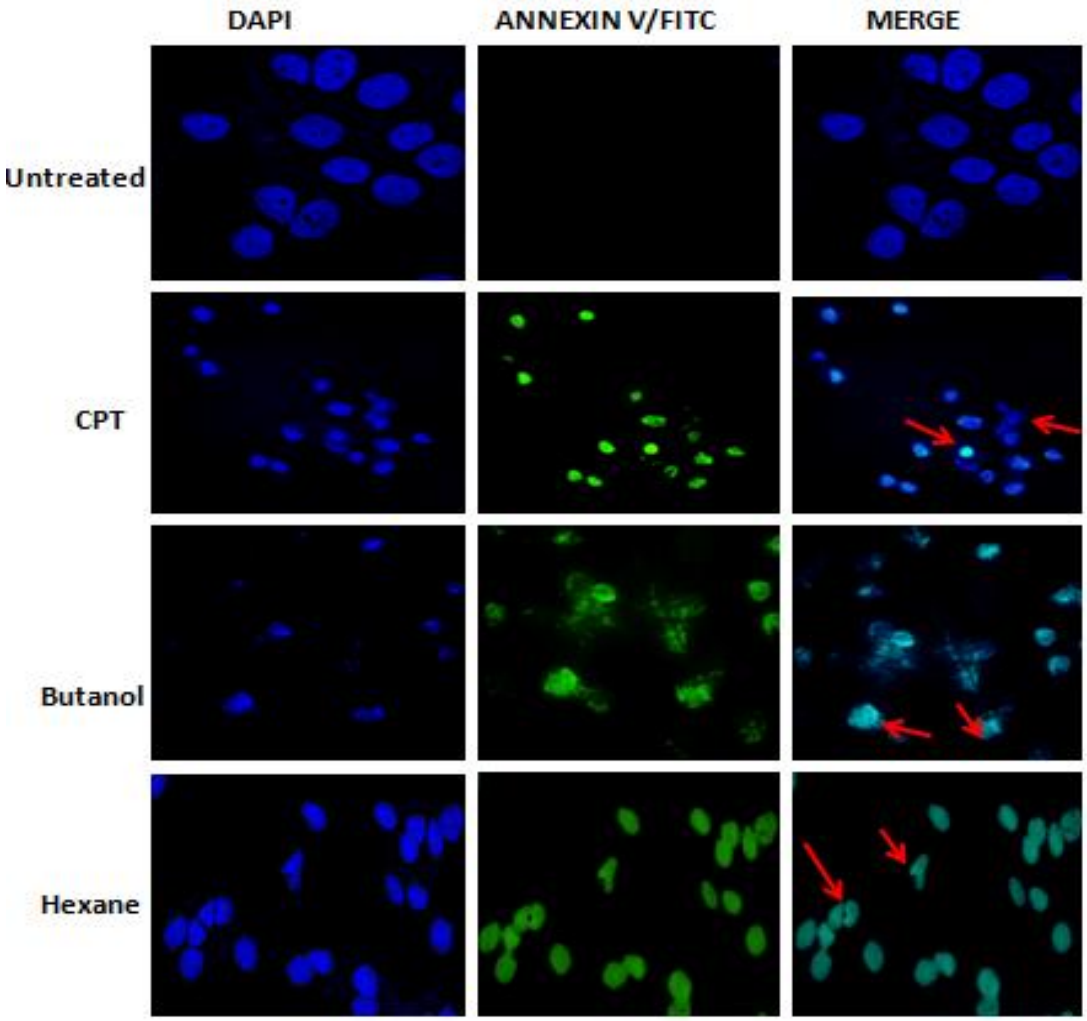

Figure 3.12: Morphological analysis and assessment of apoptosis in SiHa cells stained with DAPI and Annexin V dye**.** Cells were incubated with IC50 of *Cannabis sativa* extracts for a period of 24hrs. Cells were stained with Annexin V and counterstained with DAPI. Fluorescence confocal microscopy was used to visualize the cells.

3.8 ***Effect of Cannabis sativa crude extracts on the expression of upstream and downstream target proteins.***

To further validate whether the mitochondrial pathway was involved in the *Cannabis sativa* induced apoptosis, Western blot was conducted. Protein lysates were prepared following treatment with camptothecin and the IC_{50} of butanol and hexane extracts. Untreated protein lysates were used as a control. Expression of anti- (RBBP6 and Bcl-

2) and pro- apoptosis (Bax and p53) related antibodies involved in the mitochondrial pathway were evaluated.

Treatment of cervical cancer cell lines with *Cannabis sativa* confirmed the induction of apoptosis via the mitochondrial pathway. Upon exposure of SiHa cells to the butanol extract, the expression level of p53 protein was up-modulated and RBBP6 protein was down-modulated **(Figure 3.13A**). Butanol extract had a similar effect upon treatment of HeLa cells, wherein up-modulation of p53 and down-modulation of RBBP6 protein was observed. This was not the case following treatment of ME-180 cells with IC_{50} of the butanol extract (**Figure 3.13C**). At a concentration of 100µg/ml, there was little to no significant difference in the up-modulation of p53 between the untreated and the butanol extract, however, down-modulation of RBBP6 was observed. The hexane extracts effectively up-modulated p53 and down modulated RBBP6 in all three cells than the butanol extract. Since Bcl-2 family members, Bax and Bcl-2, play a vital role in the control of cell death via the mitochondrial pathway, their expression levels were evaluated following treatment with both extracts and camptothecin. Bax acts as an antiapoptotic protein while Bcl-2 is an anti-apoptotic protein. Following treatment of cervical cancer cells, Bax protein was up-modulated and Bcl-2 was down-modulated. Interesting to note was that up-modulation of Bax and down-modulation of Bcl-2 in ME-180 cells was independent of p53. This might mean that activation of the Bcl-2 family members might be via another pathway independent of p53.

Caspases play an effective role in the execution of apoptosis Therefore, we evaluated for their involvement during the execution of apoptosis via the mitochondrial pathway. An effector caspase-9 and executor capsase-3 were included, elucidate their role in *Cannabis sativa* induced apoptosis. Caspase-9 protein was effectively up-modulated by both butanol and hexane extracts when compared to untreated (**Figure 3.14F**, **Figure 3.15L**, and **Figure 3.16R**). The results further demonstrate that the hexane extract was more effective in the up-modulaton of caspase-3 in all three cell lines. Butanol extract had a similar effect in only two cell lines (SiHa and HeLa), however, failed to up-modulate caspase 3 (**Figure 3.13C** and **Figure 3.16Q**) In ME-

180 cells.. Therefore, we can conclude that execution of apoptosis in ME-180 cells might have been independent of caspase-3.

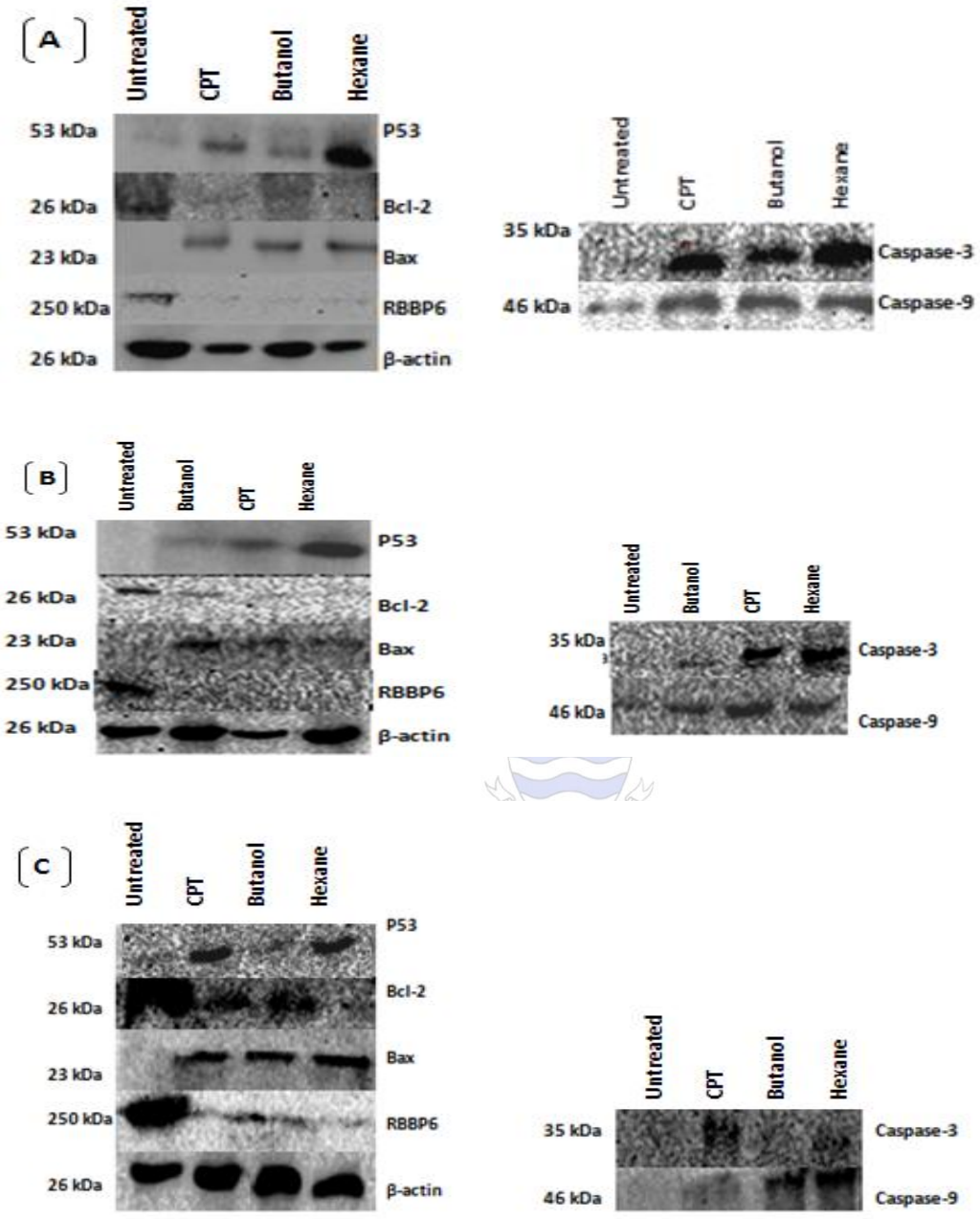

Figure 3.13: Western blot analysis of the protein expression before and after 24hour treatment with IC_{50} of *Cannabis sativa* butanol and hexane extracts. A) SiHa, B) HeLa, and C) ME-180 cells were treated for a period of 24 hrs and protein lysates were separated using SDS-PAGE gel. Untreated protein was used as a control. Antibodies against pro-apoptotic proteins (p53 and Bax) and anti-apoptotic proteins

(Bcl-2 and RBBP6), Initiator caspase-9 and effecter caspase-3 were included to elucidate apoptosis induction.

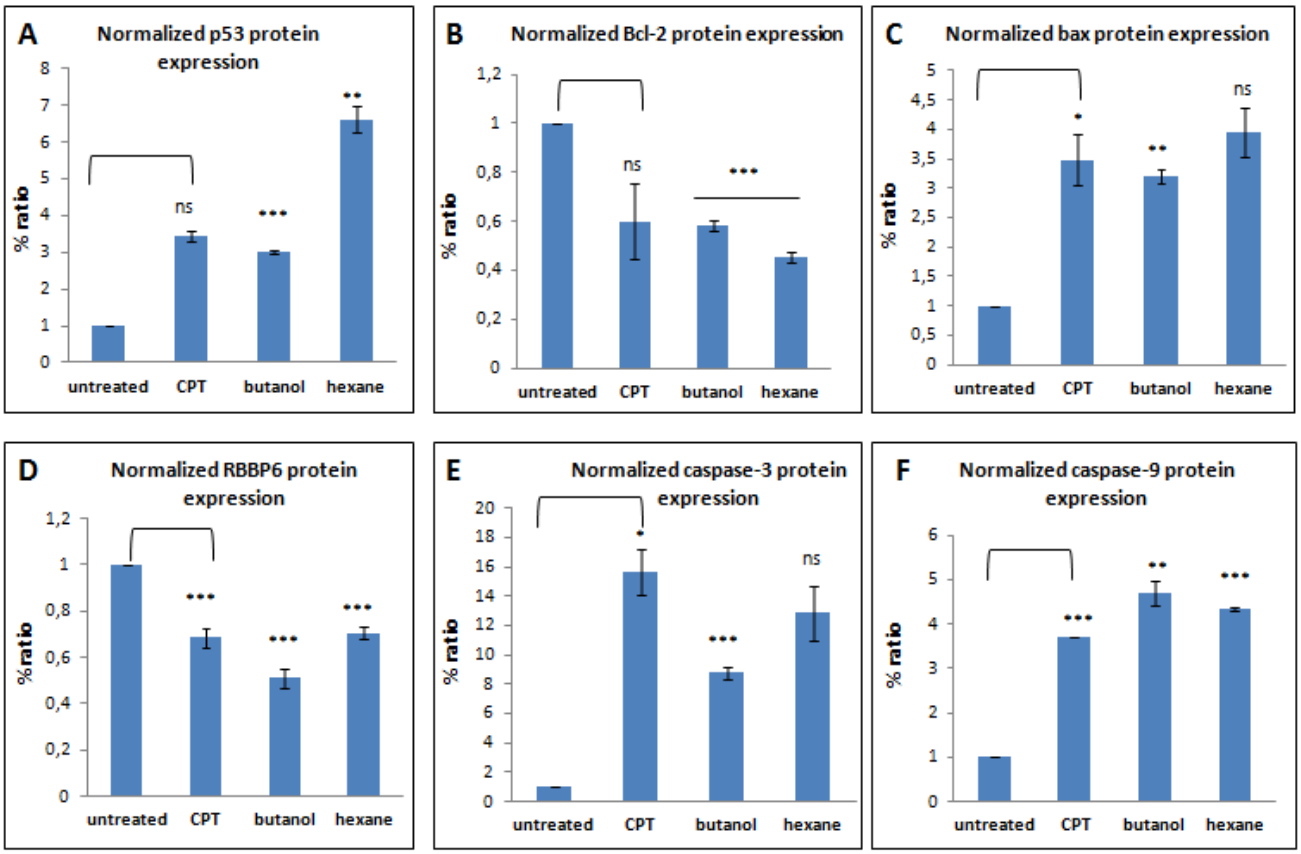

Figure 3.14: A densitometry analysis SiHa protein was performed using ImageJ quantification software to measure the relative band intensity. CPT represents camptothecin. Data represented as mean ± standard deviation with ***p<0.001, **p<0.01 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

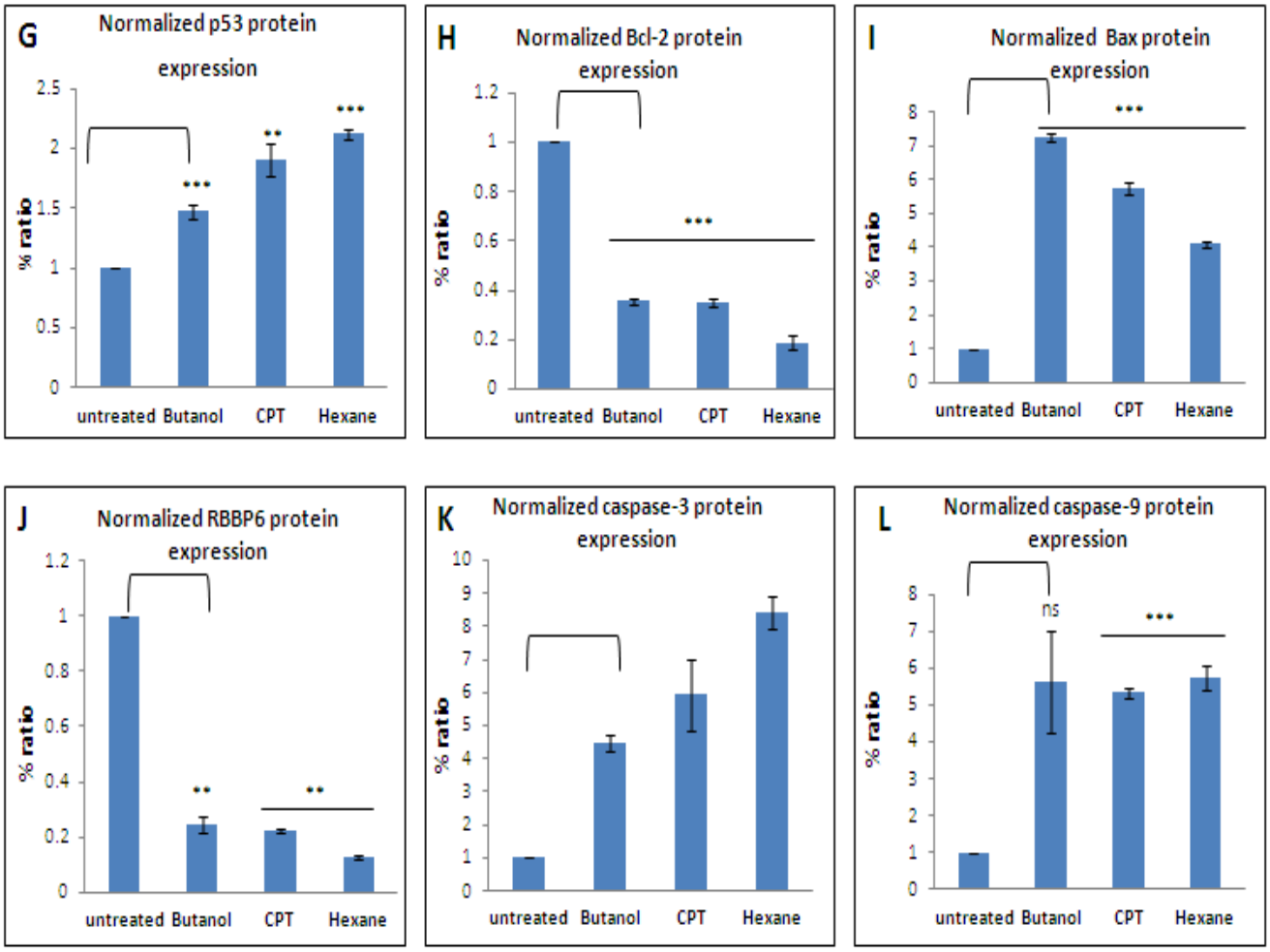

Figure 3.15: A densitometry analysis of HeLa protein was performed using imageJ quantification software to measure the band intensity. CPT represents camptothecin. Data represented as mean ± standard deviation with ***p<0.001, **p<0.01, *p<0.05 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

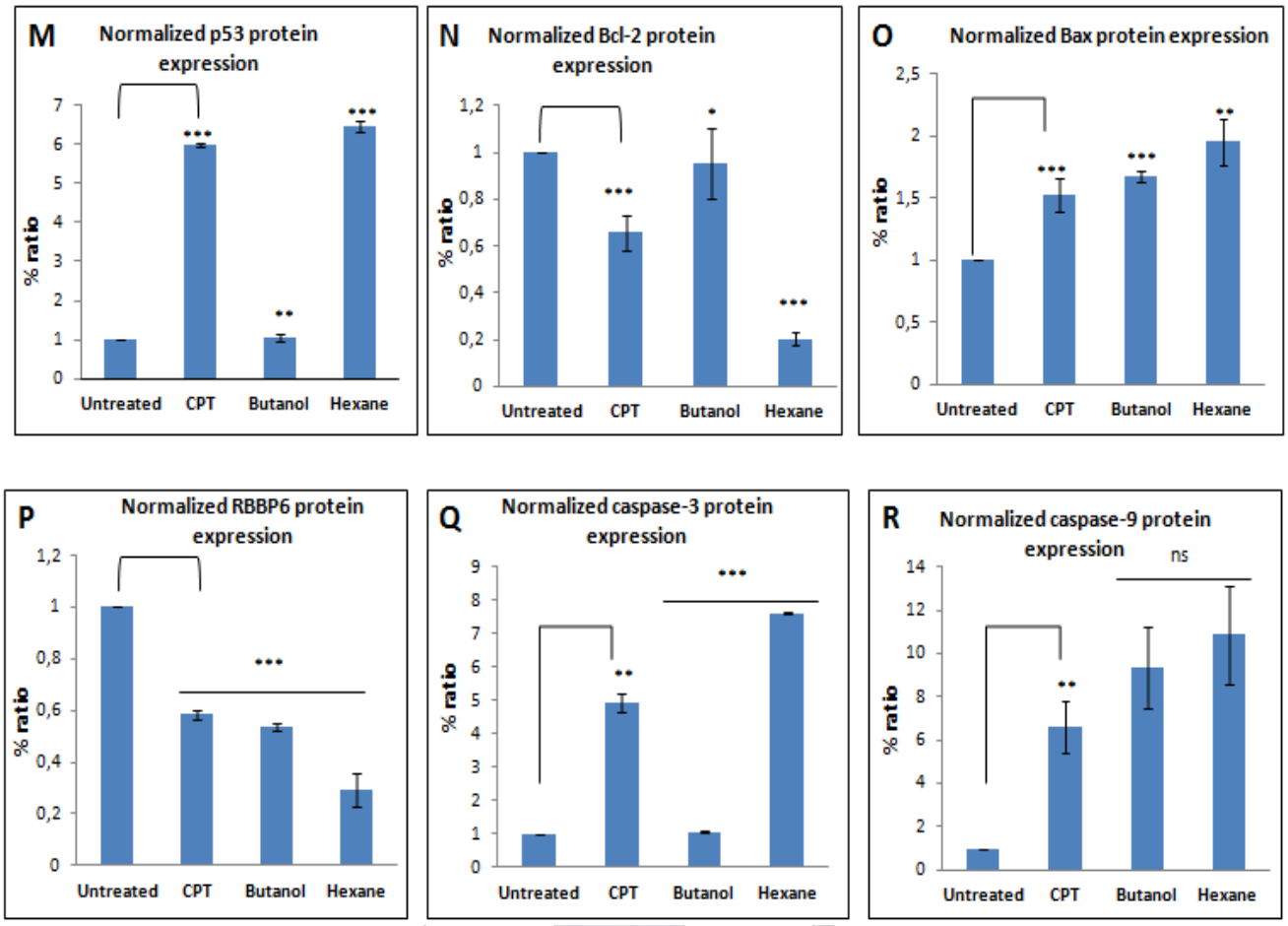

Figure 3.16: A densitometry analysis of ME-180 protein was performed using imageJ quantification software to measure the band intensity. CPT represents camptothecin. Data represented as mean ± standard deviation with ***p<0.001, **p<0.01, *p<0.05 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

3.9 *Effect of Cannabis sativa crude extracts on caspase 3/7 activity*

Since caspases are known to be mediators of apoptosis, it was vital to check for the involvement of caspase 3/7 in executing this mode of cell death. A caspase 3/7 assay was performed on cervical cancer cells following treatment with IC_{50} of *Cannabis sativa* extracts for a period of 24 hrs. Cells were further exposed for 1 hr to caspase 3/7 DEVD-aminoluciferin substrate to initiate caspase activity and determined caspase 3/7 activity by measuring luminescence. As shown in **Figure 3.17a, b, c**, we observed an increase in caspase 3/7 activity in three cell lines following treatment with 0.3µM of camptothecin. Similar results were obtained during treatment of SiHa and HeLa

cells with IC_{50} of butanol and the hexane extract. An increase in the caspase 3/7 activity confirms the results obtained in **Figure 3.13A** and **B**. An increase in the activity of caspase 3/7 confirms the results obtained during western blot, suggesting that the induction of apoptosis by *Cannabis sativa* might be as a result of the activation of caspases. On the other hand, treatment of ME-180 with IC_{50} of butanol and hexane extract slightly increased caspase 3/7 activity. However, on the basis of the results obtained during Western blot (**Figure 3.16C**), wherein butanol extract failed to up-modulate caspase-3 protein expression; we can conclude that caspase 7 was responsible for the reported apoptosis induction.

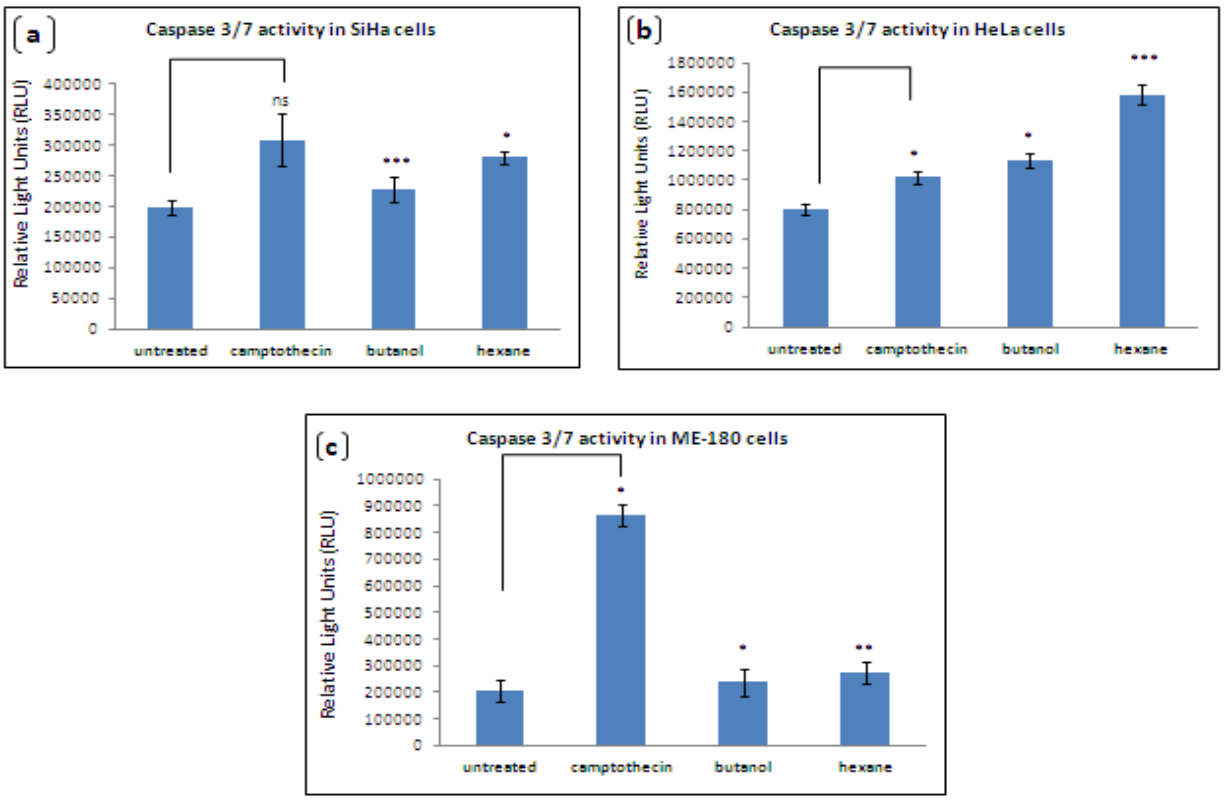

Figure 3.17: Caspase 3/7 activity after treatment of SiHa, HeLa, and ME-180 cells with IC_{50} of *Cannabis sativa* butanol and hexane extract. Cells were treated with IC50 of butanol and hexane extracts for a period of 24 hrs. Caspase 3/7 reagent was added to the treated cells for 1 hr. Luminescence -was measured using GLOMAX instrument in RLU. Data represented as mean ± standard deviation with ***p<0.001, **p<0.01, *p<0.05 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

3.2 **Cannabidiol effect in cervical cancer**

Medicinal plants possess secondary metabolites that display chemopreventative and therapeutic potential against various disorders including cancer. However, it is also known that these metabolites have synergistic effect to one another. Therefore, screening of compounds individually or in combination is of importance, in order to evaluate for their effect on cancer. In this study, cannabidiol was used as a standard reference in order to determine whether the reported pharmacological activities displayed by *Cannabis sativa* crude extracts may be attributable to the presence of this compound or not.

3.2.1 ***Cannabidiol reduces cell viability of cervical cancer cells***

To evaluate the cytotoxic effect of cannabidiol upon treatment of cervical cancer cells, MTT assay was conducted. Cells were treated with various concentrations of cannabidiol (0.8, 1.5, and 3.2 µg\ml) for a period of 24 hrs, followed by staining with MTT reagent for 4hrs. As shown in **Figure 3.18A, B, C**, cannabidiol reduced cell proliferation in a dose dependent manner. The cytotoxic activity of cannabidiol was effective at smaller dose when compared to *Cannabis sativa* extracts (50-100µg\ml) (**Figure 3.1** and **Figure 3.2**) SiHa and HeLa were sensitive at the highest concentration (3.2 µg\ml), wherein cell viability was significantly reduced ($p<0.001$) to 51% and 50% in comparison to the untreated. On the other hand, ME-180 cells were found to be sensitive at an IC_{50} concentration of 1.5 µg\ml wherein cell viability was reduced to almost 56%. Cannabidiol was maintained in absolute ethanol. Therefore, ethanol was included as a control to check whether the observed cell death was as a result of cannabidiol or ethanol. There was little cytotoxic effect (7.3%, 7.8%, and 5.6%) following treatment of SiHa, HeLa, and ME-180 cells with absolute ethanol. Cell viability was reduced to 92.6%, 92.5%, and 94.3%.

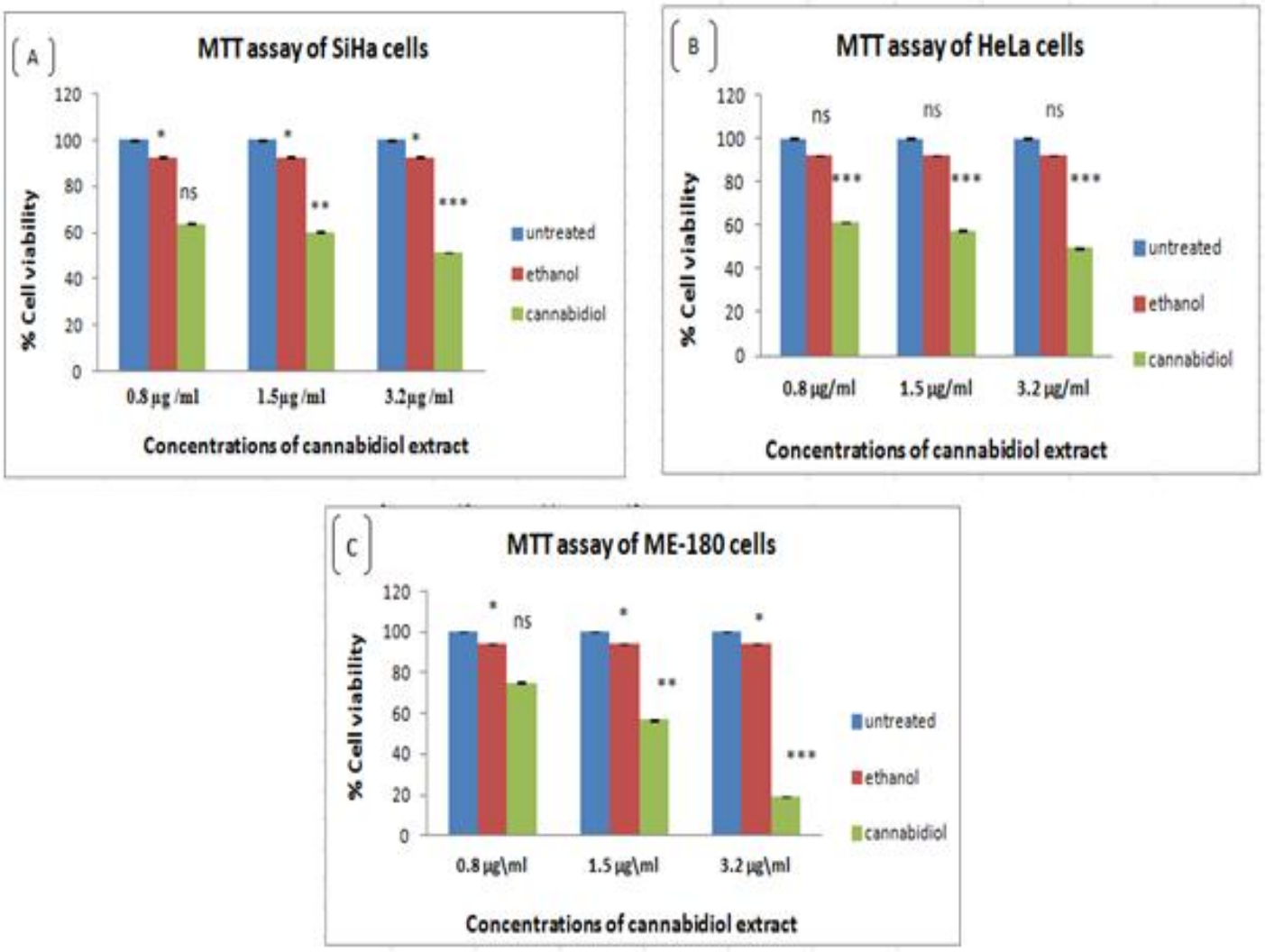

Figure 3.18: Inhibitory effects of cannabidiol on cervical cancer cell lines**.** SiHa (**A**), HeLa (**B**) and ME-180 cells (**C**) were treated with various concentrations (0.8, 1.5 and 3.2 µg/ml) of *Cannabis sativa* crude extracts for a period of 24 hrs. Untreated and ethanol were included as controls. Data are expressed as the mean ± SD and ***p<0.001, **p<0.01, and ns p>0.05 representing the level of significance in comparison to the untreated.

3.2.2 ***Cannabidiol reduces ATP levels in cervical cancer cell lines.***

Since treatment of cervical cancer cell lines (SiHa, HeLa, and ME-180) with cannabidiol reduced cell viability, we wanted to check the levels of ATP following the detected cell death. The results indicate that cannabidiol reduced the levels of ATP in cervical cancer cell line. At 2hrs, treatment of SiHa, HeLa, and ME-180 cells with

IC_{50} led to a reduction of ATP levels by ~ 61% (from 4704419 to 1802508 RLU), 93% (from 627621 to 40371 RLU), and 8% (from 798688 to 734039 RLU). A prolonged incubation period (24 hrs) of cells with IC_{50} led to a further decrease in the ATP levels of SiHa, HeLa, and ME-180 cells by ~66% (from 4486150 to 1497648 RLU), 97% (from 601694 to 13426 RLU), and 8.5%.(from 790757 to 723039 RLU) . This could mean that cannabidiol depletes ATP levels more than *Cannabis sativa* extracts after treatment of SiHa and HeLa cells.

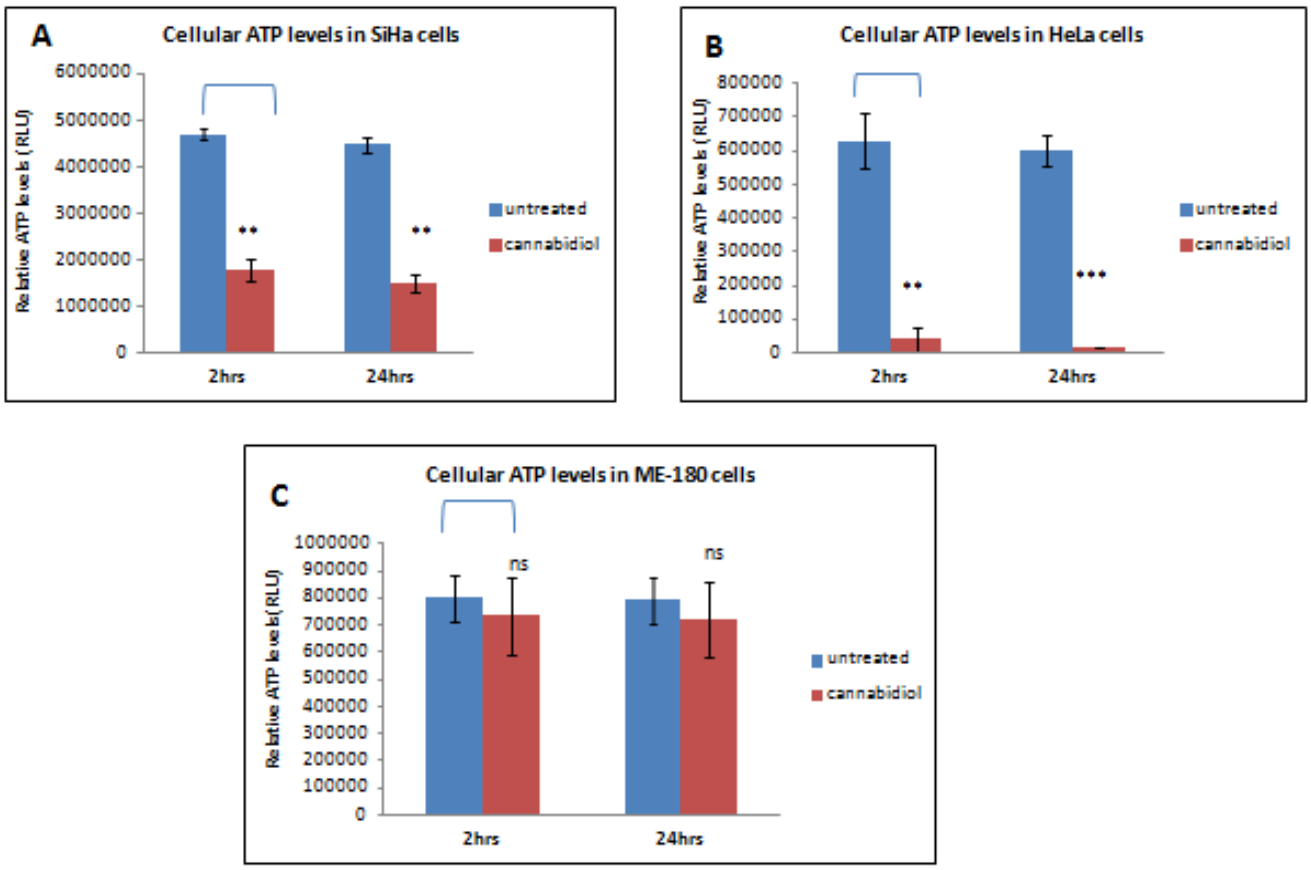

Figure 3.19: Representative bar graphs of changes in the ATP levels following treatment of cervical cancer cells with cannabidiol. Cells were treated with IC_{50} of butanol and hexane extract for a period of 24 hrs. Untreated was included as a control for omparative purposes. Data represented as mean ± standard deviation with *** $p<0.001$, ** $p<0.01$, and ^{ns} $p>0.05$ representing the level of significance in comparison to the untreated.

3.2.3 ***The effect of cannabidiol on cell growth of cervical cancer cells***

The IC_{50} obtained during MTT assay was used to treat cells for further analysis using the xCELLigence system. This was done to monitor the anti-growth effects of cannabidiol on cervical cancer cells. Optimal densities of SiHa, HeLa, and ME-180 cells were plated on a 16-well plate for a period of 22-24hrs, depending on the doubling time of each cell line. Results show that the IC_{50} of cannabidiol inhibit cell growth in a cell type and concentration dependent manner. In comparison to *Cannabis sativa* extracts, cannabidiol exhibited slightly similar anti-growth effects in all three cell lines. The cell index of SiHa continuously increased following treatment with IC_{50} concentration (3.2µg/ml). At a similar IC_{50} of cannabidiol (3.2µg/ml), the cell index of HeLa cells slightly reduced indicating the inhibition of cell growth over a period. On the other hand, IC_{50} of cannabidiol (1.5µg/ml) resulted in the reduction of the cell index therefore altering the growth curve of ME-180 cells. In all three cell lines, camptothecin altered the growth curve and reduced cell index causing inhibition of cell growth. Ethanol, highlighted in red (**Figure 3.20**), did not have an effect on cells. This means that cannabidiol IC_{50} of 3.2µg/ml and 1.5µg/ml is responsible for the observed effects on cervical cell lines.

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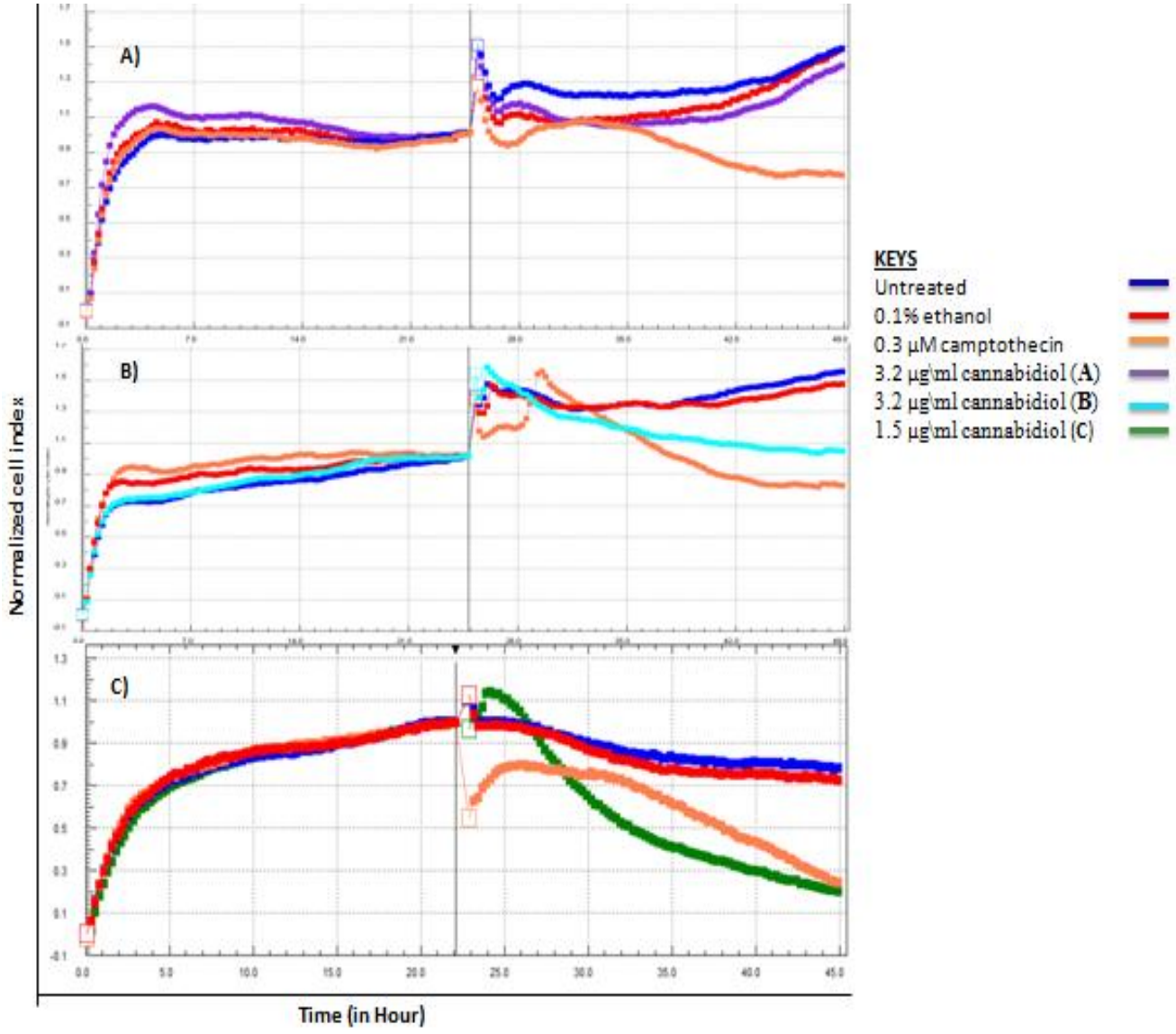


Figure 3.20: Cell growth analysis of SiHa (**A**), HeLa (**B**), and ME-180 (**C**) cells using an impedance-based system, xCELLigence. Cells were seeded for a period of 22-24 hrs, followed by treatment with IC_{50} concentrations of cannabidiol. Untreated, ethanol, and camptothecin curves highlighted in blue, red and choral were included as controls for comparative purposes.

3.2.4 *The effect of cannabidiol on the cell cycle of cervical cancer cells*

For cell cycle analysis, SiHa, HeLa, and ME-180 cells were treated with IC_{50} of cannabidiol for a period of 24 hrs and stained with PI. This was done in order to distinguish a population of cells and evaluate the effect of cannabidiol on cells in the different phases of the cell cycle. Before treatment, most of the cells were

accumulated at G0/G1 phase, indicating that they were undergoing cell division (**Figure 3.21A, C, and E**). Each cell line responded differently after treatment with cannabidiol. Almost 42.2% of SiHa cells were accumulated at the cell death phase, sub-G0 while significantly ($p<0.001$) reducing cells in the G0/G1 phase, from 57.9% to 42.8% (**Figure 3.22A**). A similar trend was observed following treatment of HeLa cells with 3.2 µg/ml of cannabidiol. Cells in the sub-G0 (from 5.1% to 17.4%) and S phase (from 4.8% to 11.2%) increased (**Figure 3.21B**). From this data, we can conclude that cannabidiol induced cell death without cell cycle arrest. A similar event was observed during treatment of ME-180 cells. Cannabidiol significantly increased ME-180 cells in the cell death phase (34.3%) as shown in **Figure 3.20F** and **Figure 3.21C**.

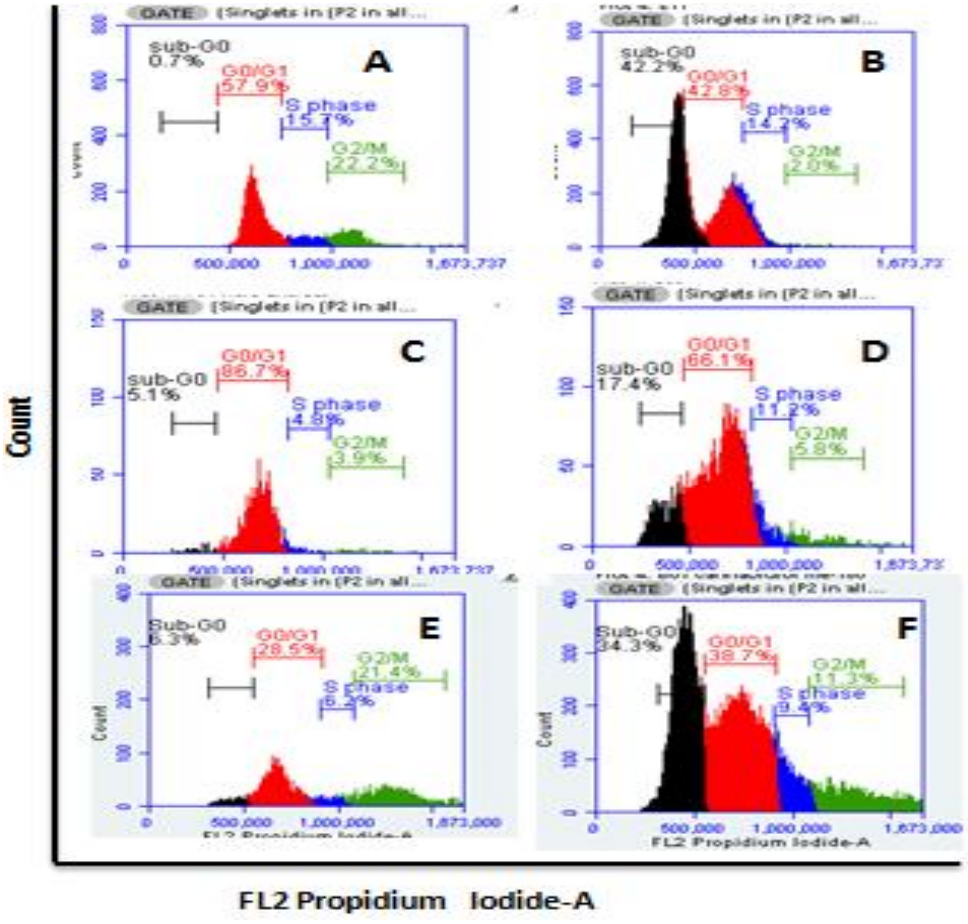


Figure 3.21: Representative histograms of SiHa (**A** and **B**), HeLa (**C** and **D**), and ME-180 cells (**E** and **F**) cell cycle, before and after treatment with IC_{50} concentrations of *Cannabis sativa* extracts. Cells were harvested and treated with IC_{50} concentrations of

Cannabis sativa extracts followed by staining with PI. Data represented as mean ± standard deviation with *** p<0.001, **p<0.01, *p<0.05 representing the level of significance in comparison to the untreated.

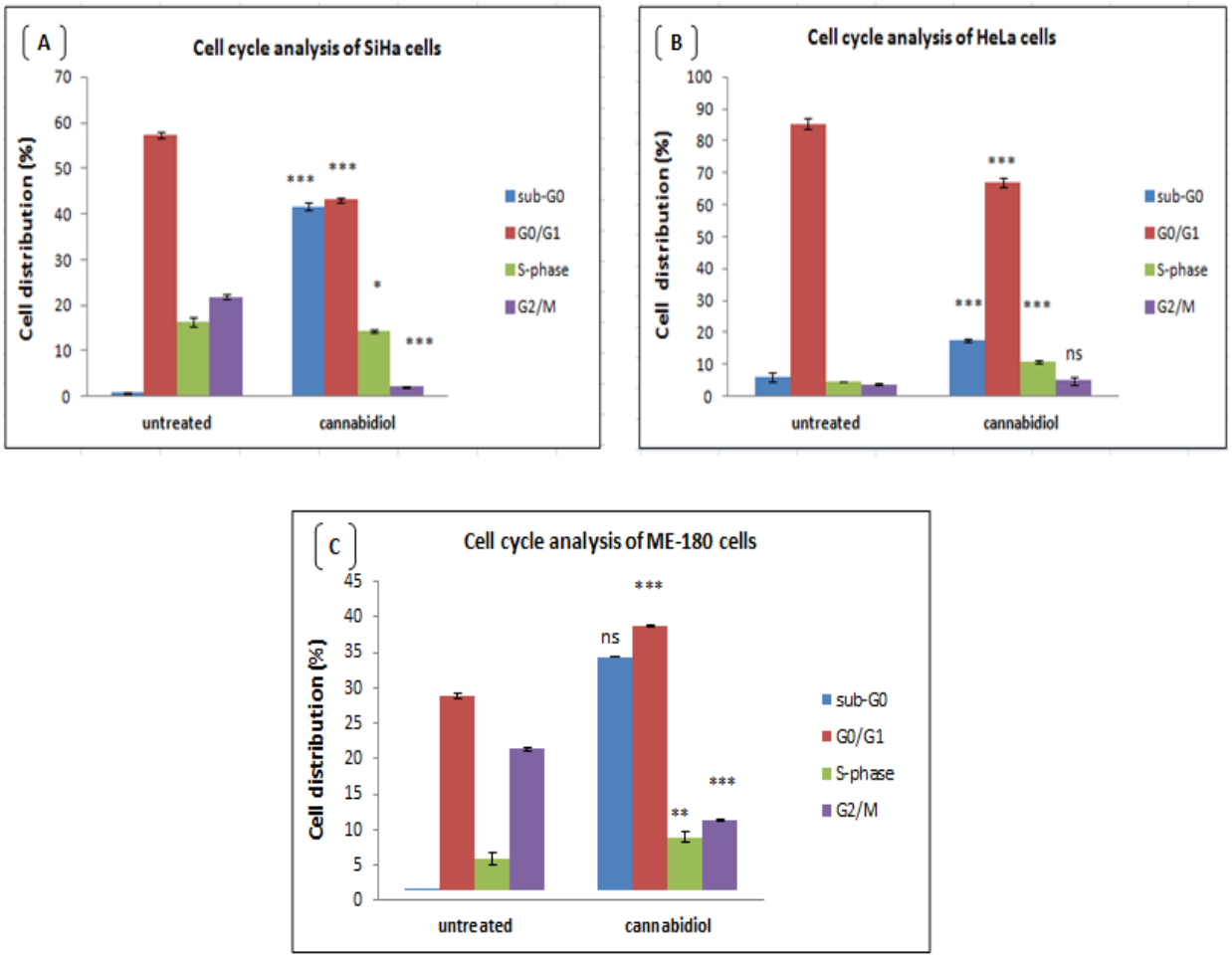


Figure 3.22: Representative bar graphs of SiHa (A), HeLa (B), and ME-180 (C) cell cycle, before and after treatment with IC_{50} concentrations of *Cannabis sativa* extracts. Data represented as mean ± standard deviation with ***p<0.001, **p<0.01, *p<0.05 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

3.2.5 ***Cannabidiol induces apoptosis in cervical cancer***

Following accumulation of cells in the cell death phase and the severe depletion of ATP levels during treatment of cells with an IC_{50} of cannabidiol, an apoptosis assay was conducted. This was done to further validate whether cannabidiol induces apoptosis. The results further confirmed that the type of cell death induced was

apoptosis. **Figure 3.23B**, **D**, **F** shows that cannabidiol induced the early onset of apoptosis in all three cell lines. Cannabidiol was more effective in inducing apoptosis In comparison to both extracts of Cannabis sativa. Treatment of SiHa cells with IC_{50} of cannabidiol induced 51.3% apoptosis (**Figure 3.24a**), while hexane and butanol induced 17.2% and 28.5% (**Figure 3.8D** and **C**). Cannabidiol exhibited almost similar activity during treatment of HeLa, wherein 43.3% of cells underwent apoptosis when compared to 15.9% and 31.9% apoptosis induced by hexane and butanol extracts as shown in **Figure 3.22D** and **Figure 3.9H** and **G**. On the other hand, there were more live cells (70.8%) than dead cells (28.6%) after treatment of ME-180 cells (**Figure 3.24c**).

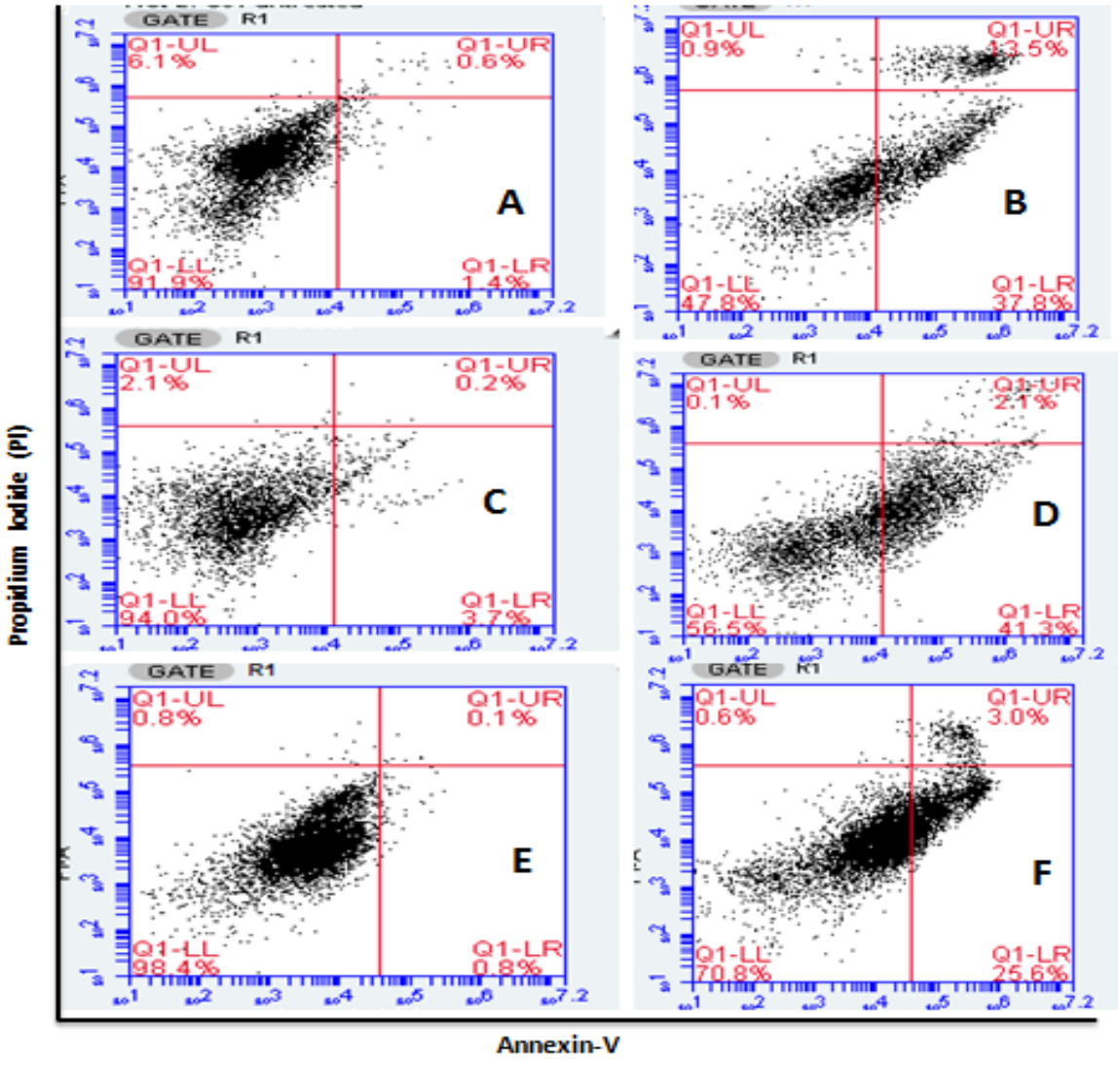


Figure 3.24: Apoptosis assessment following treatment of cervical cancer cells with IC_{50} concentrations of cannabidiol. Dot plots illustrating the percentage of live,

apoptotic, and necrotic cells, in comparison to the untreated cells and cells treated with camptothecin. Q1-LL represents live cells, Q1-LR is early apoptosis, Q1-UR is late apoptosis and Q1-UL is necrosis. **A**, **C**, and **E** represent untreated SiHa, HeLa, and ME-180 cells and their respective treatments **B**, **D**, and **F**.

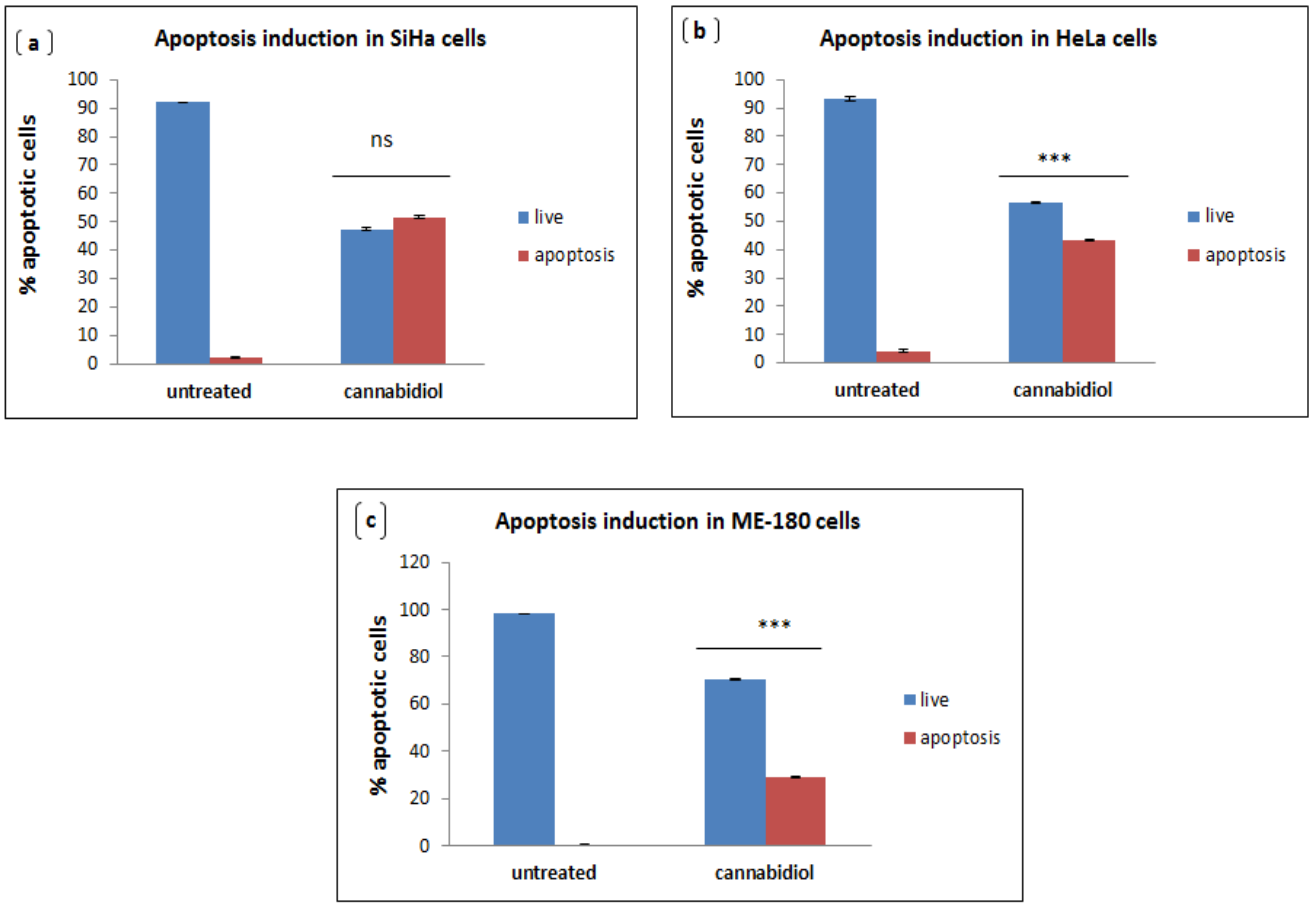


Figure 3.25: Statistical analysis of (**a**) SiHa, (**b**) HeLa, and (**c**) ME-180 cells following treatment with cannabidiol. Data represented as mean ± standard deviation with ***$p<0.001$, **$p<0.01$, *$p<0.05$ and ^{ns} $p>0.05$ representing the level of significance in comparison to the untreated.

3.2.6 *Effect of Cannabis sativa extracts on the morphology of SiHa cells.*

We further assessed whether cannabidiol changes the morphology of SiHa cells in a similar manner to that of *Cannabis sativa* extracts. Fluorescence confocal microscopy was conducted by staining cells with nuclear stain DAPI and Annexin V/FITC.

Apoptotic cells were assessed for alterations caused by treatment with Cannabidiol. The results in **Figure 3.26** show that before treatment, no apoptotic cells were detected. However, in camptothecin treated cells, both DAPI and Annexin V/FITC resulted in the reduction of cell size and loss of shape of the cells. Similar results to that of butanol and hexane treated cells (**Figure 3.12**) were obtained during treatment of SiHa cells with cannabidiol. DAPI and Annexin V/FITC staining revealed a population of cells with a reduced cell size, blebbling, and loss of shape, which are morphological characteristics of apoptosis induction. In comparison to camptothecin treated cells, cannabidiol slightly increased co-localization of DAPI and Annexin V/FITC dye in SiHa cells.

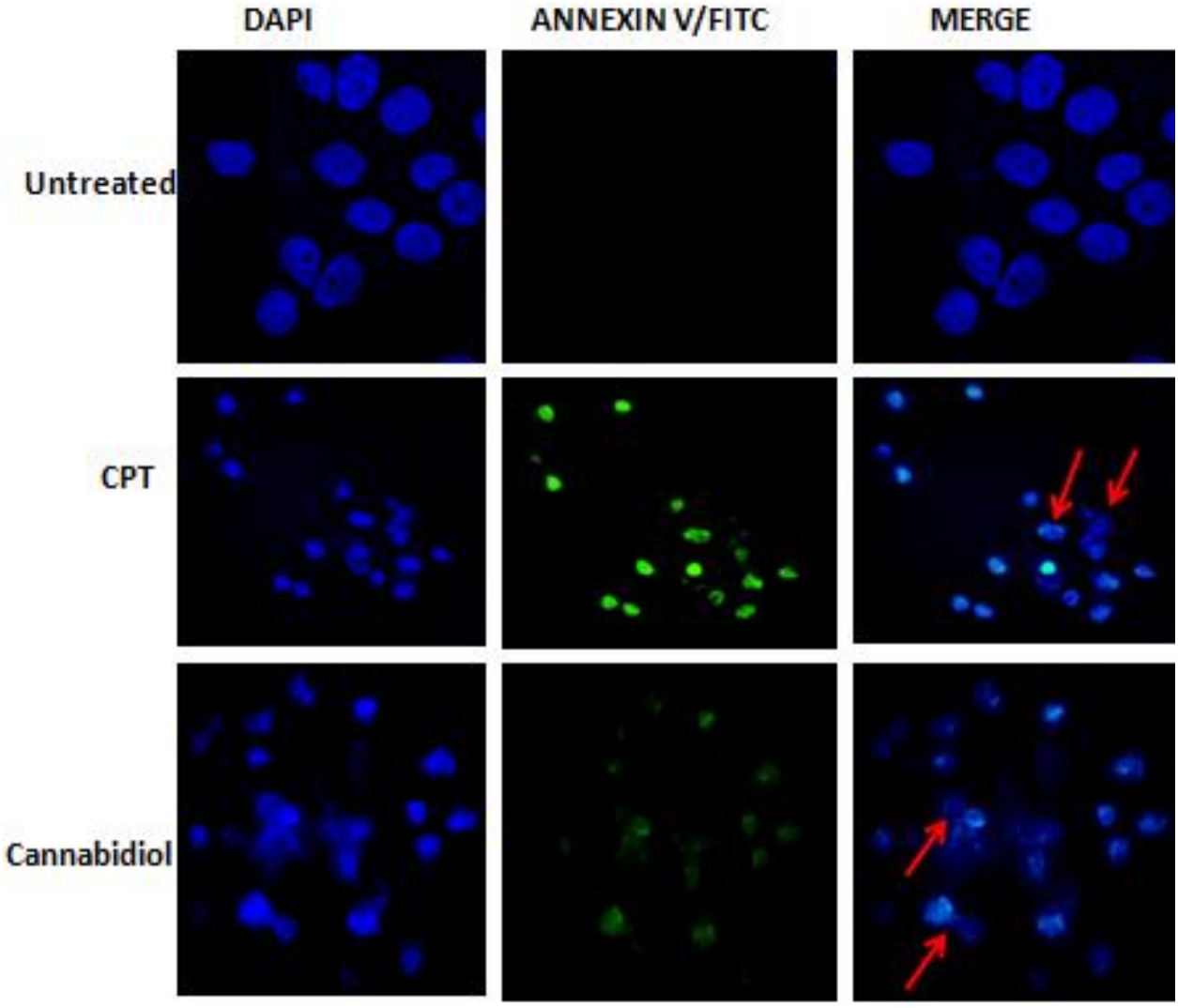
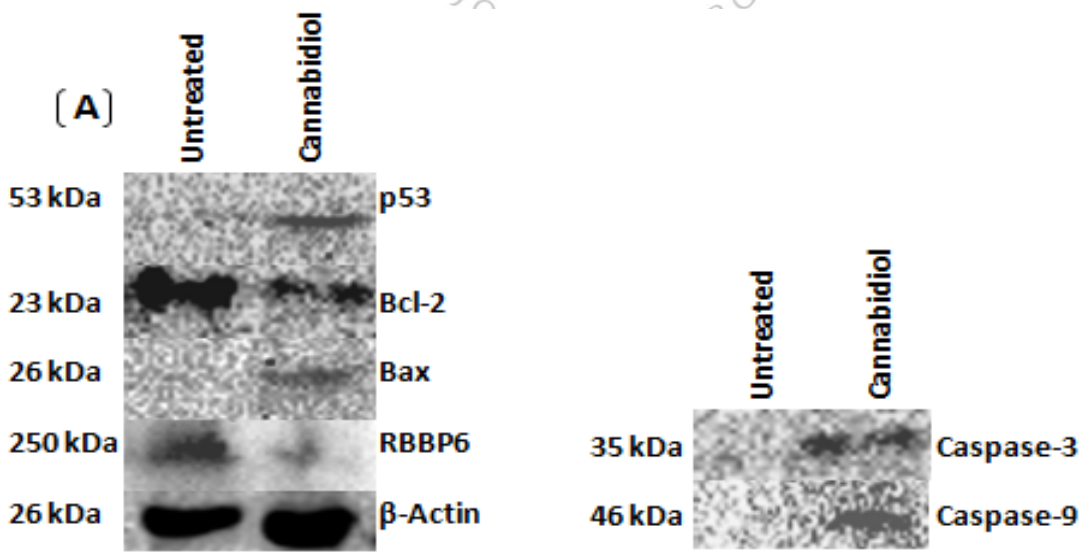


Figure 3.26: Morphological analysis and assessment of apoptosis in SiHa cells stained with DAPI and Annexin V dye after treatment with IC_{50} of Cannabidiol. Cells

were incubated with IC_{50} of cannabidiol for a period of 24hrs. Cells were stained with Annexin V and counterstained with DAPI. Fluorescence confocal microscopy was used to visualize the cells.

3.2.7 *Cannabidiol up-modulates the expression of upstream and downstream target proteins*.

To determine whether cannabidiol induces apoptosis via the mitochondrial pathway, western blotting was conducted. Cells were treated for 24 hrs with cannabidiol and protein lysates were separated on a 12% SDS-PAGE gel. Beta actin was included as a loading control. Western blot analysis revealed that cannabidiol effectively caused an increase in the expression pro-apoptosis proteins, p53 and Bax, while simultaneously decreasing the anti-apoptosis proteins, RBBP6 and Bcl-2 in all three cervical cancer cell lines (SiHa, HeLa, and ME-180 cells). Inhibition of Bcl-2 causes the expression of Bax protein to significantly increase, which has been implicated to be form part of the mitochondrial proteins that regulate apoptosis occurring via the mitochondria. **Figure 3.27** also showed that cannabidiol increased the expression of caspase-9 and caspase-3 when compared to untreated. The results suggest that apoptosis induction by cannabidiol involved the activation of caspase-3 and caspase-9.



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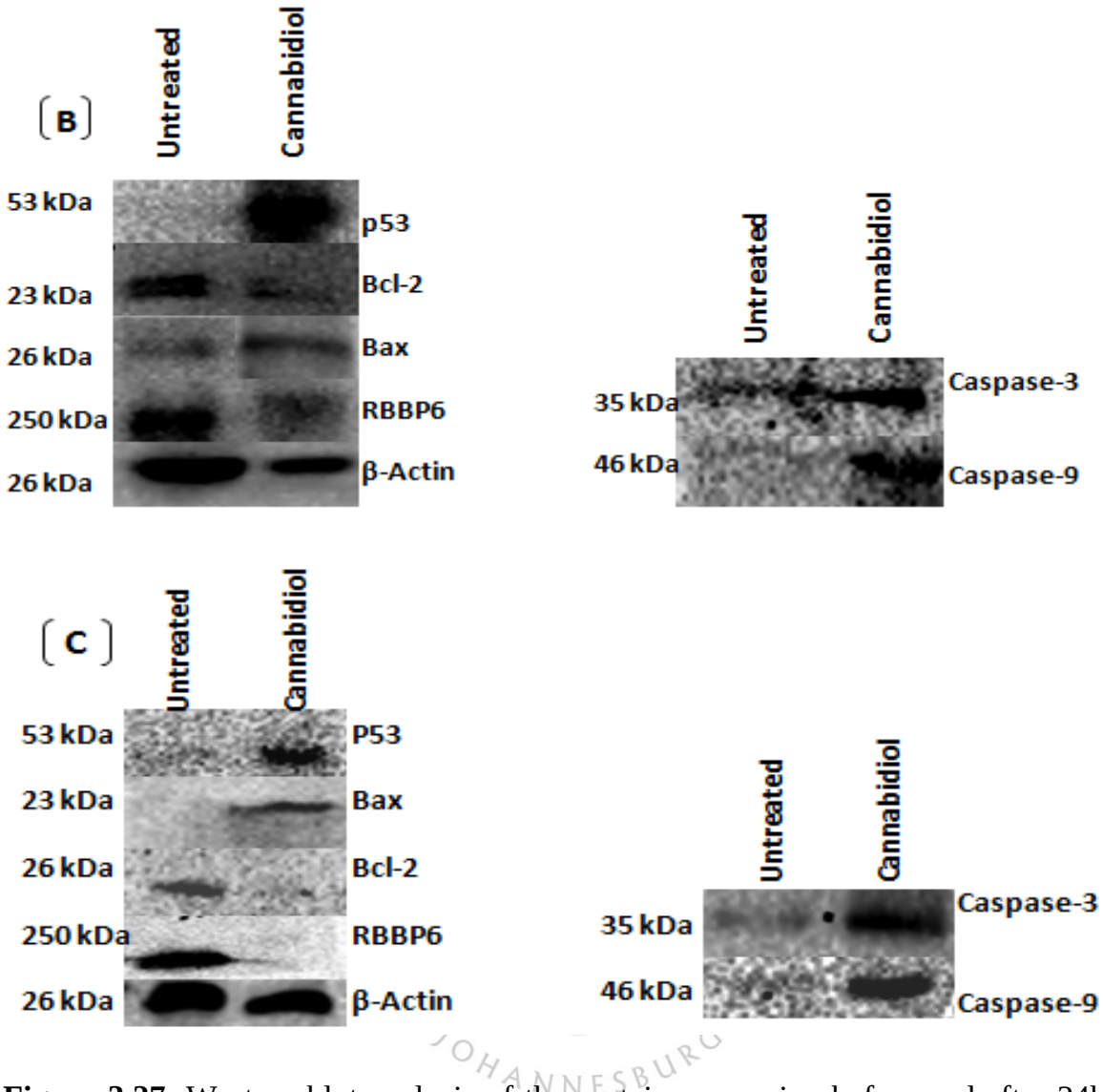

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Figure 3.27: Western blot analysis of the protein expression before and after 24hour treatment with IC_{50} of cannabidiol. A) SiHa, B) HeLa, and C) ME-180 cells were treated for a period of 24 hrs and protein lysates were separated using SDS-PAGE gel. Untreated protein was used as a control. Antibodies against pro-apoptotic proteins (p53 and Bax) and anti-apoptotic proteins (Bcl-2 and RBBP6), Initiator caspase-9 and effecter caspase-3 were included to elucidate apoptosis induction.

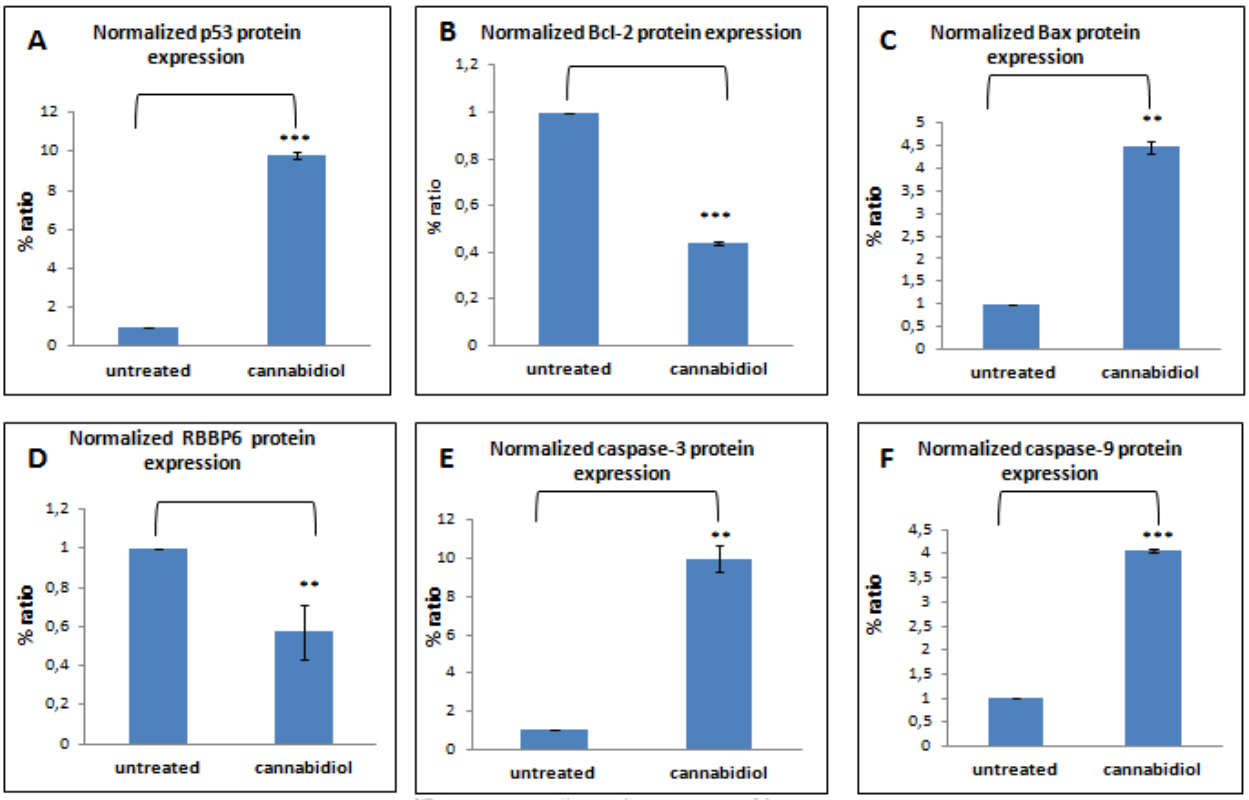

Figure 3.28: Densitometry analysis of SiHa protein following treatment with cannabidiol CPT represents camptothecin. Data represented as mean ± standard deviation with ***p<0.001 and **p<0.01 representing the level of significance in comparison to the untreated.

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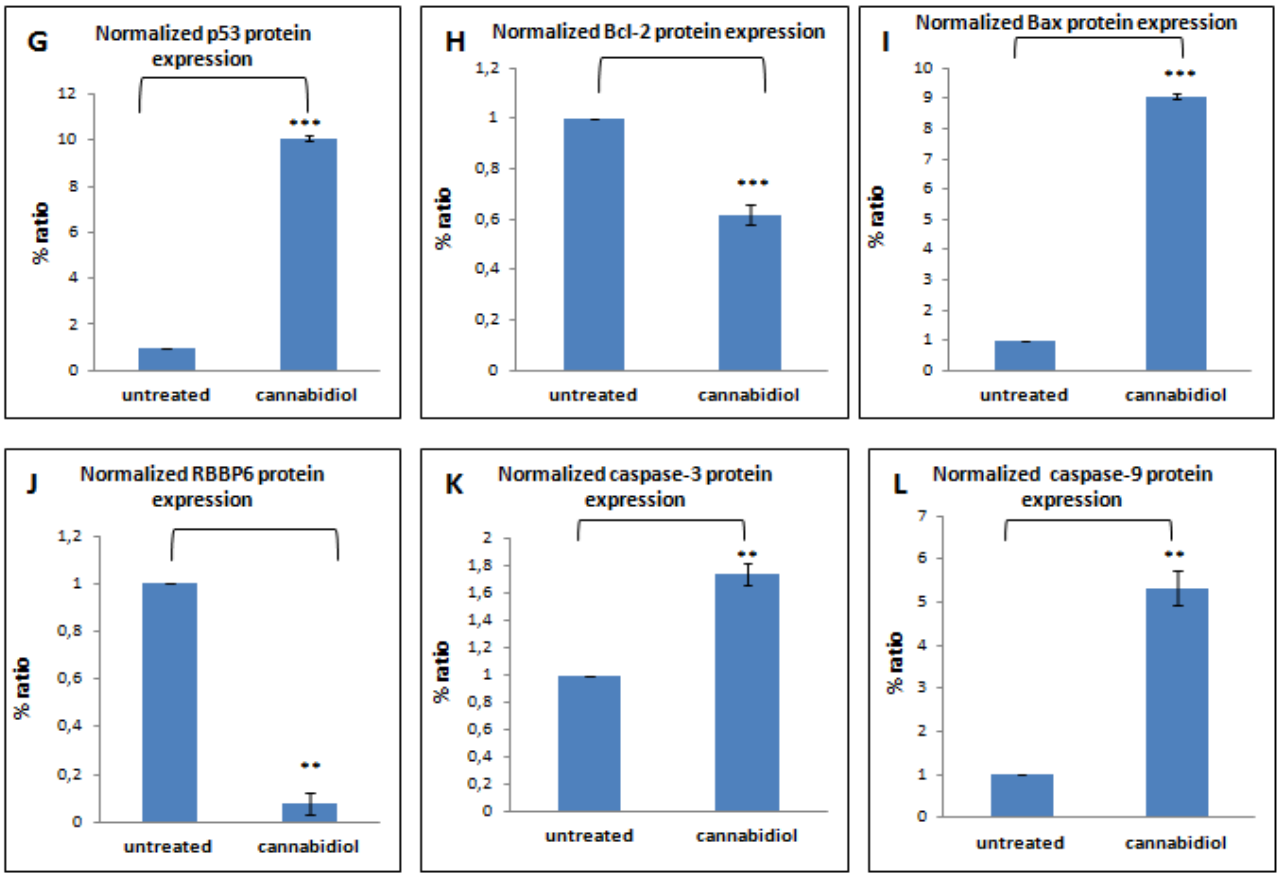

Figure 3.29: Densitometry analysis of HeLa protein following treatment with cannabidiol. CPT represents camptothecin. Data represented as mean ± standard deviation with ***p<0.001 and **p<0.01 representing the level of significance in comparison to the untreated.

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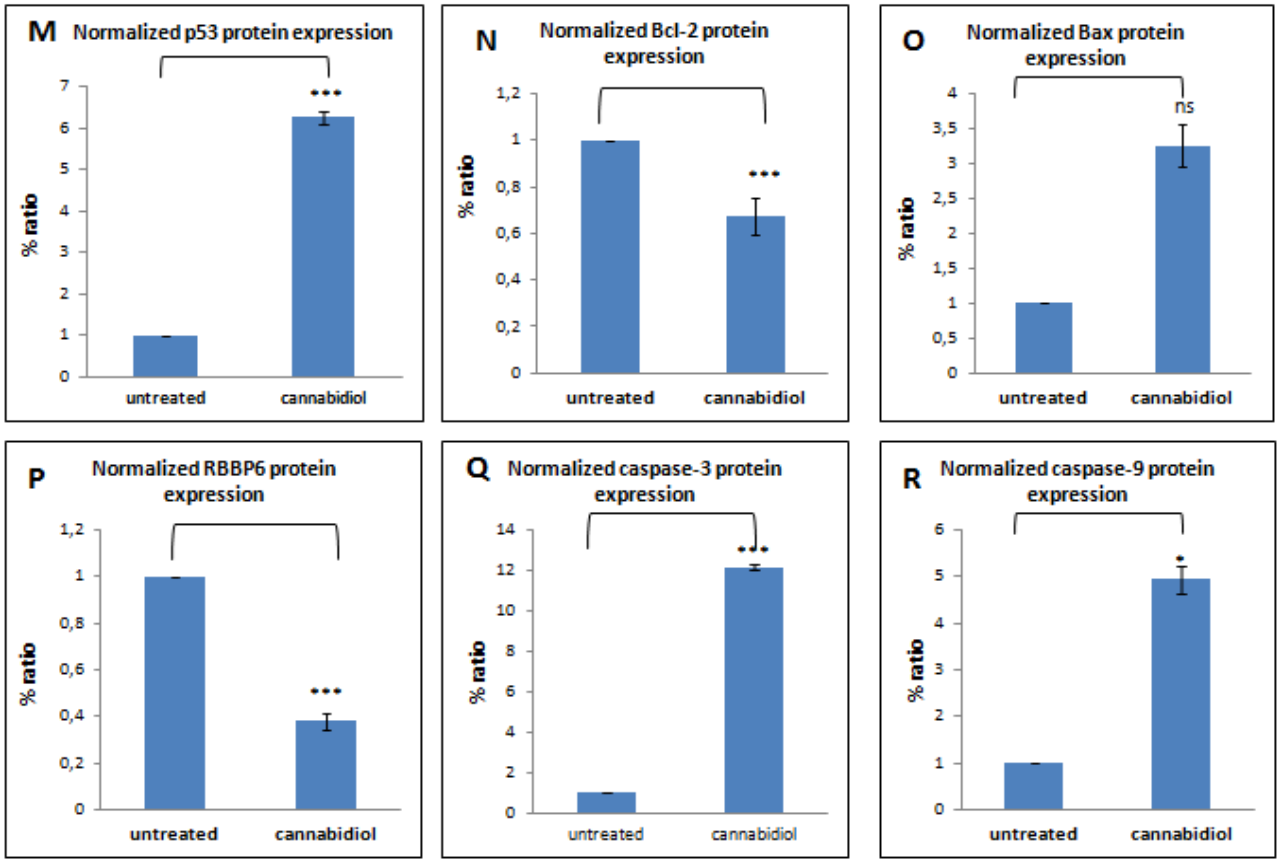

Figure 3.30: Densitometry analysis of ME-180 protein following treatment with cannabidiol CPT represents camptothecin. Data represented as mean ± standard deviation with ***p<0.001, *p<0.05 and ^{ns} p>0.05 representing the level of significance in comparison to the untreated.

3.2.8 *Cannabidiol increases caspase 3/7 activity in cervical cancer cells*

Caspase 3/7 play an effective role in the execution of apoptosis. Hence, the effect of treatment with cannabidiol was tested on caspase 3/7 activity. Human cervical cancer cell lines (SiHa, HeLa, and ME-180) were treated with cannabidiol. The activity of caspase 3/7 was compared to untreated. The results show that cannabidiol increased the activity of caspase 3/7 in all three cell lines (**Figure 3.31**). Interesting to note was that, treatment of ME-180 cells was almost compatible to those of *Cannabis sativa* extracts. This could mean that, the activity of *Cannabis sativa* might be due to the presence of cannabidiol. These results confirm the up-modulation of caspase-3 protein obtained in Western blot, meaning that cannabidiol-induced apoptosis might be caspase 3/ 7 dependent.

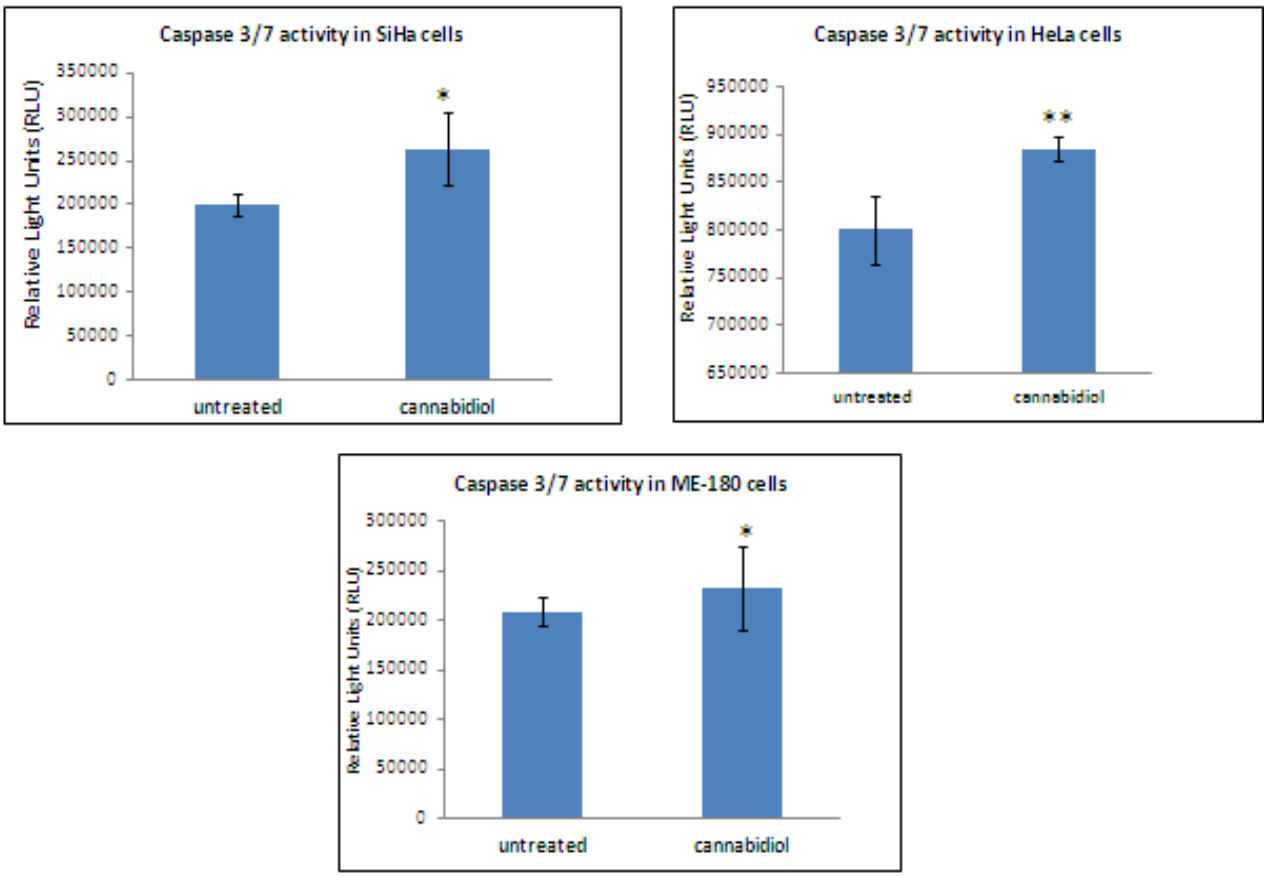

Figure 3.31: Caspase 3/7 activity after treatment of SiHa, HeLa, and ME-180 cells with IC_{50} of cannabidiol. Cells were treated with IC_{50} of butanol and hexane extracts for a period of 24 hrs. Caspase 3/7 reagent was added to the treated cells for 1 hr. Luminescence -was measured using GLOMAX instrument in RLU. Data represented as mean ± standard deviation with **p<0.01and *p<0.05 representing the level of significance in comparison to the untreated.

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

Cervical cancer remains a burden for women of Sub-Saharan Africa. Half a million new cases of cervical cancer and a quarter of a million deaths are reported annually due to lack of effective treatment. Currently, recommended therapeutic regimens include chemotherapy, radiation therapy, and surgery. However, they present several limitations including side effects or ineffectiveness, if the disease is metastatic in case of surgery. Therefore, it is important to search for new novel therapeutic agents that are naturally synthesized and cheaper, but still remain effective. Plants have been used for decades for health benefits and to treat several different diseases. Many communities are still dependent on medicinal plants to maintain their mental and physical health (Street and Prinsloo, 2012). However, some of the medicinal plants used by these communities are not known to be effective and their safety is still unclear. Therefore, it is important to scientifically evaluate and validate their efficacy and safety. In the present study, cervical cancer cell lines (SiHa, HeLa, and ME-180) were exposed to different concentrations of *Cannabis sativa* extracts and that of its compound, cannabidiol, with the aim of investigating their antiproliferative activity.

The first objective was to determine whether *Cannabis sativa* butanol and hexane extracts had important constituents that might impact on the survival of cervical cancer cells. The importance of performing phytochemical screening was to identify and select for the crude extract that consists of potentially useful secondary metabolites. It is one of the most effective ways to discover new phytochemicals for the development of new drugs. Analysis detected for the presence of various medicinally important secondary metabolites including flavonoids, glycosides, phenols, quinones, steroids, saponins, tannins, triterpenes, and triterpenoids in the hexane extract. All the other phytochemicals, except saponins, were detected in the butanol extract of *Cannabis sativa*. These findings correlate with those of Audu *et al* (2014) and Zade *et al* (2013), whereby they found that Cannabis sativa dissolved in petroleum ether and chloroform also contained flavonoids, alkaloids, tannins, cardiac glycosides, balsam, phenols, terpenes, steroids and resins, with or without the

presence of saponins. This could mean that saponins might be solvent specific. All these components have been shown to possess anti-inflammatory, anti-cancer, anti-viral, and anti-microbial effects. Batra and Sharma (2013) suggested that flavonoids are active against different human cancers including colon, rectal, breast, and lung cancer.

Following detection of important secondary metabolites, we tested for the ability of *Cannabis sativa* extracts to induce cell proliferation using MTT assay. MTT assay determines the inhibitory concentration that induces 50% cell death upon treatment of cancer cell lines. As reported in Chapter 3.2 (Figure 1.7 and Figure 1.8), *Cannabis sativa* extracts were able to reduce cell viability and increase cell death in SiHa, HeLa, and ME-180 cells. At an IC_{50} of 50µg/ml, butanol and hexane extract exhibited 56.6% and 48.4% cell viability in SiHa cells. For HeLa cells, the concentration of 100µg/ml of butanol extract reduced cell viability to 52.7% and hexane reduced it to 54%. On the other hand, ME-180 cells were sensitive to an IC_{50} ranging from 100µg/ml of butanol extract to 50µg/ml of hexane extract. Butanol and hexane extracts were able to reduce cell viability to 48.6% and 54%, respectively. These results correlate with the findings obtained by Romano *et al* (2014), whereby they reported that *Cannabis sativa* was able to reduce cell proliferation in two colorectal cancer cell lines (DLD-1 and HCT 116). Sharma *et al* (2014) further proved that *Cannabis sativa* extracts rich in cannabidiol were able to induce cell death in prostate cancer cell lines LNCaP, DU145, and PC3 at low doses (20-70µg/ml).

According to Ligresti *et al* (2006), the activity of *Cannabis sativa* may be attributed to the presence of active compounds such as cannabinoids. *Cannabis sativa* is rich in delta-9-tetrahydrocannabinol (Δ^9-THC) followed by its inhibitor, cannabidiol. However, *in vitro* screening has revealed that Δ^9-THC exhibited little to no potency against cancer cell lines (Ligresti *et al*., 2006). It was suggested that cannabidiol might be responsible for the reported activities. Therefore, in this study, cannabidiol was included as a reference standard in order to determine whether the reported pharmacological activities displayed by *Cannabis sativa* extracts might have been due to the presence of this compound. We treated cervical cancer cells with lower doses,

since cannabidiol is present in very low concentrations in *Cannabis sativa*. Shrivastava *et al* (2011) showed that cannabidiol effectively induced cell death at an IC_{50} of 5-10µM during treatment of breast cancer cell lines such as MDA-MB-231, MCF-7, SK-BR-3, and ZR-75-1.We were able to show that cannabidiol reduces cell viability of SiHa, HeLa, and ME-180 cells at almost similar IC_{50}. At an IC_{50} of 3.2µg/ml (~ 10µM), almost 51% and 50% cell death was induced in SiHa and HeLa cells. On the other hand, ME-180 cells were responsive at an IC_{50} of 1.5µg/ml (~ 5µM), whereby cell viability was reduced to almost 56%. The activity of cannabidiol was similar to that of *Cannabis sativa*.

To compare the activity of *Cannabis sativa*, camptothecin was included as a positive control. Camptothecin functions as an inhibitor of an enzyme that regulates winding of DNA strands. This in turn causes DNA strands to break in the S-phase of the cell cycle. A study conducted by Moela *et al* (2013), exhibited the ability of camptothecin to be cytotoxic against MCF-7 breast cancer cell line and also induce apoptosis as a mode of cell death at 0.25µM. We also observed a similar cytotoxic pattern, whereby treatment of HeLa, SiHa, and ME-180 cells with 0.3µM of camptothecin induced almost 40.36%, 47.19%, and 32.25% cell death, respectively.

Cell death can be characterized by a decrease in the energy levels as a result of dysfunction of the mitochondria (Chen *et al*., 2011). Therefore, to evaluate the effect of treatment on the energy content of the cells, we conducted mitochondrial assay. This assay uses membrane integrity and ATP to detect for defects in the mitochondria. We only detected for ATP after treatment of cervical cancer cells with IC_{50} predicted in MTT. ATP acts as determinant of both cell death and cell proliferation (Lemasters *et al*., 1998). Cancerous cells use ATP to maintain their metabolic status and continuously divide to make copies of their genetic material (Lemasters *et al*., 1998). Therefore, a reduction of the ATP levels compromises the status of cell and often leads to cell death either by apoptosis or necrosis, while an increase is indicative of cell proliferation (Lemasters *et al*., 1998). Levels of ATP were high in untreated cells, which was evident that ATP was continuously produced to aid in the survival of the cells. Exposure of SiHa, HeLa, and ME-180 cells to the IC_{50} of *Cannabis sativa*

extracts caused a reduction in the ATP levels (Figure 1.10). Treatment of cells with cannabidiol either slightly or severely depleted the ATP levels. In this case we concluded that depletion of ATP was cell line dependent.

We further evaluated for the anti-proliferative effects of the IC_{50} on cervical cancer cell lines using the impedance-based xCELLigence system. xCELLigence continuously monitors cell growth, adhesion, and morphology in real-time in the presence of a toxic substance. Upon treatment of SiHa and HeLa cells with IC_{50} of butanol extract, we noted that there was little to no inhibitory effect observed on cell growth (Figure 3.4A, B, C, and D). The growth curve continued in its exponential growth in all cells including the treated, untreated and 0.1% DMSO. The same phenomenon was observed when SiHa cells were treated with 50µg/ml of hexane extracts and 3.2µg/ml of cannabidiol. Similar events were obtained in a study by Gumulec *et al* (2013), wherein the IC_{50} obtained via MTT slightly reduced or increased the viability of doxorubicin treated prostate cancer cell lines PNT1A, 22Rv1, and LNCaP. However, at a similar IC_{50} of 100µg/ml, a reduction in cell viability was observed following treatment of HeLa cells with hexane extract. This showed that the cells were responsive to hexane extract leading to a reduction in the cell index. On the other hand, ME-180 cells responded after a period of 2hrs following treatment with the IC_{50} of butanol and hexane extract. In comparison to butanol and hexane extracts, cannabidiol reduced the cell index of ME-180 cells after 2hrs of treatment, signalling growth inhibition.

Differences in the findings could be attributable to the fact that both methods have different principles and mechanism of action. MTT assay is an end-point method that is based on the reduction of tetrazolium salt into formazan crystals by mitochondrial succinate dehydrogenase enzyme. Mitochondrial succinate dehydrogenase is only active in live cells with an intact metabolism (Gumulec *et al*., 2013). Induction of cell death by *Cannabis sativa* crude extracts decreases the activity of the enzyme following treatment of HeLa, SiHa, and ME-180 cervical cancer cell lines. On the other hand, xCELLigence system is a continuous method that relies on the use of E-plates engraved with gold microelectrodes at the bottom of the plate. The

xCELLigence system is based on the changes in impedance influenced by cell number, size and attachment (Gumulec *et al*., 2013). Therefore, we concluded that it was possible that dead cells might have been attached at the bottom of the E-plate after treatment. Treatment of cells with 0.3µM of camptothecin led to a decrease in cell viability of HeLa cells after a period of 12hrs. A decrease in the cell index might be signal of treatment inhibiting cell growth and promoting death of HeLa cells. Post exposure of SiHa cells to camptothecin resulted in no alteration of the growth pattern. However it was noted that after 4hrs of treatment, cells reached a cytostatic phase, whereby the growth curve neither decreased nor increased. ME-180 cells responded to treatment after 3hrs, which saw a reduction in the cell index throughout the period of treatment. Even though all three cell lines were treated with similar concentration of camptothecin, however, we observed that cell growth inhibition was cell type dependent.

Following confirmation that *Cannabis sativa* and cannabidiol have anti-proliferative activity, we had to verify whether both treatments have the ability to induce cell cycle arrest in all three cell lines. Cell cycle analysis is an important method used to determine cell status and to distinguish between populations of cells in different stages of the interphase. This method uses a PI stain and flow cytometry to measure the relative amount of DNA present in the cells. In this study, propidium iodide (PI) was used to stain cells. Propidium iodide can only intercalate into the DNA of fixed and permeabilized cells with a compromised plasma membrane or cells in the late stage of apoptosis. Viable cells with an intact plasma membrane cannot uptake the dye. The intensity of stained cells correlates with the amount of DNA within the cells. HeLa, SiHa, and ME-180 cervical cancer cells were stained with PI and analysed using flow cytometry.

Prior to the addition of *Cannabis sativa* crude extracts, untreated SiHa (63.9%) and HeLa (71.6%) cells were accumulated in the G_0/G_1 phase, signalling cell growth and division. As cells were treated with IC_{50} of hexane and butanol extracts, changes were noted in the cell cycle. Treatment of SiHa cells with butanol (50µg/ml) and hexane (100µg/ml) extracts led to an accumulation of cells in the cell death phase (sub-G0

phase), without cell cycle arrest. When compared to the S-phase (9.4%) and G2/M (15.8%) phase of untreated cells, exposure of HeLa cells to *Cannabis sativa* butanol (100µg/ml) extract resulted in the accumulation of cells in the S-phase (22.1%) of the cell cycle and slight cell death induction (2.1%). And thus, according to Armania *et al* (2013), signals DNA synthesis and cell cycle proliferation. Results also indicate a decrease in the S-phase (8.45%) and an increase in the G2/M (19.7%) phase of HeLa cells following treatment with hexane (100µg/ml) extract, suggesting blockage of mitosis and cell cycle arrest. Interesting to note was that, the untreated metastatic cell line (ME-180) had the lowest number of cells committed to G_0/G_1 phase. However, when cells were exposed to hexane (50µg/ml) and butanol (100µg/ml) extracts, an increase in the G_0/G_1 phase was observed, with 29.8% and 31.9% coupled by an increase in the S-phase population which favours replication and duplication of DNA. A slight increase in the cell death phase (sub-G_0). This was not the case following treatment of cells with cannabidiol. Cannabidiol resulted in the accumulation of cells in the cell death phase of the cell cycle. Almost 42.2%, 17.4%, and 34.3% of SiHa, and HeLa, and ME-180 cells were commited to the cell death phase. In summary, *Cannabis sativa* induces cell death with or without cell cycle arrest while cannabidiol induces cell death without cell cycle arrest.

Since treatment caused the accumulation of cells in the sub-G_0 phase, also known as the cell death phase, and the severe depletion of ATP levels by cannabidiol, we further conducted an apoptosis assay. This was done to characterize the mode of cell death induced. Apoptosis plays a major role in determining cell survival .Annexin V/FITC and PI were used to stain the cells to be able to distinguish between viable, apoptotis and necrotic cells. Annexin V/ FITC can only bind to phosphotidylserine residues exposed on the surface of the cell membrane while PI intercalates into the nucleus and binds to the fragmented DNA. Viable cells cannot uptake both dyes due to the presence of an intact cell membrane. Treatment of all three cell lines with camptothecin, IC_{50} of *Cannabis sativa* and cannabidiol exhibited the type of induced cell death as apoptosis. As shown in Figure 3.11, camptothecin resulted in 30.4%, 31.3% and 45% apoptosis induction in SiHa, HeLa, and ME-180 cells. In comparison

to camptothecin, the IC_{50} of butanol and hexane extracts resulted in 28.5% and 17.2% apoptosis in SiHa cells. For HeLa cells, 100µg/ml of butanol and hexane extracts resulted in an increase of in number of apoptotic cells to 31.9% and 15.3%. On the other hand, almost 44.8% and 43.2% of apoptosis was induced following treatment of ME-180 cells with butanol and hexane extract. In a study by Sharma *et al* (2014), showed a similar pattern of cell death, wherein treatment of a prostate cancer cell lines, LNCaP and PC-3, with *Cannabis sativa* resulted in the induction of 40% and 39.4% of apoptosis at a concentration of 60µg/ml.

Apoptosis is characterized by morphological changes and biochemical features which include condensation of chromatin, convolution of nuclear and cellular outlines, nuclear fragmentation, formation of apoptotic blebs within the plasma membrane, cell shrinkage due to the leakage of organelles in the cytoplasm (Bortner *et al*, 2005). This study also investigated for induction of apoptosis upon treatment of cervical cancer cell lines with camptothecin, cannabis sativa extracts and cannabidiol. Annexin V/FITC and DAPI were used to visualize the cells under a fluorescence confocal microscopy. DAPI stained the nucleus of both live and dead cells while Annexin V/FITC binds to the phosphotidylserine residues present on the surface of dead cells. In contrast to untreated cells, morphological changes including the reduction of cell size, cell shrinkage, loss of shape of the cell, a fragmented nucleus, and blebbling of the membrane were observed during visualization of SiHa cells after treatment. An uptake of Annexin V/FITC suggests the induction of apoptosis, since it can only bind to externalized PS residues. This also proves that during cell growth analysis, SiHa cells were undergoing cell death while still attached to the surface of the flask.

Apoptosis is known to occur via two pathways, the death receptor pathway and the mitochondrial pathway (Zangemeister-Wittke and Simon, 2001). *Cannabis sativa* isolates including cannabidiol have been implicated to induce apoptosis via the death receptor pathway, by binding to Fas receptor or through an activated Bax triggered by the synthesis of ceramide in the cells (Bla´zquez *et al*., 2000). However, not much has been reported on the induction of apoptosis via activation of p53 by *Cannabis sativa*. In this study, we wanted to evaluate for the ability of *Cannabis sativa* and its isolate to

induce apoptosis via the mitochondrial pathway. Pro-apoptosis (p53 and Bax) and anti-apoptosis proteins (RBBP6 and Bcl-2) of all three cell lines were subjected to Western blot. P53 acts as a transcription factor for a number of target genes (Wong, 2011). Under normal conditions, p53 levels are maintained through constant degradation MDM_2 and its monomers (Vassilev *et al.*, 2012). RBBP6 is one of the monomers that helps degrade p53, due the presence of Ring finger domain that promotes the interaction of both proteins (Kappo *et al*., 2012). In response to stress stimuli such as DNA damage, hypoxia, UV light, and radiation light, p53 becomes activated and causes MDM2 expression to decrease (de Bruin and Medema, 2008). Mutation of p53, implicated to be associated with 50% of all human cancers, promote the tumorigenesis.

Bax and Bcl-2 form part of the proteins that regulate apoptosis via the mitochondria (O' Brien and Kirby, 2008). Following activation, p53 translocates into the cytosol and triggers the oligomerization of Bcl-2 with BAD, resulting in the inhibition of Bcl-2 activity (Li-Weber, 2013). This in turn allows Bax protein to be translocated to the mitochondria and participate in the release of cytochrome c through poration of the outer mitochondrial membrane (Li-Weber, 2013; Chipuk *et al*., 2004). An imbalance between Bax and Bcl-2 has been linked to the development and progression of tumours through the resistance of apoptosis (Li-Weber, 2013).

It is therefore crucial to design drugs that would effectively target these genes involved in the execution of apoptosis via the mitochondrial pathway. Camptothecin, hexane extract, and cannabidiol effectively up-modulated the expression of p53 in all three cell lines, leading to a decrease in RBBP6 protein expression. Apart from SiHa and HeLa, butanol extract failed to up-modulate p53 in ME-180 cells. Interesting to note was that butanol extract reduced the expression of RBBP6 protein in ME-180 cells. The mechanism behind failure of butanol to up-modulate p53 while down-modulating RBBP6 is unclear. However, we came to a conclusion that butanol induces apoptosis independently of p53. We further demonstrated that *Cannabis sativa* extracts, cannabidiol, and camptothecin were able to down-modulate the expression of Bcl-2 protein and up-modulate Bax expression.

Caspases play an effective role in the execution of apoptosis either through the extrinsic or intrinsic pathway. In this study, we wanted to validate whether caspase-9 and caspase-3 were involved in the initiation and execution of apoptosis. We demonstrated the ability of *Cannabis sativa* to initiate apoptosis by activating caspase-9. However, execution of apoptosis was either with or without the presence of capsase-3, depending on each cell line. Western blot revealed that *Cannabis sativa* hexane extract induced apoptosis via the activation of caspase-9 and caspase-3 when compared to untreated in all three cell lines. Similar results were obtained during treatment of all three cell lines with camptothecin. This was not the case with butanol. Butanol extracts up-modulated caspase-9 and caspase-3 in SiHa and HeLa cells only. Caspase-3 was not up-modulated in ME-180 cells. Caspase 3/7 activity assay revealed the up-modulation of caspase 3/7 following treatment of cervical cancer cells. However on the basis of the Western blot results, wherein butanol extract failed to up-modulate caspase-3, we can conclude that caspase-7 was responsible for the reported activity. cannabidol effectively up-modulated caspase-9 and caspase-3 in all three cell lines, when compared to the untreated and *Cannabis sativa* extract,. From the results we can conclude that, apoptosis induction was caspase dependent.

Conclusion

The aim of this study was to evaluate for the anti-growth effects of *Cannabis sativa* extracts and to also determine the mode of cell death following treatment. The activity of *Cannabis sativa* extracts was compared to that of cannabidiol, in order to verify whether the reported results were due to the presence of the compound. The study showed that the activity of one of the extracts might have been due to the presence of cannabidiol. It further demonstrated the ability of *Cannabis sativa* to induce apoptosis with or without cell cycle arrest and via mitochondrial pathway. More research needs to be done elucidating the mechanism between the active ingredients and molecular targets involved in the regulation of the cell cycle.

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References

1. Alexander, A., Smith, P. F., and Rosengren, R. J. (2009). Cannabinoids in the treatment of cancer. *Cancer letters*, vol. 285, pp. 6-12.
2. Amundson, A. S., Myers, T. G., and Fornace Jr, A. J. (1998) Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene* (17), pp. 3287-3299.
3. Arbyn, M., Castellsague, X., de Sanjose, S., Bruni, L., Saraiya, M., Bray, F., and Ferlay, J. (2011). Worldwide burden of cervical cancer in 2008. *Annals of oncology* 22, pp. 2675- 2686.
4. Audu, B.S., Ofojekwu, P.C., Ujah, A., and Ajima, M.N.O. (2014). Phytochemical, proximate composition, amino acid profile and characterization of Marijuana (Cannabis sativa L.).*The Journal of Phytopharmacology*, vol. 3 (1), pp. 35-43.
5. Bla´zquez, C., Galve-Roperh, I., Guzma´n, M., 2000. De novo-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase. *FASEB Journal*, 14, pp. 2315 – 2322.
6. Bortner, C.D., Oldenburg, N.B., Cidlowski, J.A. (1995). The role of DNA fragmentation in apoptosis. *Trends. Cell. Biol*. 5, pp. 21–26.
7. Braicu, C., Pileczki, V., Irimie, A., and Berindan-Neagoe, I. (2013). p53 siRNA therapy reduces cell proliferation, migration and induces apoptosis in triple negative breast cancer cells. *Mol Cell Biochem*, 381, pp. 61-68.
8. Bray, F., Ren, J., Masuyer, E., and Ferlay, J. (2013). Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int. Journal of cancer*, 132, pp. 1133-1145.
9. Burd, E.M. (2003). Human papillomavirus and cervical cancer. *Clinical Microbiology Reviews*, vol. 16, pp.1-17.
10. Caffarel, M. M., Andradas, C., Perez-Gomez, E., Guzman, M., and Sanchez, C. (2012). Cannabinoids: Anew hope for breast cancer therapy? *Cancer Treatment Reviews*: 38, pp. 911-918.

11. Caldon, C.E., Daly, R.J., Sutherland, R.L., and Musgrove, E.A. (2006). Cell cycle control in breast cancer. *Journal of Cellular Biochemistry*, vol. 97, pp. 261-274.
12. Cárdenas-García, M., and González-Pérez, P. P. (2013) .Integration of Pro-Apoptosis and Pro-Survival Signalling Pathways: A Useful Approach to In silico Biomedical Research. *Comput Sci Syst Biol*, vol. 6 (4), pp. 1-9.
13. Chakrabarti, O., Krishna, S., (2003). Molecular interactions of high risk human papillomaviruses E6 and E7 oncoproteins: implications for tumour progression. *Journal of Biosciences*, 28, pp. 337-348.
14. Chang, H.Y and Yang, X. (2000). Proteases for cell suicide: functions and regulation of caspases. Microbiol. *Mol Biol Rev*, vol. 64 (4), pp 821-46.
15. Chen, P., Yu, J., Chalmers, B., Drisko, J., Yang, J., Li, B., Chen, Q. (2011). Pharmacologic ascorbate induces cytotoxicity in prostate cancer cells through ATP depletion and the induction of autophagy. *Anticancer drugs Preclinical reports*, vol.23, pp. 437-444.
16. Chen, X., Niu, H., Chung, W. H., Zhu, Z., Papusha, A., Shim, E. Y., Lee, S. E., Sung, P. and Ira, G. (2011). Cell cycle regulation of DNA double-strand break end resection by Cdk1-dependent DNA2 phosphorylation. *Nat. Struct. Mol. Biol*, vol.18, pp.1015-1019.
17. Chen, J., Tang, H., Wu, Z., Zhou, C., *et al*. (2013). Overexpression of RBBP6, alone or combined with mutant p53, is predictive of poor prognosis in colon cancer. *PLoS ONE*, vol.8(6).
18. Chibi, M., Meyer, M., Skepu, A., Rees, D. J. G., Moolman-Smook, J. C., and Pugh, D. J. R. (2008). RBBP6 Interacts with Multifunctional Protein YB-1 through Its RING Finger Domain, Leading to Ubiquitination and Proteosomal Degradation of YB-1. *J. Mol. Biol*., vol. 284, pp. 908-916.
19. Chipuk, T.E., Kuwana, T., Bouchier-Hayes, L., Droin, M.N., Newmeyer, D.D., Schuler, M., and Green, D.R. (2004). Direct activation of Bax by p53 mediates Mitochondrial Membrane Permeabilization and Apoptosis. *Science journal*, vol. 303, pp. 1010.

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

20. Chung, S., Franceschi, S., and Lambert, P.F. (2010). Estrogen and ER-alpha: culprits in cervical cancer. *Trends Endocrinol Metab*, vol.21 (8), pp. 504-511.
21. Côté-Martin, A., Moody, C., Fradet-Turcotte, A., D'Abramo, C. M., Lehoux, M., Joubert, S., Poirier G. G., Coulombe, B., Laimins, L. A., Archambault, J. (2008) Human papillomavirus E1 helicase interacts with the WD repeat protein p80 to promote maintenance of the viral genome in keratinocytes. *J Virol*, 82, pp. 1271–1283.
22. Cragg, G. M. And Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology*, vol. 100, pp. 72-79.
23. diCiommo, D., Gallie, B. L., and Bremner, R. (2000). Retinoblastoma: the disease, gene and protein provide critical leads to understand cancer. *Cancer biology*, vol. 10, pp. 255-269.
24. Di Domenico, F., Foppoli, C., Coccia, R., and Perluigi, M. (2012). Antioxidants in cervical cancer: chemopreventive and chemotherapeutic effects of polyphenols. *Biochimica et Biophysica Acta* (1822), pp. 737-747.
25. de Bruin, E. C and Medema, J. P. (2008). Apoptosis and non-apoptosis deaths in cancer development and treatment response. *Cancer Treatment Reviews*, 34, pp. 737-749.
26. Denney, L., Adewole, I., Anorlu, R., Dreyer, G., Moodley, M., Smith, T., Snyman, L., Wiredu, E., Molijn, A., Quint, W., Ramakrishnan, G., and Schmidt, J. (2013). Human papillomavirus prevalence and type distribution in invasive cervical cancer in sub-Saharan Africa. *International Journal of Cancer*: 134, pp. 1389-1398
27. Delbridge, A. R. D., Valente, L. J., and Strasse, A. (2012).The Role of the Apoptotic Machinery in Tumor Suppression. *Cold Spring Harb Perspect Biol*. Doi: 10.1101/cshperspect.a008789.
28. Dueñas-Gonzălez, A., Lizano, M., Candelaria, M., Getina, L., Arce, C., and Cervera, E. (2005). Epigenetics of cervical cancer. An overview and therapeutic perspectives, pp. 1-24.

29. Flemming, R., Muntendam, T., Steup, T., Kayser, O. (2007).Chemistry and biological activity of tetrahydrocannabinol and its derivatives. *Top. Heterocycl. Chem*., 10, pp. 1–42
30. Francis, S. A., Nelson, J., Liverpool, J., Soogun, S., Mofammere, N., and Thorpe Jr, R.J. (2010). Examining attitudes and knowledge about HPV and cervical cancer risk among female clinic attendees in Johannesburg, South Africa. *Vaccine*, vol. 28, pp. 8026- 8032.
31. Fankhauser, M. (2002). History of cannabis in Western Medicine. In: Grotenhermen F, Russo E, eds. *Cannabis* and Cannabinoids. *New York: The Haworth Integrative Healing Press*. pp. 37-51.
32. Forman, D., de Martel, C., Lacey, C.J., Soerjomataram, I., *et al*. (2012). Global burden of human papillomavirus and related diseases. *Vaccine*, 305, pp. f12-f23.
33. Fonseca-Moutinho, J.A. (2011). Smoking and cervical cancer. Obstetrics and gynaecology, pp. 1-6.
34. Freedman, D. A., Wu, L., and Levine, A. J. (1999). Functions of the MDM2 oncoprotein. *Cell. Mol. Life Sci*., vol. 55, pp. 96-107.
35. Fulda, S., and Debatin, K. M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* (25), pp. 4798–4811.
36. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer. Available from http://globocan.iarc.fr. Accessed
37. Garcia-Garbonero, R and Supko, J.G. (2002). Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. *Clin Cancer Res*, vol. 8 (3), pp. 641-61.
38. Go´mez del Pulgar, T., Velasco, G., Sa´nchez, C., Haro, A., Guzma´n, M. (2002). De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis. *Biochemical Journal*, 363, pp. 183 – 188.
39. Green, J., Berrington de Gonzalez, A., Sweetland, S., *et al*. (2003). Risk factorsfor adenocarcinoma and squamous cell carcinoma of the cervix in

women aged 20-44 years: the UK National case control study of cervical cancer. *British Journal of Cancer*, vol. 89, pp. 2078-2086.

40. Gurib-Fakim, A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular aspects of medicine*, vol. 27, pp. 1-93.
41. Gumulec, J., Balvan, J., Sztalmachova, M., Raudeska, M., *et al*. (2013). Cisplatin-resistant prostate cancer model: differences in antioxidant system, apoptosis, and cell cycle.international journal of oncology. DOI: 10.3892/ijo.2013.2223.
42. Hainaut, P. & Hollstein, M. (2000). p53 and human cancer: the first ten thousand mutations. *Advances in Cancer Research*, vol. 77, pp. 81-137.
43. Happyana, N., Agnolet, S., Muntendam, R., Van Dam, A.,Schneider, B., and Kayser., O. (2013). Analysis of cannabinoids in laser-microdissected trichomes of medicinal *Cannabis sativa* using LCMS and cryogenic NMR. *Phytochemistry*, vol. 87, pp. 51-59.
44. Harris, C.C. (1996). Structure and Function of the p53 Tumor Suppressor Gene: Clues for Rational Cancer Therapeutic Strategies. *Journal of the National Cancer Institute*, vol. 88, pp. 1442-1455.
45. Hinds, P.W., and Weinberg, R. A. (1994). Tumor suppressor genes. *Current opinions in Genetics and Development*, vol. 4, pp. 135-141.
46. Hoetelmans, R. W. M., van Slooten, H. J., Keijzer, R., Erkeland, S., van de Velde, C. J. H., and van Dierendonck, J. H. (2000). Bcl-2 and Bax proteins are present in interphase nuclei of mammalian cells. *Cell Death and Differentiation*, vol. 7, pp. 384-392.
47. Horvath, C. A. J., Boulet, G. A. V., Renoux, V.M., Delvenne, P.O., and Bogers, J.J. (2010). Mechanisms of cell entry by Human papillomaviruses: an overview. *Virology journal*, vol. 7 (11), pp. 1-7.
48. Huang, S.T., Pang, J.S., and Yang R.C. (2010). Anticancer effects of *phyllanthius urinaria* and relevant mechanics. *Chang Gung Med J*, vol. 33:477-84.

49. Huertas, P., Cortés-Ledesma, F., Sartori, A. A., Aguilera, A. and Jackson, S. P. (2008). CDK targets Sae2 to control DNA-end resection and homologous recombination. *Nature*, 455, pp. 689-692.
50. Hunter, T. & Pines, J. (1994). Cyclins and cancer II: Cyclin D and CDK inhibitors. Come of age. Cell 79, 573–582.
51. Ibrahim, B., Sowemimo, A., Spies, L., Koekemoer, T., van der Venter, M., and Odukoya, O.A. (2013). Antiproliferative and apoptosis inducing activity of *Markhamiato mentosa* leaf extract on HeLa cells. *Journal of Ethnopharmacology* 149, pp. 745-749.
52. Igney, H. F and Krammer, H. P. (2002). Death and anti-death: tumor resistance to apoptosis, *Nature Reviews of Cancer*, vol. 2, pp. 277-288.
53. Itokawa, H., Morris-Natschke, S.L., Akiyama, T., and Lee, K.H. (2008). Plant derived natural product research aimed at new drug discovery. *Journal of Natural Medicines*, vol. 62, pp 263-280.
54. Jemal, A., Bray, F., Center, M.M. *et al*. (2011). Global cancer statistics. *CA Cancer J Clin*, vol. 61, pp. 69–90.
55. Jia, W., Hegde, V.L., Singh, N.P., Sisco, D., Grant, S., Nagarkatti, M., and Nagarkatti, P.S. (2006). Delta-9-tetrahydrocannabinol-induced apoptosis in Jurkat leukemia T cells is regulated by translocation of Bad to mitochondria. Mol Cancer Res 4, 549-562.
56. Joseph, B and Priya, R.M. (2010). Preliminary phytochemical of *Psidium guajava* L. Leaf of methanol extract and its cytotoxic study on HeLa cell lines. *Ethnopharmacology*, vol. 1.
57. Kastan, M.B. and Bartek, J. (2004). Cell-cycle checkpoints and cancer. *Nature*, vol. 432, pp. 316-323.
58. Kappo, M. A., Faqeer Hassem, E. A.B., Andrew Atkinson, R. A., Faro, A., Muleya, V., Mulaudzi, T., Poole, J, O., McKenzie, J. M., Chibi, M., Moolman-Smook, J. C., Rees, D. J. G., and Pugh, D. J. R. (2012). Solution Structure of RING Finger-like Domain of Retinoblastoma-binding Protein-6 (RBBP6) Suggests It Functions as a U-box. *The Journal of Biological Chemistry*, vol. 287 (10), pp. 7146-7158.

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

59. Kaur, R., Kapoor, K., Kaur, H. (2011). Plants as a source of anticancer agents. *J. Nat. Prod. Plant Resource*, vol. 1, pp. 119-124.
60. Kumar, R. V and Bhasker, S. (2014). Optimizing cervical cancer care in resource-constrained developing countries by tailoring community prevention and clinical management protocol. *Journal of Cancer Policy*, vol. 2, pp. 63-73.
61. Lavrik, I.N., Golks, A., and Krammer, P.H. (2005). Caspases: pharmacological manipulation of cell death. *J Clin Invest*, vol. 115 (10), pp. 2665-2672.
62. Ledwaba, R.J., Dlamini, Z., Naicker, S., and Bhoola, Z. (2004). Molecular genetics of human cervical cancer: role of papillomavirus and the apoptotic cascade. *Boil. Chem*., 385, pp.671-682.
63. Lemaster, J.J., Nieminen, A., Qian, T., Frost, L.C., *et al*. (1998). The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis, and autophagy. *Biochimica et Biophysica Acta*, 1366, pp. 177-196.
64. Li, L., Deng, B., Xing, G., Teng, Y., *et al*. (2007). PACT is a negative regulator of p53 and essential for cell growth and embryonic development. *Proc. Natl. Acad Sci*, 104 (19), pp. 7951-6.
65. Ligresti(2006). Anti-tumor activity of plant cannabinoids with the emphasis on the effect of cannabidiol on human breast cancer.
66. Lindsay, J., Degli Esposti, M., and Gilmore, A. P. (2011). Bcl-2 proteins and mitochondria- Specificity in membrane targeting for death. *Biochimica et Biophysica Acta* 1813, pp. 532–539.
67. Li-Weber, M. (2013). Targeting apoptosis pathways in cancer by Chinese medicine. *Cancer Letters* (332), pp. 304-312.
68. Lozano, I. (2001). The therapeutic use of *Cannabis sativa* in Arabic medicine. *Journal of Cannabis Therapeutics*, vol, 1 (1).
69. Magal, S.S., Jackman, A., Ish-Shalom, S., Botzer, E.L., Gonen, P., Schegel, R., and Sherman, L. (2005). Downregulation of Bax mRNA expression and protein stability by the E6 protein of human papillomavirus 16. *Journal of General Virology* 86, pp. 611-621.

70. Mamedov, N. (2012). Medicinal Plants Studies: History, Challenges and Prospective. *Med Aromat Plants* 1:e133. doi 10.4172/2167-0412.1000e13.
71. Mathabe, M.C, Nikolova, R.V, Lall, N., and Nyazema, N.Z. (2006). Antibacterial activities of medicinal plants used for the treatment of diarrhoea in Limpopo Province South Africa. *Journal of Ethno pharmacology*, 105, pp. 286-93.
72. Mbita, Z. (2012). Molecular analysis of the Domain With No Name (DWNN)/RBBP6 in human cancers. University of the Witwatersrand, PhD thesis.
73. McKallip, R.J., Jia, W., Schlomer, J., Warren, J.W., Nagarkatti, P.S., Nagarkatti, M. (2006). Cannabidiol-induced apoptosis in human leukemia cells: A novel role of cannabidiol in the regulation of p22phox and Nox4 expression. *Mol Pharmacol*, 70, pp. 897-908.
74. Moela, P., Choene, M.S., and Motadi, L.R. (2013). Silencing RBBP6 (Retinoblastoma binding protein 6) sensitizes breast cancer cells MCF-7 to camptothecin and staurosporine-induced cell death. *Immunology*, pp. 1-9.
75. Moitto, B., Chibi, M., Xie, P., Koundrioukoff, S., *et al*. (2014). The RBBP6/ZBTB38/MCM10 axis regulates DNA replication and common fragile site stability. *Cell reports*, vol. 7(2), pp. 575-587.
76. Moodley, M. (2009). Cervical cancer in Southern Africa: The challenges. *South African Journal of Gynaecological Oncology*, vol. 1 (1), pp. 11-13.
77. Morgan, D. O. (2007). The Cell Cycle: Principles of Control. London: New Science Press Ltd.
78. Motadi, L. R., Bhoola, K.D., and Dlamini, Z. (2011). Expression and function of retinoblastoma binding protein 6 (RBBP6) in human lung cancer. *Immunology*. vol. 216, pp. 1065-1073.
79. Mthembu, N. N., Choene, M., and Motadi, L.R. (2012). Wisdom from the forefathers' traditional medicine can induce apoptosis and inhibit cell proliferation. *Biological and Biomedical Reports* 2 (3), pp. 180 – 189.
80. Munger, K., Basile, J.R., Duensing, S., Eichten, A., Gonzalez, S.L., Grace, M., and Zacny, V.L. (2001). Biological activities and molecular targets of the

targets of the human papillomavirus E7 oncoproteins. *Oncogene*, 20, 7888 – 7898.

81. Newman, D.J., Cragg, G.M., and Snader, K.M. (2007). Natural products as sources of new drugs over the period 1981–2002. *Journal of Natural Products*, vol. 66(7), 1022–1037.
82. Nobili, S., Lippi, D., Witort, E., Donnini, M., *et al*. (2009). Natural compounds for cancer treatment and prevention. *Pharmacol Res*, vol.59 (6), pp. 365-78
83. O'Brien, M. A., and Kirby, R. (2008). Apoptosis: a review of pro-apoptotic and anti- apoptotic pathways and dysregulation in disease. *J Vet Emerg Crit Care*, vol. 18 (6), pp. 572-585.
84. Pang, C.L., and Thierry, F., (2012). Human papillomavirus proteins as prospective therapeutic. *Microbial Pathogenesis xxx*, pp. 1-11.
85. Pezzuto, M.J. (1997). Plant-derived anticancer agents. *Biochemical pharmacology*, vol. 53, pp. 121- 133.
86. Portt, L., Norman, G., Clapp, C., Greenwood, M., and Michael, T. (2010). Anti-apoptosis and cell survival: A review. *Biochimica et Biophysica Acta* (1813), pp. 238-259.
87. Pugh, D. J., Ab, E., Faro, A., Lutya, P.T., Hoffmann, E., and Rees, D.J. (2006) DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Struct Biol*, 6 (1).
88. Rahman, A.H.M.M., Anisuzzaman, M., Ahmed, F., Islam, R.A.K.M., and Naderuzzaman, A.T.M. (2008). Study of nutritive value and medicinal uses of cultivated cucurbits. *Journal of Applied Sciences Research*, vol. 4 (5), pp. 555-558.
89. Rao, G.V., Kumar, S., Islam, M. and Mansour, E.S. (2008). Folk medicines for anticancer therapy-a current status, *Cancer Therapy*, vol. 6, pp. 913-922.
90. Romano, B., Borrelli, F., Pagano, E., Cascio, M.G., Pertwee, R.G., and Izzo, A.A. (2014). Inhibition of colon carcinogenesis by a standardized Cannabis sativa extract with high content of cannabidiol. *Phytomedicine*, vol. 21(5), pp. 631-9.

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91. Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001). Regulation and function of the p53 tumor suppressor protein. *Current Opinion in Cell Biology*, 13: 332-337.
92. Safaraz, S., Adhami, V. M., Syed, D.N., Afaq, and Mukhtar, H. (2008). Cannabinoids for cancer treatment: Progress and promise. *Cancer Res*; 68 (2), pp. 339-344.
93. Sager, R. (1992). Tumor suppressor genes in the cell cycle. *Current opinions in Cell biology*, vol. 4, pp. 155-160.
94. Schwartz, G.K. and Shah, M.A. (2005). Targeting the cell cycle: A new approach to cancer therapy. *Journal of Clinical Oncology*, vol. 23 (36), pp. 9408-9421.
95. Sharma, M., Hudson, J.B., Adomat, H., Guns, E., and Cox, M.E. (2014). In Vitro Anticancer Activity of Plant-Derived Cannabidiol on Prostate Cancer Cell Line. *Pharmacology & Pharmacy*, 5, pp. 820-806.
96. Shamas-Din, A., Kale, J., Leber, B., and Andrews, D. W. (2013). Mechanisms of Action of Bcl-2 Family Proteins. *Cold spring harb. perspect biology*, vol. 5, pp. 1-21.
97. Sherr, C. J. (2004). Principles of Tumor Suppression. *Cell press*, vol. 116, pp. 235-246.
98. Soumuya, D., and Arunkumar, R. (2011), Brief assessment on cervical cancer. *J. cancer Scither* 17.
99. Solinas, M., Massi, P., Cantelmo, A.R., Cattaneo, M.G., *et al*. (2012). Cannabidiol inhibits angiogenesis by multiple mechanisms. *British Journal of Pharmacology*, vol. 167, pp. 1218-1231.
100. Stanely, M.A. (2012). Epithelial cell responses to infection with Human Papillomavirus, vol 25 (2); 215, pp. 215-220.
101. Street, R.A., and Prinsloo, G. (2013) "Commercially Important Medicinal Plants of South Africa: A Review." *Journal of Chemistry*, pp. 1-16. doi:10.1155/2013/205048.

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102. Tota, J.E., Chevarie-Davis, M., Richardson, L.A., deVries, M., and Franco, E.L., (2011). Epidemiology and burden of HPV infection and related diseases: implications for prevention strategies, *Preventive medicine* 53, pp. 12- 21.
103. Turner, C. E., Hadley, K. W., Holley, H. J., Billets, S., and Mole, L. M., Jr. (1975). Constituents of *Cannabis sativa* L. VIII: Possible biological application of a new method to separate cannabidiol and cannabichromene. Journal of Pharmaceutical Sciences, vol. 64 (5), pp. 810-14.
104. Vassilev, L. T., Vu B. T., Graves B., *et al.* (2003) In Vivo activation of the p53 pathway by small molecule antagonists of MDM2. *Science* 343:844-48.
105. Wall, M.E., Wani, M.C., Cooke, C.E., Palmer, K.H., MePhail, A.T., Sim, G.A. (1966). Plant antitumor agents I. The isolation and structure of camptothecin, a novel alkaloidal leukaemia and tumor inhibitor from *Camptotheca acuminata*. *Journal of the American Chemical Society* 88: 3888-3890.
106. Weinberg, R.A. (1995). The Retinoblastoma protein and cell cycle control. *Cell press*, vol. 81, pp. 323-330.
107. Wong, R,S,Y. (2011). Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental& Clinical Cancer Research*, vol. 30 (87), pp.1-14.
108. Wu, M., Ding, H., and Fischer, D.E. (2001). Apoptosis molecular mechanisms, *Encyclopedia of life sciences*, pp.1-8.
109. Yamaori, S., Kushihara, M., Yamamoto, I., Watanabe, K. (2010). Characterization of major phytocannabinoids, cannabidiol and cannabinol, as isoform-selective and potent inhibitors of human CYP1 enzymes. *Biochem Pharmacol*: 79, pp. 1691–8.
110. Zade, V., Wikhe, M., Dabhadkar, D., Dawada, S., and Patil, U. (2013). Antifertility efficancy of *Cannabis sativa* leaves on female albino rats. *The Journal of science inventions today*, vol. 2 (2), pp. 107-117.
111. Zangemeister-Witke, U and Simon, H.U. (2001). Apoptosis-Regulation and clinical implications. *Nature Publishing Group*: 8, pp. 537-544.

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

112. Zur Housen, H. (2000). Papillomaviruses causing cancer: Evasion from Host-cell control in early events in carcinogenesis. *Journal of the National Cancer Institute*, vol. 92 (9), pp.690-695.

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APPENDIX A

Preparation of buffers and reagents

Ferric chloride solution

Dissolve 9 g of Ferric chloride in 100 ml of distilled water.

10X PBS

8 g NaCl

0.2 g KCl

1.15 g Na_2HPO_4

0.2 g KH_2PO_4

pH 7.4

Dissolve in 800 ml distilled water.

Autoclave and store at room temperature

NB For the preparation of 1X PBS, dilute 1:10 with sterile distilled water and store at 4°C.

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10X stock solution of citric saline

1.35M potassium chloride

0.15M sodium citrate

Autoclave and store at 4° C

NB For the preparation of 1X citric saline solution, dilute 1:10 with sterile distilled water before use

Double lysis buffer

123.8 mM Tris-HCl, pH 6.8

4% SDS

20% Glycerol

10% β-Mercaptopethanol

Glucose free media preparation

500 ml RPMI 1640 with bicarbonate

10 mM galactose

10% FBS

1% Penicillin/ Streptomycin

Store at 4ºC

Western blot reagents

Laemmlie 4X sample buffer

31.5 mM	Tris-HCl, pH 6.8
10%	Glycerol
1%	SDS
0.005%	Bromophenol blue

10X Running gel buffer

25 mM	Tris base
190 mM	Glycine
0.1%	SDS

NB For the preparation of 1X Running gel buffer, dilute 1:10 with sterile distilled water before use.

10X Transfer buffer

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25 mM Tris base

380 mM Glycine

0.1% SDS

NB For the preparation of 1X Transfer buffer, dilute 1: 9 with sterile distilled water and add 20% methanol before use.

4.4. **PBS-Tween washing buffer**

1X PBS

0.1% Tween 20

Dilute in 900 ml distilled water

4.5. **5% w/v blocking buffer**

Dissolve 5g of non-fat powdered milk in a 100 ml of PBS-Tween.

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Appendix B

Protocols

1. Cell detachment procedure

Old media was discarded and cells were washed twice with 2 ml of 1X PBS (appendix 1). Two millilitres of 1X citric saline buffer (appendix 2) was added to the cells and further incubated 37°C under 5% CO_2 until the cells have detached from the surface of the flask. Ten millilitres of supplemented DMEM media was added to the cells to deactivate the action of citric saline buffer.

2. Phytochemical screening procedure

Detection of Flavonoids:

To detect for the presence of flavonoids, an alkaline reagent test was conducted. Ten drops of sodium hydroxide solution was added to 1 ml of the filtrate. The mixture was allowed to stand until a yellow colour was formed. A few drops of dilute hydrochloric acid were added to the sample. Change of colour from yellow to colourless indicated the presence of flavonoids.

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Detection of Glycosides

To test for the presence of glycosides, a modified Borntragers test was conducted. a few drops of dilute hydrochloric acid were added in 1 ml of the filtrate. Equal amounts of the filtrate and Ferric chloride solution were mixed and the sample boiled for 5 min, to allow efficient mixing. The sample was allowed to cool down and equal amounts of benzene were added to it. A few drops of ammonia solution were added until two immiscible layers were formed. Formation of a rose pink ammonia layer indicated the presence of glycosides

Detection of Phenols

To detect for the presence of phenols, a Ferric chloride test was conducted. a few drops of ferric chloride solution were added to the filtrate and the formation of a dark blue to black colour indicated the presence of phenols.

Detection of Quinones

To detect for the presence of quinines, equal amounts of filtrate and concentrated Sulphuric acid were mixed together and the formation of a brick red colour indicated the presence of quinines.

Detection of Steroids

To check for the presence of steroids, equal amounts of filtrate and chloroform were mixed together. This was then followed by the addition of 2 ml acetic anhydride and a few drops of sulphuric acid to the mixture. Formation of a brownish-red monolayer indicated the presence of steroids.

Detection of Saponins

To detect for the presence of saponin, a froth test was performed. Ten millilitres of distilled water was added to 1 ml of the filtrate. The sample was placed on a shaker for 15 min. The formation of white foam indicated the presence of saponins.

Detection of Tannins

To detect for tannins,a gelatin test was conducted. one percent gelatin solution containing sodium chloride was added to the extract and formation of a white precipitate indicated the presence of tannins.

Detection of Triterpenes

To detect for the presence of triterpenes, a Salkowski's test was performed. A few drops of chloroform were added to the filtrate and the sample was filtered, to separate the precipitate from the liquid extract. A few drops of concentrated Sulphuric acid was added and the sample was allowed to stand until a golden yellow colour was formed, indicating the presence of triterpenes

Detection of Triterpernoids

To detect for the presence of triterpenoids, 2 ml of chloroform and 3 ml of concentrated sulphuric acid was added to 5 ml of the plant extract and the formation of a reddish brown monolayer indicated the presence of triterpernoids

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APPENDIX C

Results

Titration curves

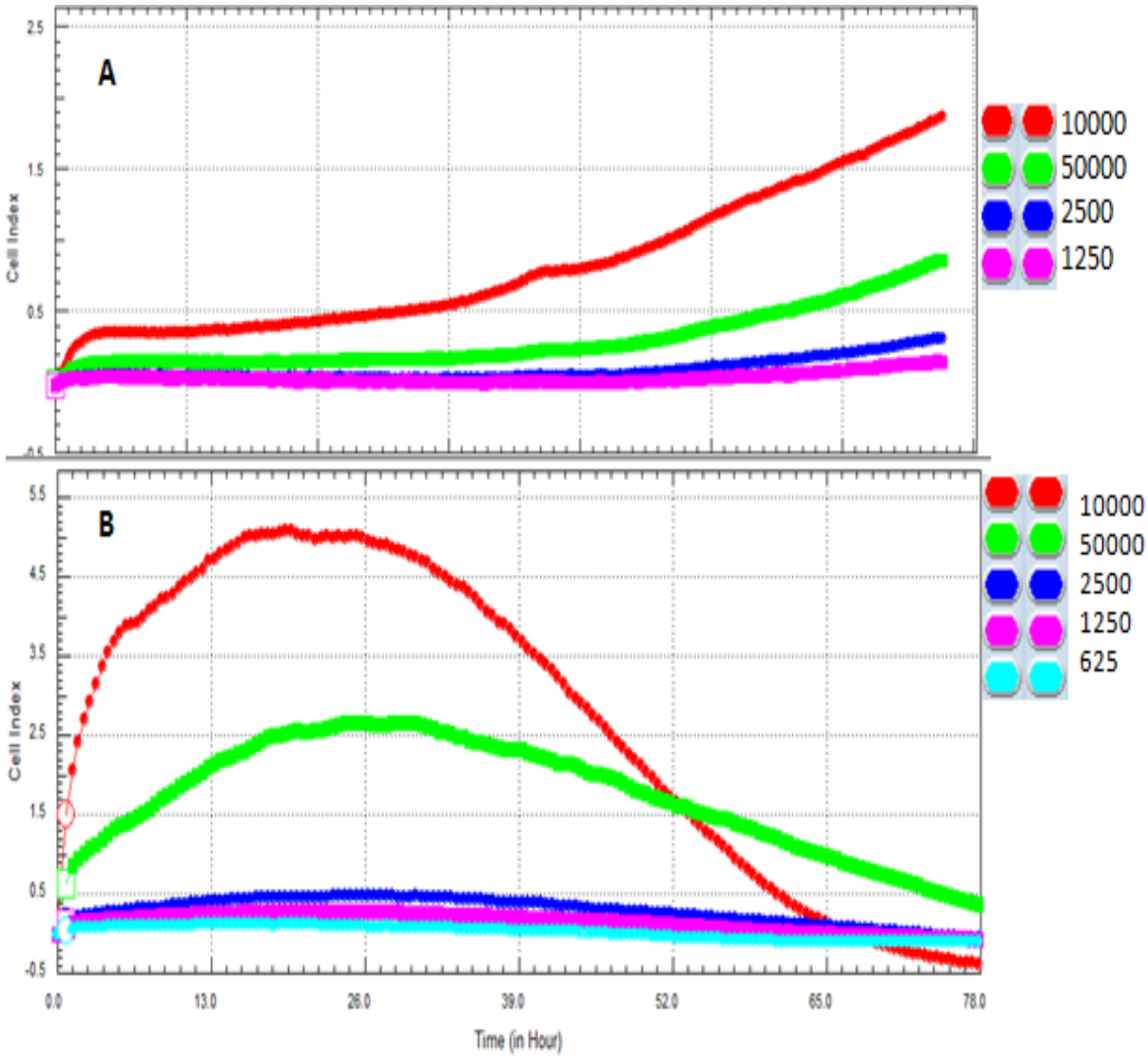

Figure 3.32: Representative optimal density titration curves of cervical cancer cell lines. A represents growth curve of SiHa cells while B was for HeLa. Cells were grown in duplicates at densities ranging from 10 000 to 625 cells per well for a period of 78 hrs.