Screening methanolic extracts of *Sutherlandia spp* as anti-tumor agents and their effects on anti-apoptotic genes.

by

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DECLARATION

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I declare that the above dissertation/thesis is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references

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ABSTRACT

Introduction: Cervical cancer is the most common malignancy after breast cancer in women worldwide. It accounts for 83% of all new cases and 85% cancer death in developing countries. In South Africa, cervical cancer is the common cancer in women with an annual crude incidence rate of 30.2 per 100,000 women and the highest rate were found to be in women between the ages of 66-69 years. Approximately 6800 women in S.A face new case of cervical cancer while accounting for 3700 cancer death annually. Because of unequal access to the health facilities, socio-economic differences, HPV and HIV infection, the rate of cervical cancer in black women is higher (42.1%) compared to the low rate in white women. Because of the name “cancer bush’ given to *Sutherlandia Frutescence(S.F)* plant by the traditional healers as well as Xhosas, Zulu, Sotho and cape Dutch for its anti-cancer activity, the plant was in this study to confirm its cytotoxic effect on the cervical cancer cell lines.

Aim of the study: to evaluate the methanolic extracts of *Sutherlandia Frutescens* on cervical cancer cell lines.

Materials and Methods: The MTT assay was performed to evaluate SiHa cell lines treated with methanolic extract of S.F (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml). The three compounds (Canavanine, GABA and Pinitol) were also evaluated for its anti-tumour activity. The cell growth was then showed in real time using Xcilligence. Flow cytometry was employed to determine the mode of action. Caspase 3/7 assay was performed to confirm if cell death was via caspase-dependent or independent and ATP was done to assess the ATP level in S.F treated cells.

Results: MTT shows a significant decline in cells treated with 50µg/ml, 100µg/ml and 200µg/ml of the extract and 50µg/ml was concluded to be the concentration at

which 50% of the cells die. The ATP results are inconsistent with MTT result; the ATP level increased in S.F treated cells. Cell index which represents the quantitative measure of cell growth in real time decline upon treatment with 50µg/ml. Flow cytometry showed cells are dying by apoptosis and the cell cycle arrest is mostly in the S phase. The cell death was caspase-dependent as it shows an increased luminescence which is proportional to the number of caspase. The concentrations of the compounds used, Canavanine (1000µM, 1500µM and 2500µM), GABA (100µM, 300µM and 500µM) and Pinitol (30µM, 90µM and 120µM) induce cell death and cell death shows to decrease after the maximum concentration.

Conclusion: *Sutherlandia Frutescence* has proven with number of research that it induces cell death via apoptosis. After evaluating its cytotoxicity, the plant shows to be a promising anti-tumor agent that needs to be clinically proven.

Keywords: Cervical cancer, *Sutherlandia Frutescence* and apoptosis

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1.1. Medicinal Plants

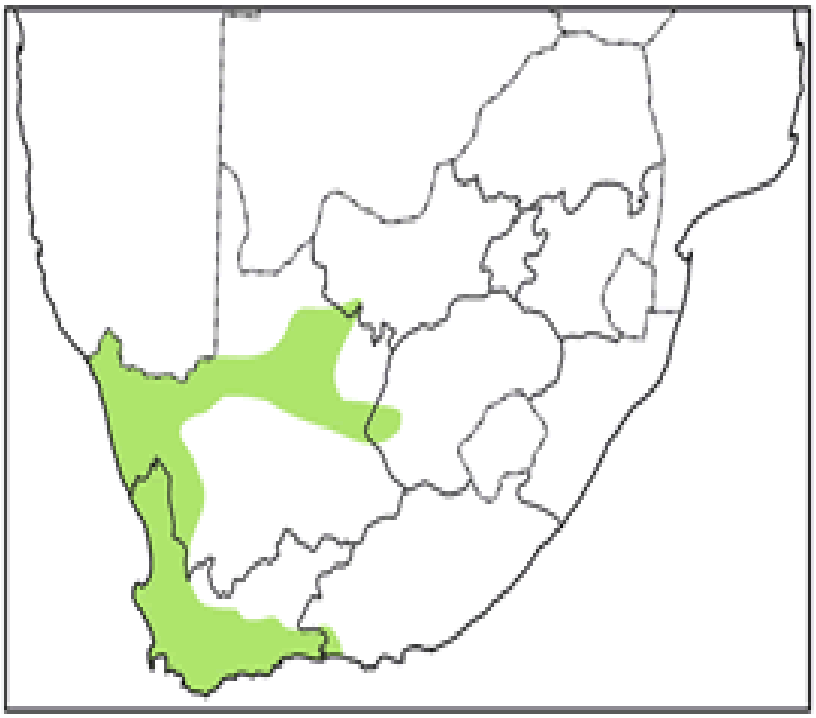
Many people worldwide especially in developing countries still trust in herbal medicines this accounts for 70-80% of the world's population as per the World Health Organisation (WHO). Traditional medicines have sustained the health of millions of Africans over hundred years and they have long history of known usage in alleviating various sicknesses and cancer is not an exception (Balunas and Kinghorn 2005).

The search for an anti-cancer drug was initiated in the 1950's by the discovery and development of vinca alkaloids, vinblastine, vincristine and podophyllotoxin (Cragg and Newman 2005) which are believed to cure various ailments such as respiratory, uterine diseases, fever, inflammation, arthritis, TB, reducing stress, relieving pain, and many other diseases. Since then medicinal plants have been selected randomly for assessment as anti-proliferating drug (Cragg and Newman 2005). More than 3000 plants have been identified as anti-cancer drugs (Balunas and Kinghorn 2005, Chan 2003). Taxol, Camptothecin, epipodophyllotocin are the anti-cancer drugs developed from *Taxus brevifolla* (Taxaceae), *Camptotheca (decuminata)* Decne (Nyssaceae) and *Podophyllum peltatum* Linnaeus respectively.

1.2. *Sutherlandia Frutescens*

Sutherlandia Frutescens (S.F), an indigenous plant to South Africa belonging to the family of Galegeae (Van Wyk and Albrecht. 2008). The xhosas, zulu, sotho and cape dutch used the plant for various illness such as stomach ache, fever, internal cancer, diabetes and wounds (Van Wyk and Gericke 2000). Moshe *et al*. (1998) found that the decoction consumed as blood tonic have been used as a treatment for cough, uterine cancer and eye infection. The plant is closely related to Astragalus and Lessertia D.C Van Wyk and Albrecht (2008) and found in southern part of Namibia, southern eastern parts of Botswana, western, central and eastern parts of South Africa and most part of Lesotho (Van Wyk and Albrecht. 2008). The other name of the plant is cancer bush or “kankerbos for its anti-cancer activity. *Sutherlandia Frutescens* is a shrub with bitter, aromatic leaves that have varying height from 0.2-2.5m (Van Wyk and Albrecht. 2008) with pinnately compound leaves compromising pubescent to white-tomentose. As the first head of the Edinburg Botanical gardens, James Sutherland was honoured by naming the plant after him whereas Frutescence means bush from a Latin word Frutex. The plant was found to contain L-canavanine, GABA, flavonoids, pinntol, tritepenoids saponnis and other compounds which have a history of boosting an immune system (Van Wyk and Albrecht 2008).

(a)



(b)

(c) 

Figure 1.1: Geographical distribution of *Sutherlandia Frutescence*, (a), Geographical distribution in South Africa (b) The Sutherlandia Frutescens plant and (c) The flowers.

Source: http://www.plantzafrica.com/medmonographs/sutherlfrut.pdf

1.2.1. L-canavanine

L-canavanine has the powerful effect on the disruption of the normal metabolic process of arginine stored in leguminous plants (Bell, 1958; Bell et al. 1978) and its anti-metabolic function as the protection against preys as well as against disease causing organisms (Rosenthal, 1977, 1992). This arginine antagonist has a role in inhibiting carcinogenesis in various cancers. The above findings was witnessed by Kruse and McCoy, 1958 who reported that canavanine together with arginine competed to ensure the growth requirements of Walker carcinosarcoma 256 cells is met. Later on Kruse *et al*. 1959 later showed that the arginine was part of the protein of the cancer cells and that the diminution content of the protein hydrolysate was the same as canavanine.

1.2.2. GABA

The relationship between cancer and GABA was first established by Masaharu Tatsuta et al. (1990) when the team evaluated the role of GABA and N-methyl-N1-nitro-N-nitrosoguanidine-induced gastric carcinogenesis. GABA acts as the gut hormone in the gastrointestinal tract known to regulate the development and growth of malignancy in gastrointestinal tumour. The diamine oxidase (DAO), an enzyme responsible for production of GABA outside CNS, is increased in various cancer and this results in high level of GABA in cancer cells. (Nicholson *et al*.2001). In addition to DAO, glutamic acid decarboxylase (GAD), an enzyme involved in GABA synthesis was found in mammary gland malignancies (Muzuikiewicz, 1999). The findings suggest that GABA could be contributing to the nine human neoplasms GABA was found in.

1.2.3. Flavonoids

Flavonoids are polyphenolic compounds that occur naturally in plants, they have been known for billions of years are mostly present in fruits and vegetables. They are also found in medicinal plants such as *Sutherlandia Frutescens*. Flavonoids function comes to play when the phase I metabolizing enzymes metabolically activates pro-carcinogens which activates other intermediates that associates with nucleophiles causing cancer. Flavonoids then prevent the activation of phase I metabolizing enzymes such as CYP1A1 and CYP1A2 Marchand *et al*. (2000) Thus; they are likely to inhibit carcinogens and therefore inhibit tumour cell proliferation (Mutoh *et al*. 2000 and Markovits *et al*. 1989).

1.3. Mechanism of medicinal plants on cancer

Despite the large commercialization of *Sutherlandia Frutescens* as informal anti-cancer drugs, there is little phytochemical studies of the plant and no detailed studies of the chemical diversity have been published (Van Wyk and Albrecht, 2008; Mncwangi and Viljoen 2012). Although Moshe 1998 found the leaves of *Sutherlandia Frutescens* to contain six flavonoids, they have been unidentified and the mechanism is still unknown (Van Wyk and Albrecht 2008).

Kundu (2005) observed an antitumor activity when methanol extracts of S.F inhibit the 12-O-tetradecanoylphorbol-13-acetate on the mouse skin. While Chinkwo (2005) has shown that the South African *Sutherlandia Frutescens* exhibited apoptotic activity on the CHO, Caski and jukart T lymphoma cells following treatment with the plant, *Sutherlandia Frutescens* extracts tested on monkey and human cells lines revealed that the plant is not toxic (Seier *et al*. 2002, Johnson *et al*. 2007).

1.4 Cervical cancer

1.4.1 Incidences

Cervical cancer is one of the common malignancy after breast cancer in women worldwide (Liu *et al*.2009) The 83% of the cancer cases are new and 85% accounts for death in developing countries (Liu *et al*. 2009). According to the American Cancer Society, 12, 340 new cases of cervical cancer will be diagnosed in the United States and about 4 030 women are estimated to die from cervical cancer. Cervical cancer

can be diagnosed as early as 15 years old and mostly the diagnosis is done between the ages of 30-69 years old (Van Schalwyk *et al*. 2008).

Cervical cancer has the largest rate in South Africa as compare to other cancers affecting women, it affects 30.2 in 100,000 women and the highest rates were found to be in women between the ages of 66-69 years (Mqoqi *et al*. 2004). Approximately 6800 women in S.A face new case of cervical cancer while accounting for 3700 cancer death annually (Moqoqi *et al*. 2004). Because of unequal access to health facilities, socio-economic differences, HPV and HIV infection, the rate of cervical cancer in black women is higher (42.1%) compared to the low rate in white women (14.5%) (Mqoqi, 2004). South African women undergo cervical excision of premalignant condition and experience the premature delivery as a result of HPV infection (Talip *et al*. 2010).

1.4.2 Causes of Cervical cancer

It is difficult to pinpoint the major cause of cancer but it is believed to be caused by various factors from environmental factors such as radiation, a transition of lifestyle to the western life such as high consumption of alcohol and fatty food, oral contraceptives, hormonal and inheritance of mutant genes (Choene *et al*. 2012). There are two main types of cervical cancer, a squamous cell carcinoma and adenocarcinoma. Of all the cervical cancer cases, 80-90% of the cases are as a result of squamous cell carcinoma.

Oral contraceptives have been found to be the risk factor in the development of cervical cancer. Studies have shown that the chances of a woman who uses birth

control pills to develop cervical cancer are double than those who do not use the oral contraceptives (Moreno *et al.* 2002). The risk increases as the number of years of using the contraceptive pills increases (Moreno *et al*. 2002).

Tobacco smoke is a promoter of cancer (Coker *et al*. 2002, Castle *et al*. 2002). Although the mechanism of how it affects the HPV during cervical cancer development is unclear, however it is believed that the tobacco by-products entering the blood stream damages the DNA of the cervix, while the HPV oncoprotein block the apoptosis and a cell cycle arrest (Cooker *et al*. 2002). HPV infected women who smoke are 4times more likely to die from cervical cancer than in a non-smoking woman (Wright *et al*. 2005).

1.4.3 Human Papilomavirus

Papillomaviruses are non-enveloped viruses with 8000 base pairs that are commonly transmitted sexually (Doorbar 2007). The virus has two stages, the early and late stages which are responsible for DNA replication, transcriptional regulation and transformation. The late gene controls the formation of capsid coat (Frazer, 2008). HPV has the ability to transform normal cells to cancer cells. The virus oncoprotein either promote cell proliferation and or loss of tumour suppressor gene function. Because of the high rate of HPV infection and low rate of cervical cancer development indicate there are additional genetic factors in progression of malignant phenotype (Ho *et al*, 1998).

E6 and E7 are the HPV oncoprotein involved in cancer development by turning-off tumour suppressor genes p53 and pRb gene function respectively (Botha and Dochez, 2012). The HR-HPV oncogene protein E6 bind to the p53 tumour suppressor gene in combination with the cellular ubiquitin ligase E6AP and causes p53 degradation through the ubiquitin-proteasome pathway (Massimi *et al*. 2008). E6 can interfere with apoptosis through the association with the Bcl-family (Vogt *et al*. 2006; Thomas and Banks, 1998) and again through the up-regulation of apoptosis protein inhibitors c-IAP2 and survivin by the mechanism involving NF-κb (James *et al*, 2006, Yuan *et al*. 2005). E7 is defined by Di Domenico et al, (2012) as a small protein that induces cell cycle arrest at the S phase by binding to the retinoblastoma protein (pRb) which releases the E2F. Dyson (1998) confirmed the degradation of pRb through the proteasome dependent pathway.

1.4.4. Prevention of Cervical cancer

Since the infection with HPV takes 10 to 20 years to progress to cervical cancer with different stages of cervical intraepithelial lesions (CIN) as the pre-cancer (zur Hausen. 2002), cervical cancer is easy to prevent. This can be done by various methods and the most common being a Papinicolao test (Pap smear) which is defined as the screening test to check the abnormality in the cervical canal of female reproductive organ. Pap smear is then followed by various diagnostic procedures depending on the nature of the abnormalities. One of the sensitive diagnostic procedures after Pap smear is Colposcopy defined by Chase et al. (2009) as a diagnostic procedure to illuminates the view of the cervix, the tissue of a vagina and

vulva to examine them for signs any signs of abnormalities. It differentiates the normal from abnormal cervix tissues and suggests biopsies for further examination.

Cervical cancer is highly prevented with 90% success rate through Pap smear screening and vaccination. (Zhu et al. 2009) indicated that although Pap smear screening is playing an effective role in decreasing cervical cancer incidences, it does not assist when the HPV infection has progressed to CIN or cervical cancer. The screening helps in finding the pre-cancers before they develop into cervical cancer. There are still limitations in the screening hence there is an effort of improvement by using biological or molecular markers as adjunction in the existing screening procedure. Because of incorrect detection of cervical cancer by HPV-DNA testing, the expression of E6/E7 is believed to be a promising method of detection in cervical cancer (Clavel *et al*. 2001). The finding was supported by Castle *et al*. (2007), Lie *et al*. (2005) & Molden *et al*. (2005) who also demonstrated that the expression of E6/E7 will also be a reliable method compared to the Pap smear and HPV-DNA testing.

The other method used is genotyping which is the preferred method as it distinguish a benign serial transient infection from type-specific infection, HPV type of differing oncogenic potential (Shiffman *et al*, 2005), and it provides epidemiological information. Treatment facilities for progressed cervical cancer is limited in S.A, There are only 20 gynaecological oncologists in the country and a high demand for radiotherapy (Botha and Dochez 2012).

1.4.5. Treatment

Once the CIN has progressed to cancer, no vaccine or screening will assist in getting rid of the tumour. There are various treatment methods that are effective from treating CIN to advanced cervical cancer (Green 2006). The choice of the treatment depends on the stage of the tumour, the size, how far it has spread and the depth and often the treatment is combined. There are three most common treatments, this includes: surgery, radiotherapy and chemotherapy. In surgery, cryosurgery is often used in the pre-cancerous stage and not invasive cancer. The method uses intense cold to kills the abnormal cells by freezing them. Another surgery for pre-cancer treatment is the laser surgery where laser beam is used to burn off the abnormal cells and remove a surface lesion.

Loop electrosurgical excision procedure (LEEP) uses an electric current passed through a wired loop to remove cancerous cells from the cervix. Hysterectomy is also used as a treatment of cancer to remove the uterus or cervix usually at the stage 1AI of the cervical cancer. However, most treatments are limited due to site effects that follow the treatment which leads to an alternative treatment that is safe for patients. And the results of many cancers is due to failed apoptosis and cell cycle machinery and by restoring the functioning of these process through agents, cancer can be combated.

1.5. Apoptosis

1.5.1. Introduction

Cell proliferation is required to renew tissues for various purposes such as healing wounds, fighting infections or removing unwanted cells that may never had a

function or lost function due to mutation. The cell proliferation goes through stages and along the way mutation can results due to environmental and genetical stimuli. The mutation or damage in the DNA signals the tumor suppressor genes to arrest the proliferation allowing the repair process to take place. The proliferation will proceed after the damages have been completely repaired. If it is impossible to repair the damages, the cell is subjected to cell death via apoptosis. This is to ensure the integrity of the cell is maintained at all times.

Cell death occurs in both unicellular and multicellular organisms but it is understood more in multicellular organisms where it aid in sculpting the body parts during development, controlling the number of cells and eliminating the damaged cells.it has been defined by Kroemer *et al*, (2005) as an irreversible loss of plasma membrane integrity. Cell death occurs due to number of reasons including mutation which causes damages in the cell. Cell damage can be caused by various exogenous stimuli such as UV radiation which mutate the DNA and stabilize the p53, a tumor suppressor gene which play a role in inhibiting the formation of tumor (Batista 2009). Martin (2012) indicated that 50 to 70 and 20 to 30 billion cell dies in an adult and a child respectively. According to Lawen, (2003), Apoptosis occur 20 times faster than mitosis and is it therefore very crucial to maintain the balance between the two processes. An 80 year old person would have 1814kg of bone marrow and lymph nodes and a gut 16 km long if mitosis proceeds without cell death (Lawen, 2003). Apoptosis and Necrosis are the main types of cell death; both methods have an important role not only in the development and homeostasis of the cells but also in the pathogenesis of several diseases processes.

1.5.1.1 Apoptosis

Apoptosis in Greek expresses falling off of petals from the flower and was first defined by Ker *et al.* (1972) as the cell death that results in apoptotic bodies that are destroyed by phagocytic bodies. It involves number of biochemical and physical changes in the nucleus, cytoplasm and plasma membrane (Lawen, 2003). According to Golstein and Kroemer (2006), all the mentioned morphological and biochemical characteristics of apoptosis occur before plasma membrane integrity is lost. Apoptosis is characterised by cell shrinkage, nuclear condensation, membrane blebbing as well as DNA fragmentation into approximately 200 base pairs fragments (Majno, 1995) and the apoptotic bodies are then engulfed by phagocytic cells (Chritop, 2003).The process usually results without inflammatory because cell contents are not released in to the neighbouring cells (King and Cidlowski, 1998) as compared to Necrosis where the release of necrotic cells alerts the innate immune system resulting in inflammation (Golstein and Kroemer, 2006).

The biochemical characterization of apoptosis is the DNA cleavage in the nucleus leading to number of number of nucleosomal pieces exposure of phosphatidylserine and proteolytic cleavage various intra-cellular substrate (Zimmermann *et al*, 2001). Impairment of apoptotic pathway results in pathological defects such autoimmune diseases, neurodegeneration or cancer (Reed *et al*. 2001). Two types of apoptosis exists which are the mitochondrial pathway known as the intrinsic pathway and the receptor-mediated pathway known as extrinsic pathway.

1.5.2 Intrinsic Pathway

Intrinsic pathway occurs inside the mitochondria, it is controlled by the pro-apoptotic and anti-apoptotic proteins. Anti-apoptotic genes are found on the outer membrane

of the mitochondrion and the pro-apoptotic genes are found on the inner membrane of the mitochondrion as well as in the cytosol where they are inactive. Upon activation by the membrane pro-apoptotic protein in the cytosol, conformational changes results exposing the BH3 binding through proteolytic and dephosphorylation (Hengartner, 2000). The pathway can be triggered by the stimuli within the cell; this can be the oxidative stress, cytotoxic drugs, high level of cystolic Ca+ or DNA damages (Karp 2008). The pro-apoptotic protein stimulates the opening of mitochondria to release cytochrome c as well as SMAC/DIABLO which binds to the apoptotic protease activating factor 1(Apaf-1).ATP is the driving force in the function of cytochrome c and APaf-1. Without ATP, cytochrome c and Apaf-1 becomes inactive therefore no apoptosis. The Binding of Apaf-1 triggers the formation of apoptosome which consist of Apaf-1, cytochrome c, ATP and procaspases 9 on a ratio of 1:1.The activated caspase 9 is released from the complex to cleave and activate the caspase 3 (Budihardjo *et al*.1999) which then cleave the cellular substrate needed for processes leading to apoptosis (Ghobrial *et al*. 2005). The pathway is controlled at various steps, including the release of cytochrome c, the binding and hydrolysis of dATP by Apaf-1 and the inhibition of caspase activation by the proteins that belong to the inhibitor of apoptosis (IAP) (Budihardjo *et al*. 1999).

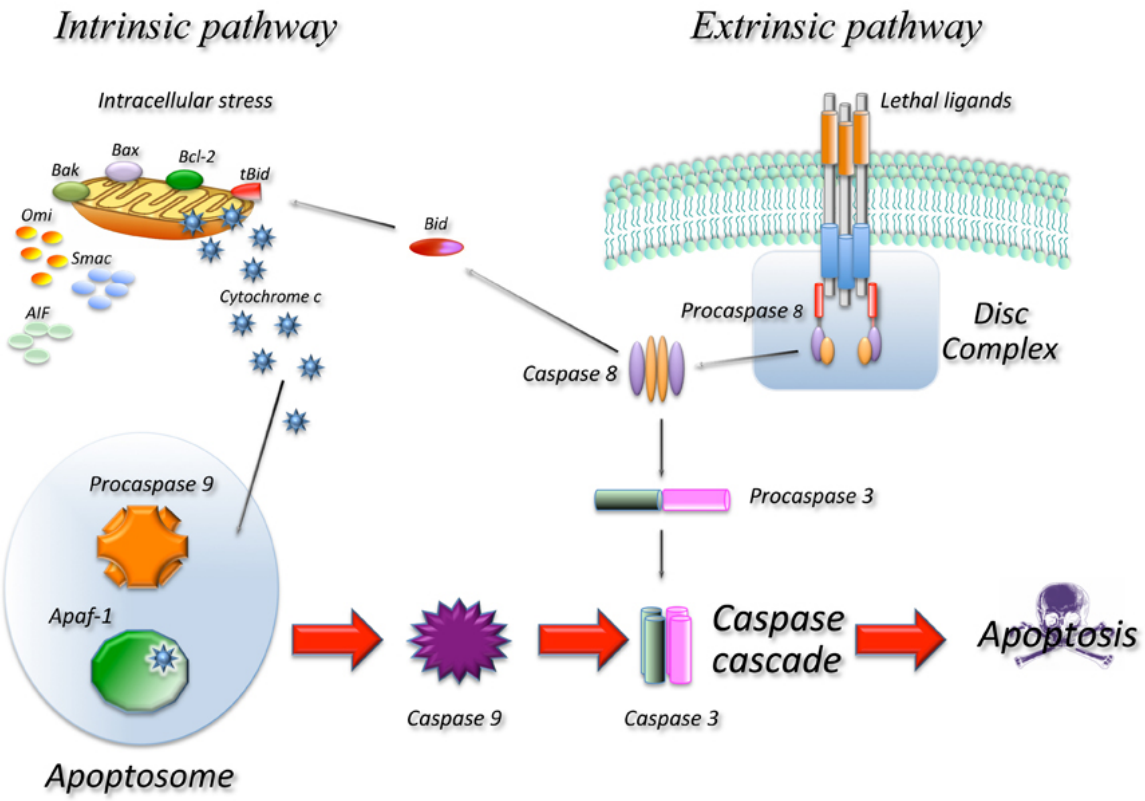


Figure1.2. The Schematic representation of the pathways leading to cell death by apoptosis (Favaloro *et al*. 2012**)**

The apoptotic stimuli activates the release of cytochrome c which binds to Apaf-1.The apoptosome consisting of cytochrome c, Apaf-1 and ATP triggers the recruitment and activation of pro-caspase-9 to caspase-9 which cleaves and activated caspase 3 leading to apoptosis.

1.5.3. Extrinsic Pathway

The pathway as the name implies, initiates at the extracellular space where the death signals causes the ligand TNF1 (Tumor necrosis factor 1) and Fas among other ligands to bind to the receptor on the membrane. There are several cell surface that play a role in the extrinsic pathway of apoptosis which have a conserved cysteine repeats at their outer domain (Budihardjo *et al*.1999). Fas and TNFR1, the most studied death receptors in apoptosis, have the same region named the death domain. The death domain; TRADD (TNF receptor associated death domain) and FADD (Fas associated death domain) binds to the TNF and Fas respectively (Schneider, 2000) resulting in Death inducing signal complex (DISC). The complex activates pro-caspases 8 to caspases 8 which then cleaves the executioner caspase 3 after the aspartate residue. The activated caspase 3 can cleave other cellular substrate including ICAD (inhibitor of caspase-activated DNase), ROCKI (Rho-associated coiled-coil forming kinase I), poly polymerase and many other (Lawen, 2003).

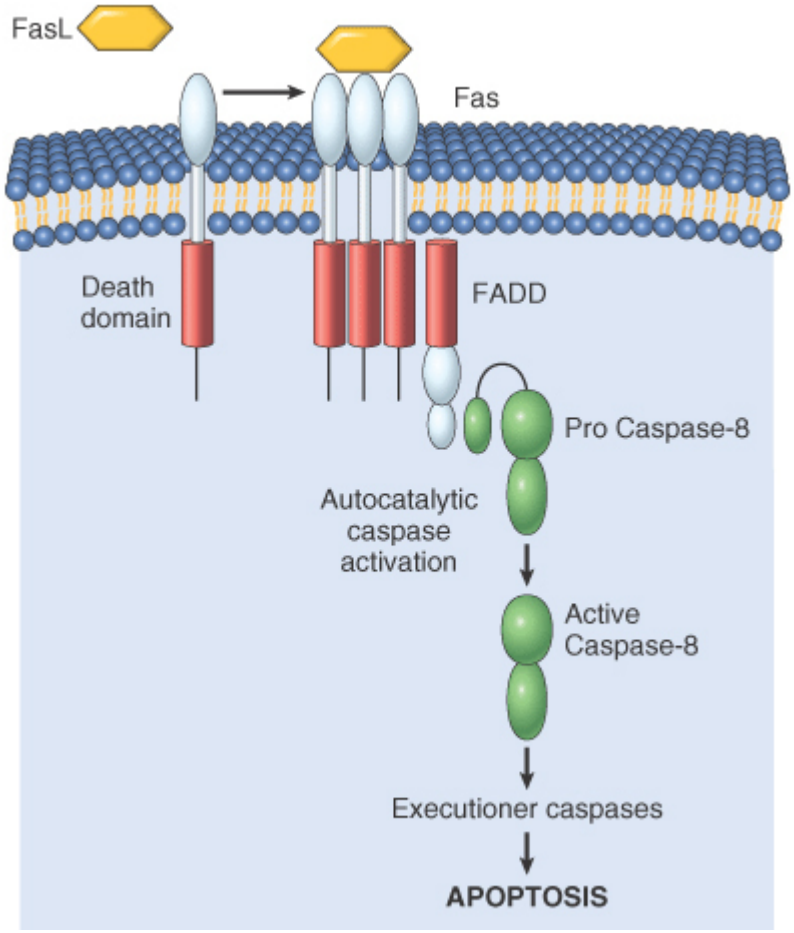


Figure 1.3. Simple schematic representation of extrinsic pathway.

Fas ligand outside the membrane attach to Fas on the cell membrane and recruits death domain specific for Fas called FADD. The complex called DISC activates pro-caspases 8 to caspases 8 which then cleaves the executioner caspase 3.

1.6. Bcl-2 Family

Reed (1997) discovered that intrinsic pathway is controlled by the balance of the anti-apoptotic and pro-apoptotic protein. Anti-apoptotic protein regulates apoptosis by blocking the permeability of mitochondrial thus preventing the release of

cytochrome c. High concentration of pro-apoptotic will stimulate the release of intermembrane protein and the mitochondrion membrane will not widen to release any protein in a case of low concentration as compared to high concentration of anti-apoptotic protein.

There are more than 20 members of Bcl-2 family (Kuwana and Newmeyer 2003) who are classified into three groups, the Bcl-2, Bax and BH3 subfamily. The Bax and BH3 subfamily are pro-apoptotic which are Bax, Bak and Bok and Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL. The Bcl-2 subfamilies, the Anti-apoptotic are Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1. The pro-apoptotic and anti-apoptotic proteins have a key role in the decision of cell to undergo cell death. Bax/bak, bad and bid are amongst the well-studied pro-apoptotic proteins in cancer research, they promote cell death by stimulating permeability of the mitochondria which releases cytochrome c upon stimulation. Mitochondrial permeability is made possible by two mechanisms; firstly by the permeability of mitochondrial mediated by Bcl-2 family protein and secondly by the induction on the mitochondrial permeably transition mainly through the release of Ca^{2+} from the endoplasmic reticulum stores (Newmeyer and Kuwana, 2003).This results when bax foes under post translation modification translocate from the cytosol to the outer membrane of the mitochondria, oligomerize and leads to the permeability of the mitochondria (Singh, 2007).

Bcl-2 family protein bax is believed to be the main pro-apoptotic protein to promote mitochondrial permeability releasing cytochrome c (Gross *et al*. 1999 and Newmeyer & Ferguson-Miller, 2003).The space in the mitochondria as a result of Bax is small to allow tiny proteins such as cytochrome c to pass through. Shimuzi *et al*. (1999)

believe that the membrane permeability occurs through the widening of voltage-dependent anion channel (VDAC) enough for small protein to pass through. Wei *et al*. (2001) argued that bax alone is not enough to stimulate mitochondrial permeability. For mitochondria to be permeable the two proteins; bax and tbid are required. Bcl-2, Bcl-xl and Bcl-w are the anti-apoptotic genes; they function as the inhibitor of mitochondrial permeability. A central checkpoint of apoptosis is the activation of Caspase-9 by mitochondria. The BH4 domain of Bcl-2 and Bcl-XL can bind to the C terminal part of Apaf-1, thus inhibiting the association of Caspase-9 with Apaf-1 (Hu *et al*. 1998).

1.7. Caspases

The specific feature of apoptosis is the cystein proteolytic enzymes called caspases; the C part of caspase refers to the cystein protease whereas the aspase refers to the specific location where the enzyme will cleave (Kumar *et al*. 2010). Although this is the case in apoptosis, Caspases should be removed from the definition of apoptosis as the protein is not universal for all the apoptosis process. Fourteen caspases have been identified so far, they all share some of their properties. All are aspartate-specific cystein protease, have conservative pentapeptide active site "QACXG" and their precursors are zymogens known as pro-caspases (Fan *et al*.2005).The N terminal of the procaspases has a structure responsible for caspase activation (Hermine *et al*.2005), has two DED(death effector domain) which make the N terminal a critical site recruiting the death domain.

Figure 3

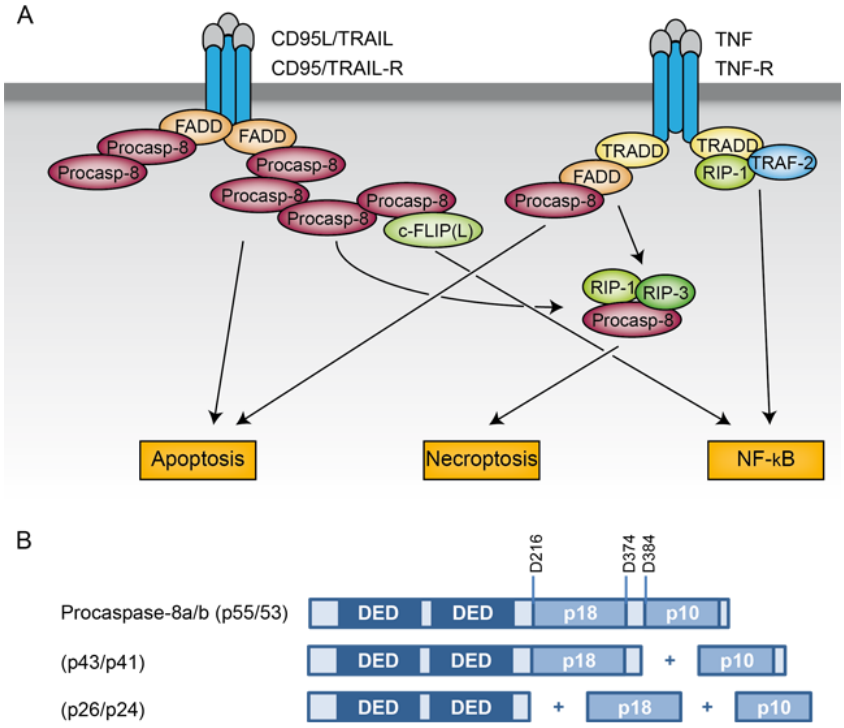


Figure1.4: Involvement of procaspase-8 in death-receptor signalling. (A) Procaspase-8 is recruited to the CD95 or TRAIL DISC through the adaptor protein FADD.

According to Fan *et al*. (2005), Caspases have been divided into three subfamilies, Subfamily I, II and III. Subfamily I consist of Caspase-2, 8, 9 and 10, they are known to be the activator of apoptosis and are capable of autolytic activation. Subfamily II consist of caspase-3, 6 and 7, they play a role in execution of apoptosis and require activation by initiating caspase cleavage (Ricci and El-Deiry) and subfamily III made up of caspase-1, 4, 5,11,12,13 and 14 which function to mediate inflammation. According to Wang *et al*. (1998), caspase 1 and 11 play a role in the cytokine processing and function of other caspases of the subfamily are unknown (Budihardjo *et al*. 1999). Caspases called interleukin-1 beta-converting enzyme (ICE) proteases, family of cysteine proteins that act as effector of the mammalian cell death pathway are the first caspases initially discovered as a cytokine-processing enzyme

(Budihardjo *et al*.1999). The structural changes of a cell as a result of apoptosis are due to activation of ICE.

At times apoptosis processes occurs faster than phagocytic cells can cope with. Because of this, apoptotic cells are released to neighboring cells resulting in Necrosis (Trump *et al.* 2001). Another reason for a switch from apoptosis to necrosis can occur when the proteins involved in apoptosis are removed or inhibited (Golstein and Kroemer, 2006). Because of ATP is required for the optimal activation of caspase, the depletion of ATP is one of the factors that lead to necrosis.

1.8. Necrosis

Necrosis comes from the Greek word 'necros' meaning death. Walker *et al*. (1988) defined necrosis as the cell death that occurs due to unfavorable environmental conditions or genetically encoded insults. In 2006, Golstein and Kroemer defined Necrosis as a lytic destruction of individual or group of cells and have been considered to be uncontrolled. The dominated concept in necrosis has been specified as a chaotic breakdown of cell under intolerable conditions (Syntichaki and Tavernarakis, 2002).The loss of cells ability to regulate homeostasis is the beginning of necrosis, this result in water retention and excretion of ions. This mode of cell death results as the consequences of internal stress such as heat, osmotic shock, freeze thawing and high concentration of hydrogen peroxide as well as the physiological conditions such as hypoxia, ischemia, hypoglycemia, toxin exposure, exposure to ROS metabolites and nutrient deprivation (Nicotera *et al*. 1999). According to Mayhew *et al*. (1999) renewal of small intestine is a physiological evidence that necrosis can occur as part of the normal cell loss (Murdoch *et*

al.1999). It often results in rupture of the plasma membrane and the organelle breakdown (Majino and Joris, 1995) which may promote the tumour progression through degenerative proliferation and invasion. Necrosis can be characterized by mitochondrial dysfunction such as production of ROS (reactive oxygen species) by mitochondria, swelling of mitochondria(oncosis), ATP depletion, failure of Ca2+ homeostasis, perinuclear clustering of organelles, activation of calpains and cathepsin, lysosomal rupture as well as plasma membrane ruptures (Dimitri *et al*, 2007).

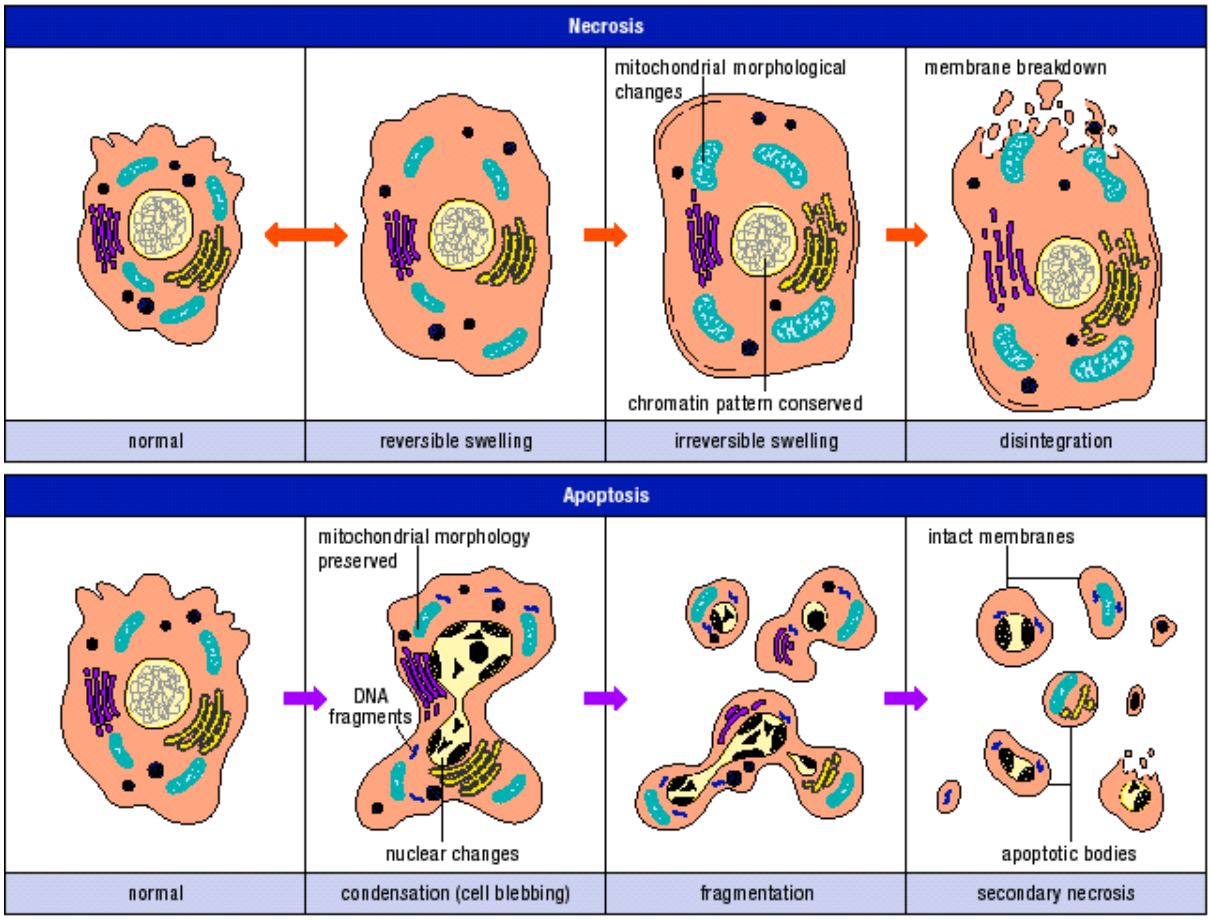


Figure 1.5. A diagram showing the biochemical and morphological characteristics of Apoptosis and Necrosis.

Apoptosis is characterized by cell shrinkage, nuclear condensation, membrane blebbing and cellular & breakage of DNA strands whereas Necrosis is characterised by mitochondrial dysfunction, swelling of mitochondria (oncosis), ATP depletion, failure of Ca2+ homeostasis, perinuclear clustering of organelles, lysosomal rupture as well as plasma membrane ruptures.

Dengenhart *et al*. (2006) believe that the inhibition of mitochondrial membrane permeability caused by the down-regulation of pro-apoptotic protein or the over expression of anti-apoptotic proteins shift the cell death from apoptosis to necrosis. There has been a debate between researchers on whether necrosis contributes to normal cell loss. Upon researches done, it was discovered that the biochemical and morphological characteristics of necrosis and apoptosis can be found in the same cell. The findings revealed necrosis contributes to normal cell loss, although it is believed necrosis only takes over after the genetic mutation that stops apoptosis before the activation of caspases. A study by Chauhan *et al*. (1999) discovered a morphological shift from apoptosis to necrosis in the interdigital of a mouse when apoptosis or caspase independent cell death is inhibited. Necrosis does not only occur due to pathological stimulus but also due to physiological events. Mutation or inhibition of caspase can signal the damaged cells to go through necrosis.

The two mode of cell death discussed occur as results of the cell damage that was impossible to repair, tumor suppressor genes play a huge role in identifying the mutation in the cell and arresting the cell proliferation to ensure the integrity of the cell is maintained.

1.9. Tumor Suppressor genes

Carcinogenesis occurs as a result of the loss of tumor suppressor genes and the gain of oncogene (Sigal and Rotter, 2000). Tumor suppressor gene has been defined by Sulina and Balan (2012), as genes whose products can transform the normal cells to tumor cell and whose loss of function can contribute to malignancy. To date there are more than 30 tumor suppressor genes identified. p53 and Retinoblastoma are the two well-studied tumor suppressor genes in cancer research.

1.9.1. Tp53

TP53 (tumors protein 53) is the synonym of p53; it is a 393-residue polypeptide and contains five functional domains. The N terminal domain involved in transcriptional activation, a proline rich domain that Mdm2 binds to, the central core domain that involves in DNA binding, the oligomerization domain that involves in p53 tetramerization and lastly the basic C-terminal domain that can inhibit sequence-specific DNA binding by the core domain (George, 2011). Cell cycle check point was first understood through King and Cidlowski, 1998 when there was no thorough checks on the apoptosis and proliferation of the mice's cells. In response to DNA damages, p53 stop the transcription at the G1-S phase to allow repair processes to restore cell integrity (Kuerbits, 1992). p53 does this by its transcriptional activation functions and binds to the transcriptional factor called E2F which prevent E2F from binding to the promoters of proto-oncogene. p53 again regulate cell cycle, this is

done by the transcription factor *GADD45* (Growth Arrest and DNA Damage), that binds to PCNA (Proliferating Cell Nuclear Antigen) and stimulates excision repair.

p53 gene was discovered in 1979 by Levine *et al*, (1979), it was first thought to be an oncogene because it portrayed an ability to promote growth in the cell lysate of simian virus (SV40) (DeLeo *et al*, 1979; Melero *et al*, 1979). This is because p53 made a stable complex with SV40 large T-antigen which has an oncogene potential. It was later discovered by DeLeo *et al*, (1979) and Lane & Crawford, (1979) that the p53 discovered initially was a mutant gene found in variety of human cancer cells. George, (2011) stipulated in one his articles about p53 that the cancer related to p53 are more aggressive and have high degree of fatalities. E. Vogelstein and White, (2010) confirmed p53 to be tumor suppressor gene after the study on colon cancer.

According to Hollstein *et al*. (1991), fifty percent of human cancer cells have the p53 that lost its main function. p53 p53 is located on chromosome 17 and composed of 11 exons. It plays a role in the negative regulator of a human cancer. According to Field, (1995), Function of p53 gene can be converted to the oncogene due to mutation on its gene. Crawford, (1984) found that the mutated p53 has long half-life as compared to the wild type, because of this the mutated p53 provide a resistance of tumor to anticancer drugs and it is mediated by hetero-oligomerization through the oligomerization domain (Rovinski and Benchimol, 1988). p53 had been known as the solitary gene, the gene homologs p73 and p63 have been discovered by Kaghad *et al.* (1997) and Yang *et al.* (1998) respectively. The collaborative study of Yang and Kaghad (1998) revealed the two homologs of p53 act as a nuclear sequence-specific

transcription factor which transactivate the overlapping set of p53 and has the ability to induce cell cycle arrest (Krysko *et al*. 2008). According to Clarke *et al*. (1993) and Lowe *et al*.(1993), p53 does not carry out its function in every form of apoptosis. It is only the activator of cell death.

Upon the DNA damage, the p53 mediated pathway is activated. The tumor suppressor gene binds to the damages DNA and causes the cell division cycle arrest to stop the proliferation allowing the repair process to take over to maintain cell function. When the process is complete and the DNA is repaired, the proliferation proceeds. If the repair is impossible, the role of p53 comes to play which leads to apoptosis (Bose and Ghosh, 2007). p53 can negatively be regulated by Mdm2; a protein that binds to the p53 and prevents p53 to carry its function. The expression of Mdm2 is activated by p53, therefore if p53 increases so does Mdm2. The protein kinase activated upon DNA damage phosphorylates p53 and reduce the affinity of Mdm2 on p53. When the p53 is phosphorylated, it does not matter the quantity of Mdm2, it will not be negatively affected by Mdm2 and thus continue its function.

1.9.2 Retinoblastoma pRb

Retinoblastoma is the tumors suppressor genes and its protein precursor pRb arrest the cell cycle on the S-phase to allow cell repair (Sunila and Balan, 2012). The existence of tumor suppressor genes was reported by Boveri (1914) and the pRb was the first TSG identified by Friend *et al*. (1986), Fung *et al*. (1987) and Lee *et al*. (1987). The gene is located on the 13q 14 chromosome, about 200 kb large and has 27 exons. pRb is a regulator of G1 phase and possess 16 sites of cdk phosphorylation. pRb is found in the G1 phase in a hypophosphrylated state and tightly bound to the E2F transcriptional factor. Upon phosphorylation by the Cdk

complex breaking the pRb-EF2 interaction. E2F becomes transcriptionally active to progress from G1 to S phase (Johnson *et al*.1993).

1.10 Aim of the study

Based on the literature and our understanding of cancer progression, the aim of this study was to evaluate the methanolic extracts of *Sutherlandia Frutescens* on cervical cancer cell lines.

Objectives

a) To use MTT and Xcillegence to check if extracts of *Sutherlandia Frutescens* can induce apoptosis in cervical cancer cell line
b) To check the expression levels of p53, bax, bcl2, RBBP6, Mdm2, Rb and caspases
c) To identify the type of cell death induced by *Sutherlandia Frutescens* using Flow cytometer.

2. Materials and Methods

2.1 Cervical cancer cell lines

SiHa was used in this study to evaluate the cytotoxicity effects of *Sutherlandia Frutescens* extracts. SiHa cell is the cervix cell line that causes grade II squamous cell carcinoma and believed to be discovered from a 55 years old Asian woman.

2.2. Plant extraction

Plant material (leaves) was dried for 3days at 50°C, powdered and autoclaved. A solution of 100mg/ml in 70% methanol was prepared and allowed to extract for 24hours.The supernatant was removed and filtered through a 0.2µm filter (Chinkwo.2005), Methanol was then evaporated. The extracted material was suspended in 10% DMSO (dimethyl sulfuroxide) to make an initial concentration of 5.0mg/ml of stock solution.

2.3 Cell culture

The cervical cancer cell (SiHa cell line) was cultured in RPMI 1640 medium supplement with 10% Foetal Bovine Serum (FBS), 1% of 2Mm L-glutamine (Keawpradub *et al*. 1999), 1% penicillin/streptomycin/fungizone cocktail and 0.5% gentamycin. The culture was grown at 37°C in a humidified atmosphere of 5 %(v/v) CO_2 in air (Chinkwo. 2005).

2.4 MTT assay

The MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide} assay measure the cells ability to survive in unfavourable conditions, it is a yellow substrate that produce a dark insoluble formazan in the mitochondria. The formation of formazan is due to the cleavage of tetrazolium ring by dehydrogenase enzymes of live cells. The formazan requires an organic solvent to be solubilised; the solubilised formazan can then be measured on a spectrophotometer (Mosmann, 1983). Since MTT can only be reduced in metabolically active cells, it has been used to determine the amount of viable cells versus the dead ones. For this study, the assay was used to check the survival of the cells when subjected to the anti-tumor agent. Cells were plated into ninety six well tissue culture plates and incubated overnight. Cells were then treated with different concentrations (50 µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) of the herbal extract. A non-treatment control and a blank were included. Cells incubated overnight, followed by addition of MTT and further incubated. The formazan crystal formed was dissolved by DMSO and the plate was read using a Bio-Rad Microplate reader at an absorbance of 570 nm.

Percentage Cell Viability was calculated as follows:

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

2.5 Flow cytometry

The flow cytometer is highly sophisticated equipment used to analyse individual cells in heterogeneous populations. It allows thousands of cells to pass through a light

beam every second (Potten and Wilson 2004), and then it can distinguish these cells based on size and health (viable or dead) amongst other parameters. This technique was employed following the MTT assay to investigate whether the herbal extract is inducing cytotoxicity to the cells by either necrosis or apoptosis. Annexin V and propidium iodide measured the number of cells undergoing apoptosis, necrosis as well as the viable cells. Annexin V has high calcium dependent affinity to phosphatidylserine residues; these residues are normally embedded in the cytoplasmic plasma membrane in healthy cells but move to the surface of the cells during apoptosis. Therefore, Annexin V can bind to these residues acting as a probe for flow cytometric analysis of cells that are undergoing apoptosis. Propidium iodide, a reagent which is used in tandem with Annexin V, is a non-cell permeable DNA dye which will stain cells undergoing necrosis. This will then distinguish between apoptotic and necrotic cells.

Cells were incubated overnight then treated with the plant extract at a concentration that induced ~50% cell death and then further incubated overnight. Cells were treated with Annexin V-FITC Apoptosis Detection Kit according to manufacturer's specifications. The cells were then analysed by flow cytometry.

2.6 Phytochemical screening

Following the extraction of crude extract using various solvents from the two plants, phytochemical screening was conducted. Phytochemical screening was carried out to identify *Sutherlandia Frutescens's* phytochemical constituents i.e. if there is presence of alkaloids, flavonoids, tannins, glycosides, phytosterols etc. (Tiwari *et al.*

2011). The method was done at the University of Fort Hare using the Time of flight Mass spectrophotometer.

2.7 Caspase 3/7 Assay

Furthermore Caspase 3/7 assay was employed to determine if cell death was induced by caspase dependent. The cells were pipetted into the 96-well plate and incubated overnight to allow cell to attach on the bottom of the plate. CaspaseGlo substrate in buffer was added on to the 50µl cultured cells mixed and incubated for an hour at 37°C in the dark. The luciferase activity was then read using the Glomax®-96 microplate luminometer.

2.8 ATP Assay

Adenosine Triphosphate is used to measure the biological activity of the cells and the amount of ATP is directly proportional to the amount of live cells. The 50µl CellTiter substrate in buffer was added on to the cells that were incubated overnight in the 96-well plate. Cells were then mixed and incubated for an hour at 37°C in the dark. The ATP level was quantified using GLOMAX (Promega, USA).

2.9 Statistical Analysis

Two-tailed student t-test was done to compare the significance difference between treated and untreated cancer cells. The probability value (p>0.05) of less than 0.05 indicates a significance difference of the treated cancer cells with respect to the untreated cancer cells.

CHAPTER 3

Results

Introduction

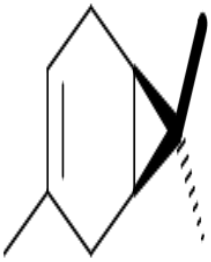
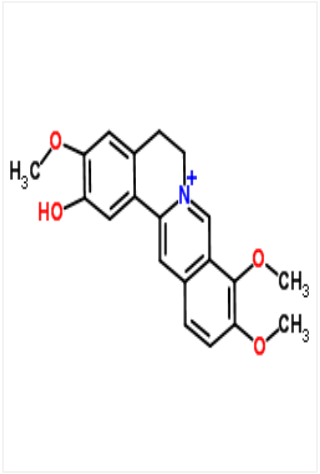
The cytotoxicity of *Sutherlandia frustescens* has been investigated on other types of cervical carcinoma but nothing was found on SiHa. The plant has been used for many years to cure various illnesses such as stomach ache, fever, internal cancer and diabetes. The plant is also called kankerbos/cancer bush due to the confidence the xhosas,zulu,sotho and cape Dutch have on the ant-cancer activity of the plant but the mode of action on cervical cancer is not well researched. Due to the cytotoxicity effect of the plant on SiHa and the mode of action on cervical cancer not well researched, the study was conducted to evaluate the cytotoxicity of the plant as well as the mode of action on SiHa and HeLa.

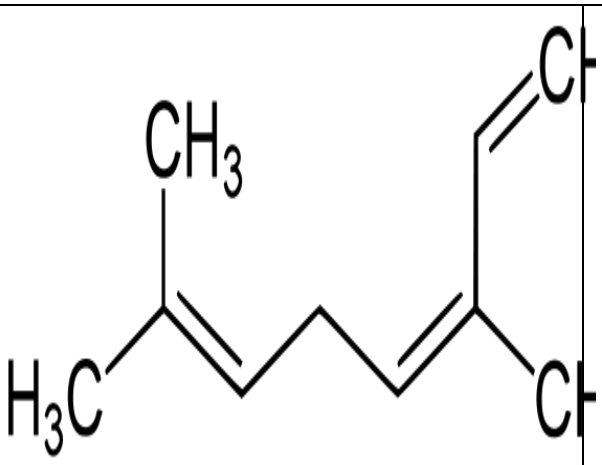
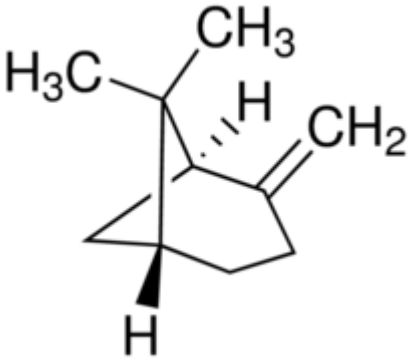
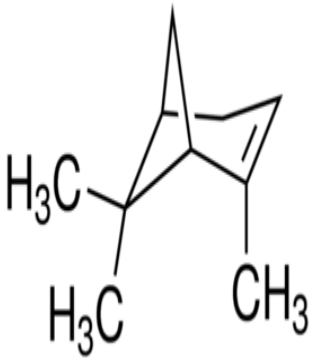
3.1. *Phytochemical compounds found from Methanol and Aqueous extracted S.F after Mass spectrophotometry*

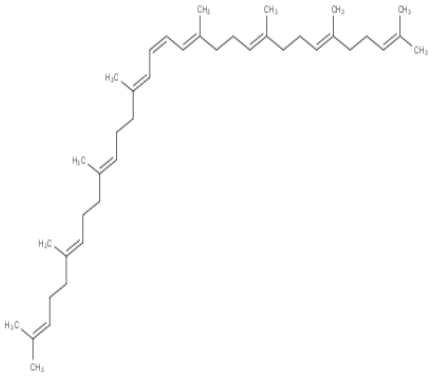
Table 3.1.

(A) Methanolic Extract

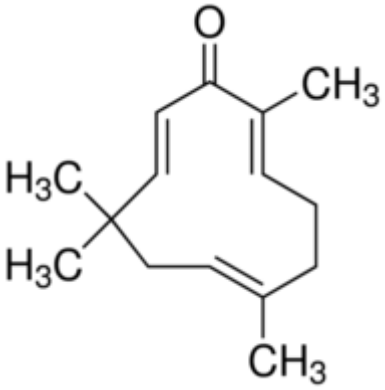
Compound	Structure	Solvent and Cancer type for anti-proliferative effect	Reference

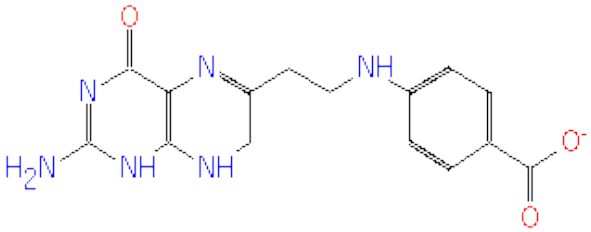
(+)-3-carene C10H16		DMS0 Solvent Breast, colon, lung and glioma cancer	Mahahpara and Washat
columbamine C15H20O2		Ethanol Pheripheral blood mononuclear blood human osteosarcoma U2OS	EL-Wahab *et al*, 2013 Bao *et al.* 2012
(E)-ß-ocimene C10H16		Colon,glioblastoma, human adenocarcinoma and hepatocellular carcinoma	De Oliveira *et al*.2015

			
(+)-ß-pinene C10H16		Ovarian and Hepatocellular carcinoma DMSO Lung,glioma,breast and colon cancer	Wang et al, 2012 Wajahaka Shah 2014
(+)-a-pinene C10H16		Ovarian and Hepatocellular carcinoma DMSO Lung,glioma,breast and colon cancer	Wang et al, 2012 and Chen et al, 2015 Wajahaka Shah 2014

15-cis-phytoene C15H22O2		Reduce risk of lung and epithelial cancers on human	Pharmacodynamic basis of herbal medicine, second edition by Manuchair Ebadi

(B) Aqueous Extract

Compound	Structure	Cancer type for anti-proliferative	Reference
zerumbone C15H26O1		HeLa and HepG2	Sakinah *et al*.2007
delphinidin 3-O-sophoroside C20H19O5		Murine melanoma,Ovarian and Cervical cancer	Diaconeasa *et al*.2015

dihydrohomopteroate C16H25N5O6			

3.2 The cytotoxic effect of Sutherlandia extract on SiHa

MTT assay was employed to determine the cell viability of SiHa using IC_{50} the concentration at which 50% of cell respond positively to the extract. Upon treatment with varying concentrations (50,100,150 and 200µg/ml), the cell viability Vs extract concentration was plotted allowing the quantification of changes in the cell proliferation.

Cells were treated with varying concentrations of the methanolic extract and incubated for 24 hours. Following the incubation period, MTT reagent was added into each well including the media only which serves as blank for comparable purpose. Untreated cells were used as a 100% cell viability and 0.5µM Taxol as a positive control. Dehydrogenase enzymes present in the live cells are responsible for the conversation of MTT to formazan, this is done when dehydrogenase cleaves tetrazolium ring. DMSO was then added after 4 hours of incubation to solubilize the formazan formed by metabolically active cells. The results were read by spectrophotometer at wavelength of 570nm and a wavelength reference of 600nm. Following treatment of SiHa, there was a reduction in cell proliferation for 50µg/ml, 100µg/ml and 200µg/ml with cell viability of 52%, 53% and 56% respectively. The concentration 150µg/ml exhibited the cell viability of 263%.This could be due to the

enzymatic reaction from MTT with no decrease in the number of cells that lead to the conclusion that the cells are alive whereas they are slowly dying. The results are depicted on the below graph **Figure 1**.The cell death statistical significance was calculated using two-tailed t-test at a 95% confidence interval. When treated-cells were compared statistically with the untreated, the P value of 0.05 was obtained on cells treated with 50µg/ml of the plant. The P value of greater than 0.05 was obtained when the standard deviation of the treated was compared with the standard deviation of the untreated cells. The SD±Error showed cell death of 0.07±0.070 of untreated cells as compared to 0.23±0.52 and the Taxol-treated showed a 0.03±0.37. The cells treated with 100µg/ml and 200µg/ml concentrations showed a cell death of 0.05±0.50 and 0.40±0.14 respectively. The cells treated with 150µg/ml showed cell death of 0.23±0.11.

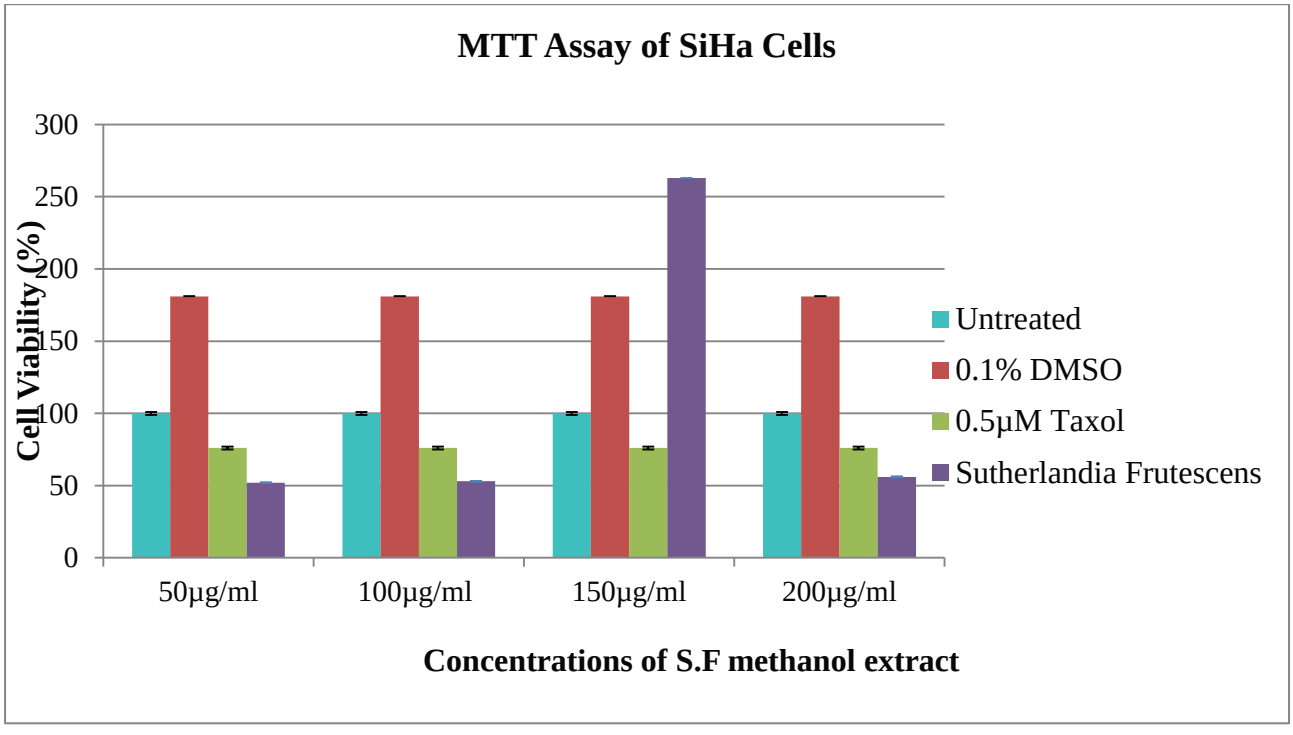


Figure 3.1: The cytotoxic effect of methanol extract of **S.F** on SiHa. Cells were plated and incubated for 24 hours to allow the cells onto the wells. Cells treated with varying

concentrations and incubated for 24hrs. MTT reagent was added followed by addition of 0.1% DMSO to solubilize the formazan. Absorbance was read which was used to calculate the cell viability.

3.3*The Effects of Sutherlandia Frutescens methanolic extract on the cell growth of SiHa cell line*.

Growth of SiHa cells was determined by Xcillegence, a new system tool to measure the cell activity in a real time. Cells were seeded into the 96 well plates and treated with varying concentrations of methanolic extract of S.F. DMSO and Taxol were used as a vehicle control and reference compound respectively to compare the level of cytotoxicity of the extract.

Cells were seeded in 96 well plates and incubated for 24 hours. The SiHa cells were then treated with plant concentrations used in MTT assay. Continuous changes in the impedance were measured and presented as cell index (CI), a dimensionless parameter reflecting the biological statue of monitored cells including cell number, cell viability, cell morphology and cell adhesion. The cell index value denotes a quantitative measure of proliferative status of cells in real time. Results are depicted in **Figure 2** where the 100µg/ml is represented by the lime graph, Untreated, DMSO and 0.5µM Taxol are represented by pink, blue and choral respectively. The 50µg/ml was depicted by the red graph; 150µg/ml and 200µg/ml were represented by the purple and turquoise respectively.

The untreated cells continued to grow as expected, cell density kept decreasing after 77 hours of treatment with 0.1% DMSO. DMSO has been documented to cause cancerous cells to become benign and has also been shown to slow the development of cancer; this has been shown on DMSO-treated cells. The cell proliferation was normal afore treatment. Upon treatment with 50µg/ml, cells decreased to less than 1.0 cell index where it stayed stable to 88 hours. The Microplate has electrodes at the bottom which detect the cell viability, as more cells are detaching the cell index decreases. This is an indication that indeed the plant has a cytotoxic effect on the SiHa cell line. Taxol as a positive control has a decrease in the impedance, this could have been because the cells slowly detaching thus reducing the cell density. The effect of paclitaxel (Taxol) on various cancers has been researched and evidence showed it disrupts microtubules dynamics by promoting tubulin polymerization and stability which leads to cell cycle arrest at the G/M phase.

Keys

Untreated 100µg/ml Methanol extracts

0.1%DMSO 150µg/ml Methanol extracts

0.5µMTaxol 200µg/ml Methanol extract

50µg/ml Methanol extract

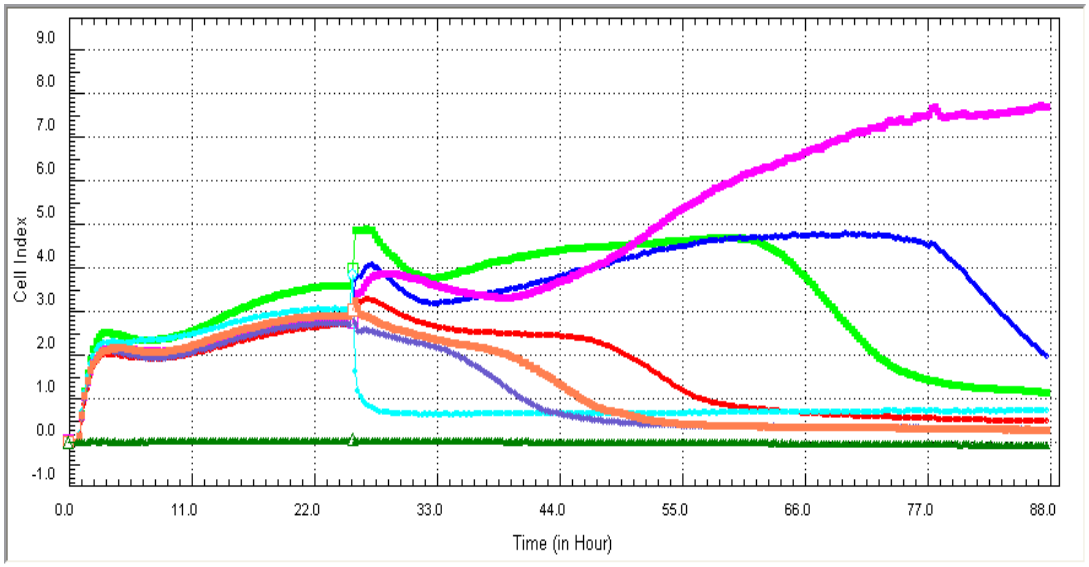


Figure 3.2: Xcelligence analysis of the effect of methanol extract on the cell growth on SiHa in real time. Cells were seeded in 96 well plates and incubated for 24 hours. The SiHa cells were then treated with the increasing concentration 50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml of methanol extract represented by the red, lime, purple and turquoise graphs. Untreated, DMSO and 0.5µM Taxol are represented by pink, blue and choral respectively.

3.4*. The Effect of Sutherlandia Frutescens methanolic extract on the Casapase activity of SiHa Cell lines.*

A positive result in apoptosis assay is not a confirmation that the drug or anti-cancer agent has an apoptotic effect on the cancer cells, Two or more assay need to be performed as necrosis and apoptosis share some of the features. The morphological changes resulting in apoptosis is caused by the caspases which either activate or deactivate specific substrate along the way. Proteolytic cleavage of effector caspases is an indication of their activation, this does nothing for initiation. Caspases 3 activation plays a role in initiation of cellular events during early apoptosis process.

Caspases has been identified as a major contribution to the execution of apoptosis. Both caspase 3 and 7 are responsible for the downstream cleavage of substrate such as PARP. During apoptosis, caspase 7 is activated by upstream caspases through proteolytic processing at Asp23, Asp198 and Asp206, thereby producing the mature subunits. Because caspase activation is the fundamental part in the apoptotic pathway, caspase activity was employed to determine if the mode of action is caspase dependent.

The caspase activity is measured by the Luminescent which is dependent on the luciferase formulated to generate the glow type luminescent. The results depicted on **Figure 3** shows that there was a slightly less difference between the caspase activity in the untreated and $IC_{50.}$ This may be because apoptosis as the normal programmed cell death try to balance the cells in the living cells.

Following the MTT assay and flow cytometry assay which indicated that the SiHa cells dying via apoptosis, the caspase activity was employed to verify results found on the above assays. There was less caspase activity as expected because the cells were not subjected to any cytotoxic agent. The caspase activity observed in untreated cell could be due to the normal programmed cells death, the same observation has been documented in lung cancer. This is believed to be the reason for reduction to treatment sensitivity. Literature reported this kind of cell death as senescence, a programmed cell death that limits tumour development.

The low levels caspase indicates a reduced apoptosis and carcinogenesis. More caspase activity was observed in cells treated with Taxol. Taxol is used as the anti-tumor agent for various cancers and it is documented to induce apoptosis through caspase and without caspase. Our results shows cell death was via caspase-dependent apoptosis. The 50µg/ml as found from MTT to induce 52% of the cell death has been used in the caspase activity and the observation on **figure 3.3** indicate there was a loss of mitochondrial membrane. The pro-apoptotic proteins were then released that trigger the caspase cascade resulting in apoptosis.

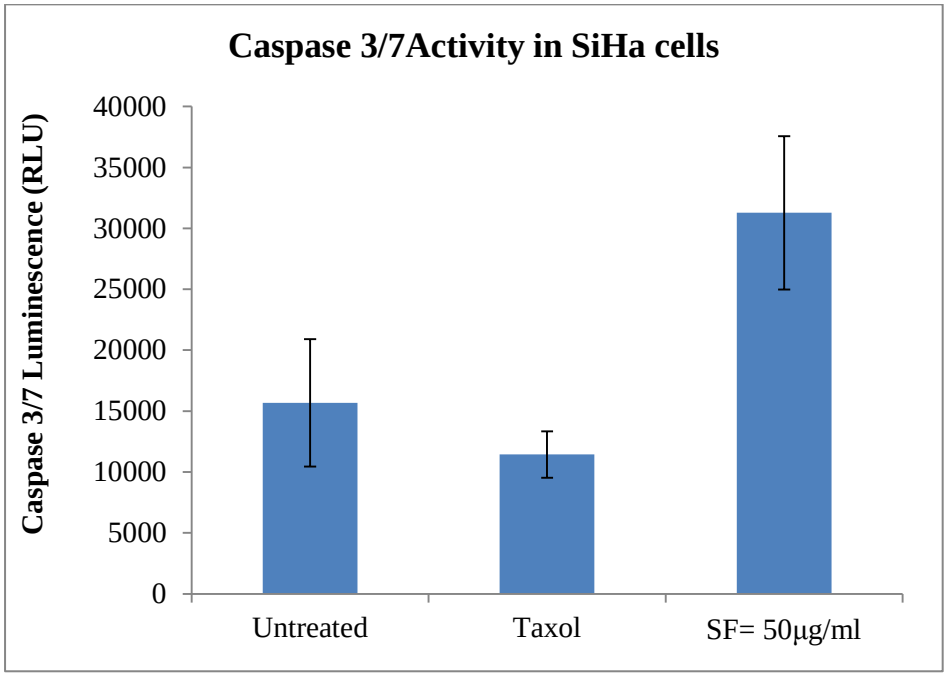


Figure 3.3: The Caspase activity graph depicting the results after the cells were treated with 0.5µM Taxol and IC_{50} from MTT. Caspase 3/7 reagent was added into the cells for cell lysis to expose caspase so luciferase which cleave the caspases resulting in a glow type luminescent. The fluorescence is proportional to the caspase activity.

3.5 *The Effect of methanolic S.F extract on cell cycle and apoptosis*

The IC_{50} found from MTT assay is expected to have the cytotoxic effect on the SiHa cells via apoptosis by arresting the cell cycle before mitosis. To achieve this, the cells were treated with IC_{50}, incubated for 24 hours and was exposed to FITC Annexin V and then Propidium iodide staining. Annexin V was used to bind to the phosphatidylserine serine to measure the early apoptosis as during this time, membrane asymmetry is lost and phosphatidylserine translocate to the leaflet. To distinguish between early apoptosis and late/necrosis, PI was used. PI does not stain the cells in early apoptosis because at this time the plasma membrane is still intact. As the cells move to the late apoptosis, the integrity of plasma membrane decreases thus allowing the PI to pass through the membrane and intercalate into the nucleic acid showing a red fluorescence.

The effect of S.F treated cells on cell cycle was measured by flow cytometry. Flow cytometry analysis revealed an increase in the apoptotic fraction with an increase in untreated cells G0/G1 in untreated (49%) and Taxol (85%) as compared to the IC_{50} (40%).High volume of Taxol and IC_{50} treated cells were residing in the S phase with 39% and 40% respectively as compared to 9% in the untreated cells. Cell cycle arrest in the G0/G1 phase of the positive control and treated cells as well as in the G2 phase was observed. The observation was as a result of a high percentage of cells in S phase as compared to the G2/M Phase. Cells normally progress to the G2/M phase and because only 27% of treated cells proceeded to the gap 2 phase, apoptosis occurred.

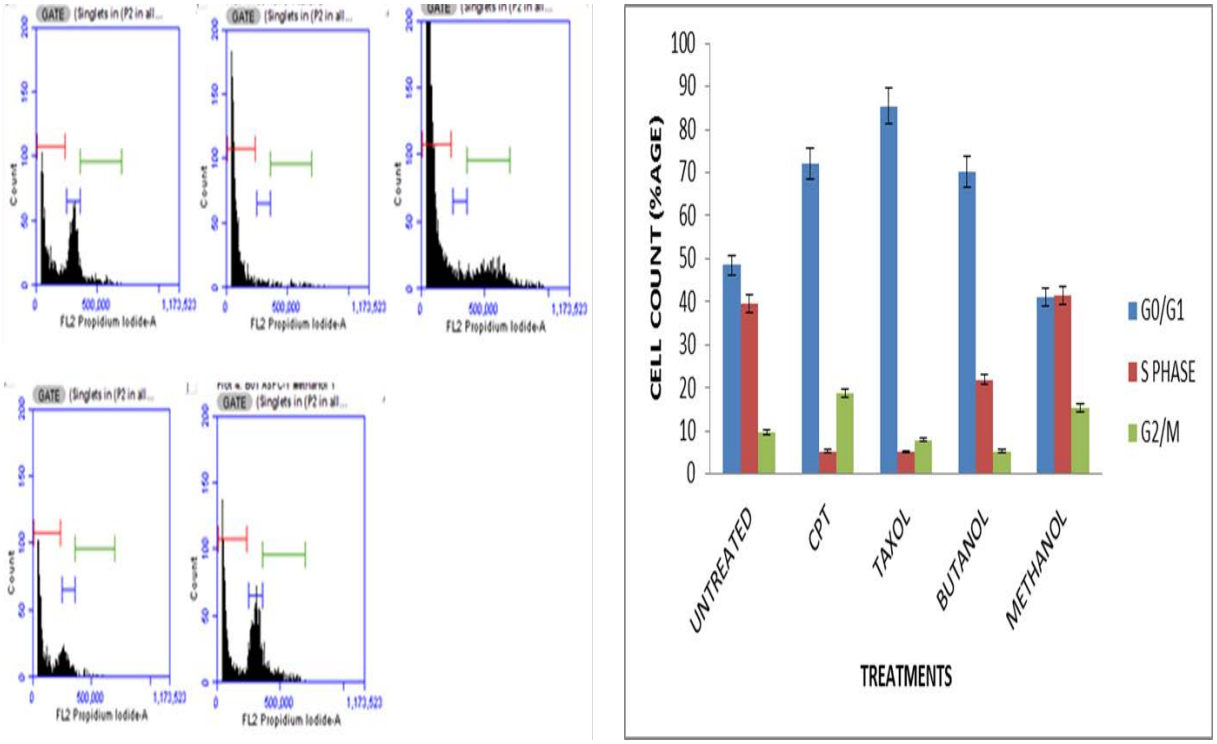


Figure 3.4(A): Flow Cytometry analysis of SiHa cell subjected to plant extract and Taxol. Cells were incubated for 24 hours to attach to the wells. Cells were then treated with IC_{50} and 0.5µM Taxol as a positive control and stained with Annexin V followed by Propidium Iodide. Cells were then analyzed by flow cytometry prior to incubation at room temperature for 15min

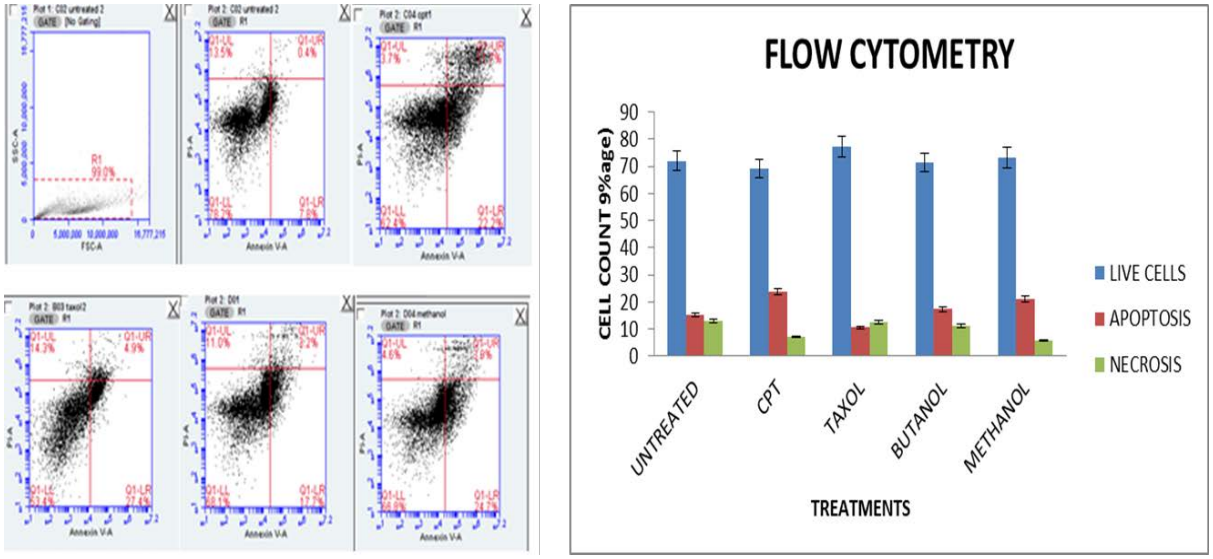


Figure 3.4(B): Represents the Flow Cytometry analysis of SiHa cell subjected to plant extract and Taxol, cells were incubated for 24 hours to attach to the wells. Cells were the treated with IC50 and 0.5µM taxol as a positive control and stained with PI and were then analyzed by flow cytometry.

Cell cycle is not sufficient to prove apoptosis, cell cycle would reveal the DNA fragmentation but this can occur in necrosis as well. To prove apoptosis, the effect of methanol extract on SiHa cells was evaluated by Annexin V/PI staining through flow cytometry. When cells are doubled stained with Annexin V/PI the different population of cells are observed. The cells that are not stained with either Annexin V or PI are viable cells and are shown in quadrant 3, cells stained with Annexin V but not PI are dying by apoptosis and resides in quadrant 4.The cells that are not stained with both the dyes are nonviable cells in the late apoptosis residing in quadrant 2 while cells scattered in quadrant 1 are necrotic cells.

On observing the results on Figure 3.5 B for S.F-treated cells, 20% of cells stained positive for externalized phosphatidylserine residues indicating that the extract induced 20% apoptosis in SiHa cells whilst 74% remained alive. Positive control Taxol-treated cells demonstrated 12% of phosphatidylserine externalization indicating the cells were dying by apoptosis and 79% of the cells remained alive. Comparing the externalization of S.F-treated cell and that of positive control, the low count of apoptotic cells in S.F-treated cells may be because of the possible error in the initial low cell count resulted prior staining when supernatant was removed. Despite the possibility of error mentioned, it is clear S.F extract induces apoptosis in SiHa cells as more cells were residing in Q2 and Q3 indicating apoptosis. The untreated 14% of cells stained positive for externalized phosphotidylserine residues whilst 72% remained alive. As previously mentioned, cells die naturally to balance the homeostasis in the cell cycle and this was indicated by less than 12 % of cell death by apoptosis.

3.6. *The effect of S.F compounds (Canavanine, GABA and Pinitol) on SiHa Cell line*

Although number of literature suggests that GABA has no other functions other than being the neurotransmitter, there was a significant cell death when SiHa cells were exposed to GABA. The concentration used was 100µM, 300µM and 500µM which resulted in 51%, 53% and 48% cell death respectively. The results shows cell death decreases after 300µM.Although the results contradicts with the study by Yue-Hui *et*

al, (2012) that shows GABA increases proliferation of HepG2 cells in a dose-dependent manner, it is supported by Chan-Ho oh and Suk-Heung Oh (2004) that proved GABA significantly induces apoptosis on the L1210(Leukemia) cells.

Canavanine is believed to be the major constituents in the S.F plant and an anti-tumor agent in various cancer cells has been proven once more in this study as the compound induced cell death by 54% for both 1000µM and 1500µM and slightly less cell death of 53% when SiHa cells were treated with 2500µM. The results shows cell death decreases after 2500µM concentration of the compound. The concentration may be too high for the cells that they could not survive. The study did not go further to analyze the mode of action of the cell death which needs to be done as the follow up to clearly understand the decrease in cell death after the specific concentration of the compound.

The observation from GABA and Canavanine is also seen in Pinitol treated cells where a slight difference in cell death was observed. The cells were treated with three various concentrations 30µM, 90µM and 120µM and the concentrations induces 53%, 50% and 44% cell death respectively. The findings indicate that the cell death decreases as concentration decreases.

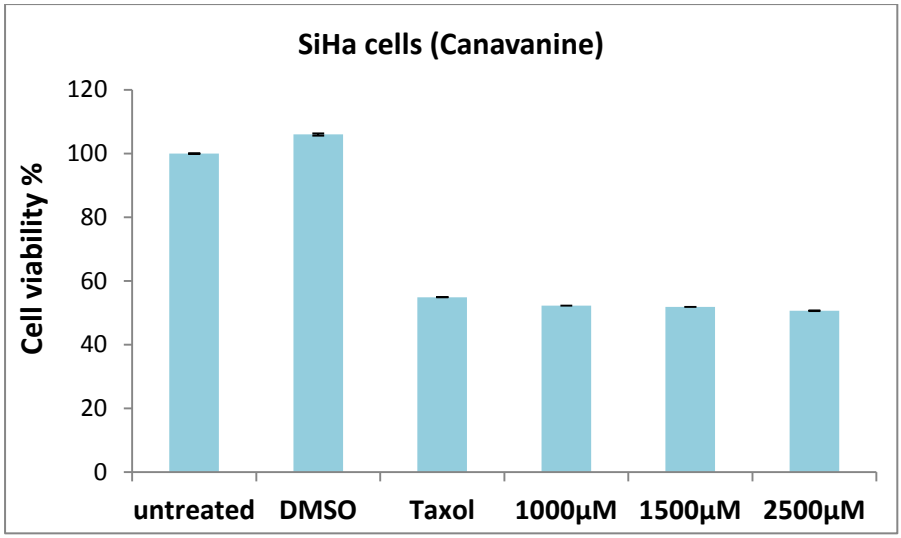


Figure 3.5: The cytotoxic effect of methanol extract of **Canavanine** on SiHa. Cells were plated and incubated for 24 hours to allow the cells onto the wells. Cells treated with varying concentrations and incubated for 24hrs. MTT reagent was added followed by addition of 0.1% DMSO to solubilize the formazan. Absorbance was read which was used to calculate the cell viability

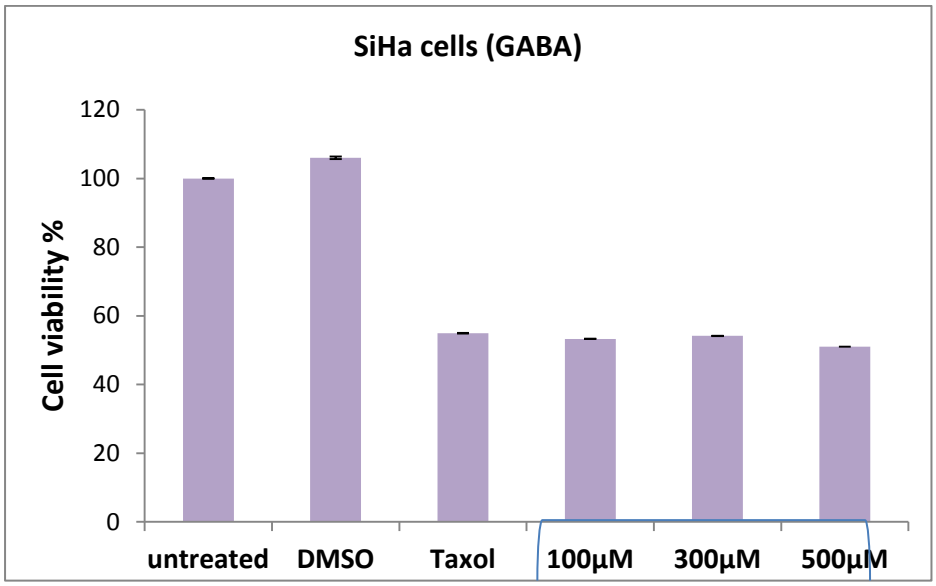


Figure 3.6: The cytotoxic effect of methanol extract of **GABA** on SiHa. Cells were plated and incubated for 24 hours to allow the cells onto the wells. Cells treated with varying

concentrations and incubated for 24hrs. MTT reagent was added followed by addition of 0.1% DMSO to solubilize the formazan. Absorbance was read which was used to calculate the cell viability

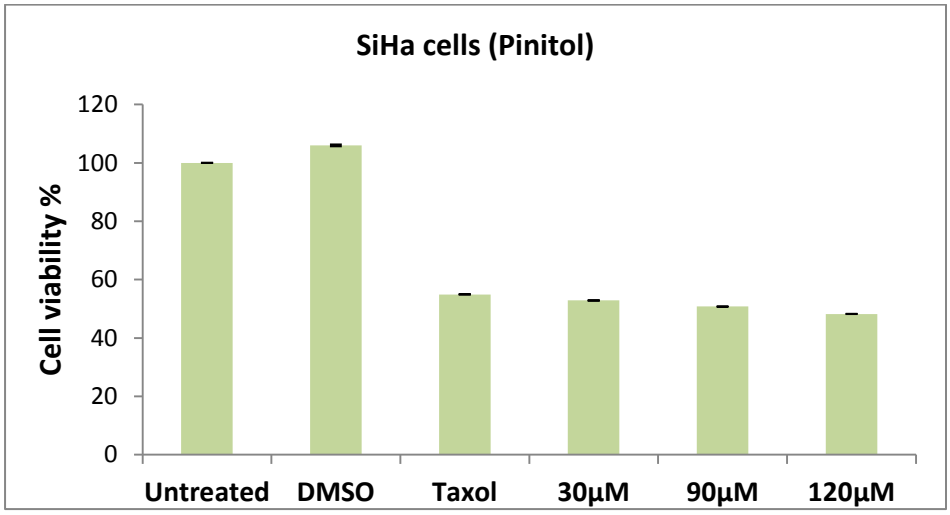


Figure 3.7: The cytotoxic effect of methanol extract of Pinitol on SiHa. Cells were plated and incubated for 24 hours to allow the cells onto the wells. Cells treated with varying concentrations and incubated for 24hrs. MTT reagent was added followed by addition of 0.1% DMSO to solubilize the formazan. Absorbance was read which was used to calculate the cell viability.

3.7 The effect of *Sutherlandia Frutescens* methanolic extract on the ATP *levels in SiHa cells.*

Adenosine Triphosphate is a biological indicator of metabolically active cells which is quantified by detecting luminescence. The assay determines the number of treated cells in comparison with untreated cells. After the SiHa cells were treated with the three compounds, IC_{50}, Taxol as the positive control, the cells were subjected to CellTiter-Glo^{TM} substrate in buffer to determine the amount of ATP in the SiHa treated cells. **Figure 8** shows a significant higher ATP levels in S.F treated cells with

more than 100000 luminescence followed by GABA, Canavanine and pinitol with 60000, 40000 and 10000 luminescence respectively. In this study we also observed increase in mitochondrial activity within 8 hours of treatment with methanolic extract followed by a drop immediately after which suggested disintegration of mitochondria and loss of cell function Because the ATP level is proportional to the amount of viable cells, the results shows there are more viable S.F treated cells at first and upon treatment, the was a loss of mitochondria and the findings is consistent with the MTT assay. Pinitol showed a significant decrease in ATP and thus cell viability, this correlate with the pinitol MTT results while Canavanine and GABA showed a decreased in ATP as compared to $IC_{50.}$ The results contradict with that of Skerman *et al*, (2011) that showed S.F extract decreases the level of ATP in Esophageal cancer cell line.

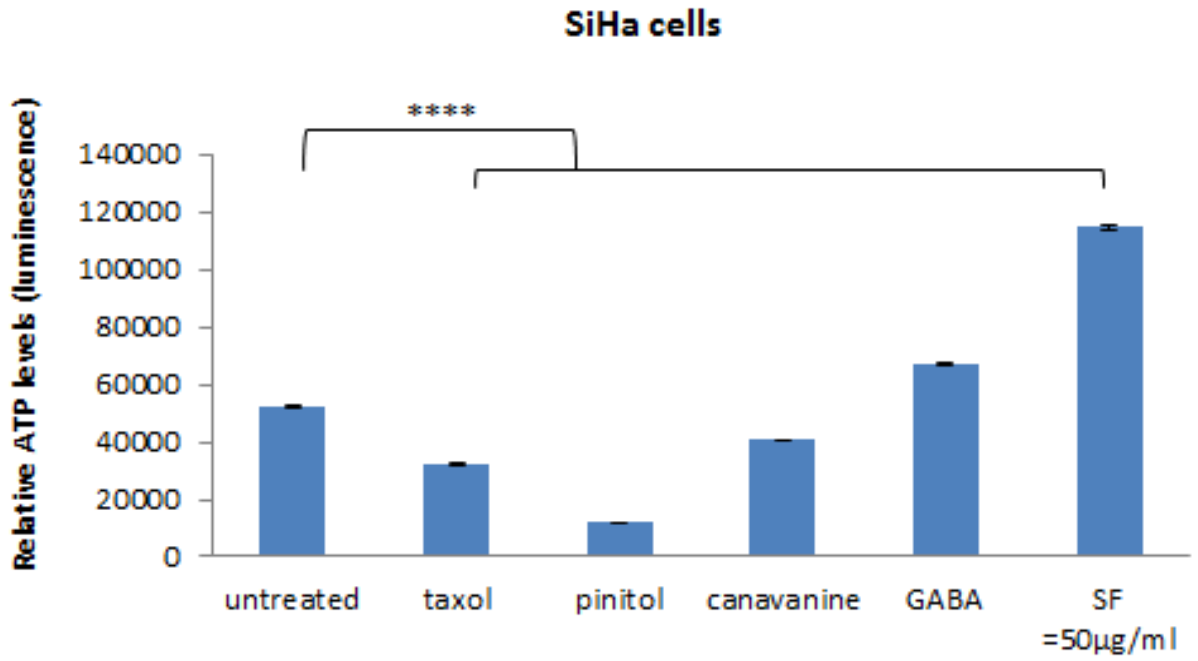


Figure 3.8: The ATP level of the cells treated with $IC_{50,}$ Canavanine, Pinitol and GABA are depicted on the graph after the luminescence was quantified.

Chapter 4

Discussion

Medical plants have been used for decades as a source of health concoctions for various ailments such as arthritis, stomach ache and cancer. It is widely known as the cancer bush as it is believed to cure cancer. Traditional healers boiled the plants to extract essential compound, which was given to patient directly to drink. The traditional healers and most Africans are still using the same methods, Smoking the plants or steaming with the plants was alternative methods. The methods were not effective as number of compounds present in the plant were not efficiently extracted and that is believed to be the drawback of the plant and the possible reason the chemical constituents or the mode of action of the plants is not clearly understood There were incidences where patients were believed to be cured of various diseases (Van Wyk and Albrecht, 2008).These claims by traditional healers risen the edge for the scientists to research the traditional medicines in the aim of finding the efficient and cheap drugs with less side effects.

Over the years various medical plants have been research for the purpose of drug discovery and among the plants *Sutherlandia Frutescens* which is mostly found in the dry parts of South Africa was among the researched. The phytochemical study done on the plant revealed various compounds that are believed by scientist to be the reason the plants is used in various ailments and was then named cancer bush. The major component being Canavanine Pinitol and GABA where canavanine is believed to be the leading compound contributing to curing cancer.

To establish whether the plants exhibited the inhibitory effect on the cancer cell line, the MTT assay was used. The results of the MTT assay was the $IC_{50,}$ the concentrations at which 50% of the cancer cells positively reacted to the extract. This is done when MTT reagent is reduced by metabolically active cells resulting in intra-cellular formazan which is then solubilized by DMSO and quantified using a spectrophotometer. The results showed the significant decrease in cell density when the SiHa cells were subjected to *Sutherlandia Frutescens* extract. The untreated cells were included as 100% cell viability, Taxol as the positive control and DMSO as the vehicle control for comparable purpose. The 50ug/ml showed 49% cell viability whereas 100 and 200µg/ml showed 38 and 37 cell viability respectively. ATP assay correlate with the MTT assay as it showed an increased ATP level in extract treated cells as compared to untreated Taxol and the compounds. Pinitol showed a low ATP level.

The MTT result indicates that the three extract concentrations induce cell death in SiHa cells therefore the 50ug/ml was concluded to be the IC_{50} resulted from the assay as the cell density decreased significantly as compared to the two concentrations (50µg/ml and 200µg/ml).MTT assay was also done on canavanine, pinitol and GABA, the results depicted in **Figure 5, 6 and 7** shows the compounds have a cytotoxic effect on the SiHa cells. The IC_{50} was 1000µM, 30µM and 100µM for canavanine, pinitol and GABA respectively with 50% cell cytotoxicity. GABA was first believed to only have function as the neurotransmitter but an enormous number of researches are being done on the GABA as the anti-cancer compound. The cell

density decreased with the concentration above the IC_{50} of the three compounds, this may be because the high concentrations were being harsh to the cells thus killing normal cells. Further research needs to be done to determine the mode of action of the compounds on the SiHa cell line.

Looking at the previous research done of the plant as the anti-cancer activity, Chinkwo, 2005 resulted in similar findings when cervical carcinoma and Chinese Hamster Ovary cancer cell lines were treated with aqueous extraction of *Sutherlandia Frutescens*. The findings on the cytotoxic of the plant indicates the plant has a potential in the drug discovery as it showed not to kill normal cells but cancer cells only. Apoptosis has been defined as the programmed cell death that occurs normally in the cell for replacement tissue remodeling and removal of damaged cells (Kerr *et al*, 1972 and Elmore, 2007).Apoptosis is the preferred method of cell death as the phygocytotic cells are engulfed by white blood cells as compared to necrosis where cells rupture and the cell content affect the neighboring cells thus causing inflammation. The S.F extract in this study achieved the aim of apoptosis.

Following the MTT assay, flow cytometry was employed to determine if the treated cells dies by apoptosis or necrosis, the two method of cell death were shown to be similar at a point in the process and the characteristics that differentiate the two are engulfment of phygocytotic cells in apoptosis and the cells swelling in necrosis which causes rupture resulting in inflammation and this may cause decreased blood flow at the affected area resulting in tissue death. Because of the above mentioned reasons, apoptosis is the preferred method of cell death as the programmed cell death

process that is regulated and controlled. Flow cytometry uses Annexin V that has a high calcium dependent affinity towards the phosphatidylserine (P.S). The stain detects the cells in the early apoptosis where cell membrane asymmetry is lost and the P.S translocate to the external membrane binding to Annexin V. Because Propidium iodide is not permeant to the viable cells, the dye intercalate into DNA base pairs when the DNA content are exposed. From **Figure 4(B)** significant high number of treated cell showed the phosphatidylserine residue externalization with an exception of Taxol treated cells that showed more cells are dying via necrosis than apoptosis. The 15% of cells were in Quadrant 4 as compared to 12% in Q1.

The DNA content of the treated and non-treated cells was measured to confirm the phase of the cell cycle the plant had effect on. The results depicted in **Figure 4(A)** shows 40% of cells in G0/G1 and S phase for IC_{50} treated cells as compared to 49% and 39% in untreated cells. In the G0 phase, cells are carrying out their normal metabolic function but not replicating therefore there is no cell cycle. In the G1 phase, cells produces proteins the cells need to grow and start replicating in the S phase .The results obtained from cell cycles shows cells are being arrested at the S phase as there are more cells in the G0/G1 Phase and less in the G2/M phase as compared to the S phase. Cells are being checked at the G1/S phase for any error and the found errors prevent cells from entering the G2/M phase where the cells are producing additional proteins, RNA and the mitotic spindle necessary for mitosis. The role of the cell cycle arrest is to prevent any errors occurred to proceed in the cell cycle as this will cause mutation which will have defects on the cells thus the

organism. Therefore the extract induced the cell cycle arrest in the S phase to allow cell repair before cell cycle continues.

Caspases are a widely known executioner of apoptosis. When these proteins are involved, cell death is believed to be through apoptosis. The caspase 3/7 was employed following flow cytometry to confirm cells death induced by the extract. Taxol was used as a positive control and media only as untreated cells. The 100µg/ml showed a significant high caspase 3/7 activity on the SiHa cells and the result shows the mode of action of the extract on the SiHa cells is caspase independent thus apoptosis. Untreated cells had a higher caspase 3/7 activity due to apoptosis being a normal programmed cell death that occur to balance the cells. Taxol widely known as paclitaxel is a known chemotherapeutic drug currently in use together with radiotherapy to reduce the risk of early-staged breast cancer re-curing as well as to treat advanced-staged breast cancer after it has stopped responding to standard chemotherapy regimens. Because Taxol has adverse side effects and is not easily soluble in water S.F becomes the promising anti-cancer agent with little side effects. Skerman *et al, (*2011) found similar results when esophageal cancer cell line (CCL-185) was treated with S.F. Further studied needs to be conducted to better understand the relationship between caspases and apoptosis as there is accumulating information that suggests apoptosis is not caspase dependent (Broker *et al*, 2005).

Conclusion: *Sutherlandia Frutescence* has proven with number of research that it induces cell death via apoptosis. After evaluating its cytotoxicity, the plant shows to be a promising anti-tumour agent that needs to be clinically proven.

References

1. Balunas, M. J. and Kinghorn, A. D. (2005).Drug discovery from medicinal plants. *Life Sciences,* 78, pp.431 – 441.

2. Bell, E. A. (1958). Canavanine and related compounds in the Leguminosae. *Journal of Biochemistry*, 70, pp. 617-619.

3. Bell, E. A., Lackey J. A. and Polhill R.M. (1978). Systematic significance of canavanine in the Papilionoideae (Faboideae). *Biochemical Systematics and Ecology*, 6, pp. 201-212.

4. Bose, I. and Ghosh, B. (2007). The p53-MDM2 network: from oscillations to apoptosis. *Journal of Bioscience*, 32, pp. 991–997.

5. Botha, M.H., Dochez C. (2012). Introducing human papillomavirus vaccines into the health system in South Africa. *Vaccines*, 3, pp.28-34.

6. Budihardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang X. (1999). Biochemical pathways of caspase activation during apoptosis. See comment in PubMed Commons below*Annual Review of Cell Developmental Biology,15, pp. 269-90.*

7. Chase DM, Kalouyan M, DiSaia PJ (2009) Colposcopy to evaluate abnormal cervical cytology in 2008.*American Journal of Obstetrics & Gynecology,* 200, pp. 472-80.

8. Chauhan, D., Hideshima, T., Pandey, P., Treon, S., Teoh, G., Raje, N., Rosen, S., Krett, N., Husson, H., Avraham, S., Kharbanda, S. and Anderson, K.C. (1999). RAFTK/PYK2-dependent and –independent apoptosis in multiple myeloma cells. *Oncogene*, 18, pp. 6733–6740.

9. Chinkwo, K.A. (2005). *Sutherlandia frutescens* extracts can induce apoptosis in cultured carcinoma calls. *Journal of Ethnopharmacology,* 98, pp.163-170.

10. Choene, M., Mthembu, N., Dlamini, Z. Mokgotho, M., Wachira, J. and Motadi, L.R. (2012). Breast cancer. Small molecules targeting apoptosis, a prospective approach to safe scientific success. *Advances in Bioscience and Biotechnology*, 3, pp. 833-844.

11. Christop, B. (2003). The Bcl-2 protein family: sensors check point for life or death decision. *Molecular Immunology*, 39, pp.615–647.

12. Clarke, A.R., Purdie, C. A., Harrison, D. J., Morris, R.G., Bird ,C. C., Hooper, M. L. and Wyllie, A.H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, 362, pp. 849–852.

13. Coker, A.L. Bond, S.M. and Williams, A. (2002). Active and passive smoking, high-risk human papillomaviruses and cervical neoplasia. *Cancer Detection and Prevention*, 26, pp.121-128.

14. Cragg, M. and Newman D.J. (2005). Plants as a source of Anti-cancer agent. *Journal of Ethnopharmacology*, 100, pp.72-79.

15. De Leo, A.B., Jay, G., Appella, E., Dubois, G.C., Law, L.W. and Old, L.J. (1979) .Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proceedings of the National Academy Science of the USA*, 76, pp. 2420-2424.

16. Doorbar, J. (2007). Papillomavirus life cycle organization and biomarker selection. *Disease Markers*, 23, pp. 297–313.

17. Fan, T.J., Han, L.H., Cong, R.S. and Liang, J. (2005). Caspase family proteases and apoptosis. *Acta Biochimica Biophysica Sinica (Shanghai),*37, pp. 719–727.

18. Gross, A., Yin, X.M., Wang, K., Wei, M.C., Jockel, J.,Milliman, C.,Erdjument-Bromage, H.,Tempst, P. and Korsmeyer, S.J.(1999) .Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *Journal of Biological Chemistry*, 274, pp.1156–1163.

19. Hengartner, MO. (2000). Review article The biochemistry of apoptosis. *Nature,* 407, pp.770–776.

20. Ho, G.Y., Bierman, R., Beardsley, L., Chang, C.J. and Burk, R.D.(1998). Natural history of cervicovaginal papillomavirus infection in young women. Journal 338(7), pp. 423-8.

21. Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991). p53 mutations in human cancers. *Science,* 253, pp. 49–53.

22. Hu, Y., Benedict, M.A., Wu, D., Inohara, N. and Nunez, G. (1998) .Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proceedings of the National Academy of. Science of the USA*, 95, pp. 4386–4391.

23. James, M. A., Lee, J. H., Klingelhutz, A. J. (2006). Human papillomavirus type 16 E6 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner. *Journal of Virology*, 80, pp.5301.

24. Johnson, Q., Syce, J., Nell, H., Rudeen, K. and Folk, W.R. (2007). A randomized, double-blind, placebo-controlled trial of *Lessertia frutescens* in healthy adults. *PLoS Clinical Trials,* 2(4), e16.

25. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J.M., Dumont. (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell*, 90, pp. 809–819.

26. Kerr, J.F.R, Wyllie, A.H. and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*. 26, pp. 239-257.

27. King, K.L, Cidlowski, J.A. (1998). Cell cycle regulation and apoptosis. *Annual Review of Physiology*, 60, pp.601-17.

28. Kroemer, M. and Jaattela, G. (2005). Lysosomes and autophagy in cell death control. *Nature Review Cancer*, 5, pp. 886–897

29. Kruse, P. R. and McCoy, T. A. (1958). The competitive effect of canavanine on utilization of arginine in growth of Walker carcinosarcoma 256 cells *invitro*. *Cancer Research. 18*, pp. 279-282.

30. Kruse, Jr., P.F., White, P.B., Carter, H.A. and McCoy, T.A. (1959). Incorporation of canavanine into protein of Walker carcinosarcoma 256 cells cultured in vitro. *Cancer Research*, 19, pp.122-125.

31. Krysko, D.V., Vanden, B.T., D'Herde, K. and Vandenabeele, P. (2008), Apoptosis and necrosis: detection, *Discrimination and phagocytosis Methods*, 44 pp. 205–221.

32. Kuerbits, S. J., Plundett, B. S., Walsh, W. V., and Kastan, M. B.Wild-type p53 is a cell cycle checkpoint determinant following irradiation.*Proceedings of the. National. Academy of Science of the USA*, *89:* 7491–7495, 1992.

33. Kuwana, T. and Newmeyer, DD. (2003). Bcl-2-family proteins and the role of mitochondria in apoptosis. *Current Opinion in Cell Biology*, 15, pp. 691–699.

34. Lane, D.P. and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature*, 278, pp. 261-263.

35. Lawen, L. (2003). Apoptosis—an introduction. *BioEssays,* 25, pp. 888–896,

36. Liu, X, Yuan, H., Fu, B, Disbrow, G.L, Apolinario, T., Tomaic, V., Kelley, M.L., Baker, C.C., Huibregtse, J. and Schlege,l.R. (2005). The E6AP ubiquitin ligase is required for transactivation of the hTERT promoter by the human papillomavirus E6 oncoprotein. *Journal Biological Chemistry*, 280(11), pp.10807–10816.

37. Liu, Y., Cherry, J.J., Dineen, J.V., Androphy, E.J. and Baleja, J.D. (2009) Determinants Of stability for the E6 protein of papillomavirus type 16. *Journal of Molecular Biology,* 386(4), pp.

38. Lowe, S.W., Schmitt, E.M., Smith, S. W., Osborne, B. A. and Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, 362, pp. 847–849.

39. Majno, G. and Joris I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *American Journal of Pathology*, 146, pp.3–16.

40. Marchand, D. A., Kettinger, W. J., and Rollins, J. D. (2000). Information Orientation People, Technology and the Bottom Line," *Sloan.*

41. Marchand, L.L., Murphy, S.P. and Kolonel LN. (2000). Intake of flavonoids and lung cancer.*Journal of the National Cancer Institute*, 92, pp.154–60. [PubMed]

42. Markovits, J. and Linassier, C. (1989). Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. *Cancer Research*, 49, pp5111–7.

43. Massimi, P., Shai, A., Lambert, P. and Banks, L. (2008). HPV E6 degradation of p53 and PDZ containing substrates in an E6AP null background. *Oncogene* 27 (12), 1800–1804.

44. Mayhew, T.M., Myklebust, R., Whybrow, A. and Jenkins, R. (1999). Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histology and Histopathology*, 14, pp. 257–267.

45. Mazurkiewicz, M., Opolski AWietrzyk JRadzikowski CKleinrok Z. (1999). GABA level and GAD activity in human and mouse normal and neoplastic mammary gland. *Journal of Experimental and Clinical Cancer Research*. 2, pp, 247-253.

46. Melero, J.A., Stitt, D.T., Mange, l .W.F. and Carroll ,R.B. (1979) .Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T antigen and present in simian virus 40-infected and transformed cells. *Journal of Virology*, 93, pp. 466-480.

47. Mncwangi and Viljoen. (2012). Devil's Claw—A review of the ethnobotany, phytochemistry and biological activity of Harpagophytumprocumbens, Journal of Ethnopharmacology,143,pp. 755-771.

48. Molden T. (2005). Comparison of human papillomavirus messenger RNA and DNA detection: a cross-sectional study of 4,136 women >30 years of age with a 2-year follow-up of high-grade squamous intraepithelial lesion. *Cancer Epidemiology.Biomarkers Prevention*, 14, pp. 367–372.

49. Moreno, V., Bosch F.X, Muñoz ,N., Meijer, C.J., Shah, K.V., Walboomers, J.M., Herrero, R. and Franceschi, S.(2002). Effect of oral contraceptives on risk of cervical cancer in women with human papillomavirus infection: the IARC multicentric case-control study, 359(9312), pp.1085-1092

50. Moshe, D., Bank, H.V., Bank, M.V. and Van Wyk, B.E. (1998). Lack of genetic differentiation between 19 populations from seven taxa the *Sutherlandia* Tribe. *Biochemical Systematics and Ecology*, 26, pp. 595–609.

51. Mqoqi, N., Kellett, P., Sitas, F., Jula, M. (2004). Incidence of histologically Diagnosed cancer in South africa,1998 – 1999.The National Cancer Registry of South Africa, National Health Laboratory Service, Johannesburg.

52. Murdoch, W.J., Wilken, C. and Young, D.A. (1999). Sequence of apoptosis and inflammatory necrosis within the formative ovulatory site of sheep follicles. *Journal Reproductive Fertility,* 117, *pp.* 325–329.

53. Mutoh, M., Takahashi, M. and Matsushima, H.Y. (2000).Suppression by flavonoids of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells: Structure-activity relationship. *Japanese Journal of Cancer Research*, 91, pp. 686–691.

54. *Newmeyer, D. D. and Ferguson-Miller, S. (2003).* Releasing Power for Life and Unleashing the Machineries of Death .*Cell,* 112(4), pp. 481–490.

55.

56. Nicolson R., Fawcett AJ. and Dean P.(2001). Dyslexia, development and the cerebellum. *Trends Neurosciences*, 24,515-516.

57. Nicotera, P., Bernassola, F. and Melino, G. (1999). Nitric oxide (NO), a signalling molecule with a killer soul. *Cell Death and Differentiation*, 6, pp. 931–933.

58. Reed, J.C. (2001). Apoptosis regulatory proteins as targets for drug discovery. *Trends in Molecular Medicine*, 7, pp.314–318.

59. *Rosenthal, G.A.* (1977). The biological effects and mode of action of L-canavanine, a structural analogue of L-arginine. Quarterly *Review of Biology,* 52, pp.155-178.

60. Rosenthal, G.A. (1992). Purification and characterization of the higher plant enzyme L-canaline reductase. *Proceedings of the National Academy Sciences of the U.S.A*, 89, pp.1780-1784.

61. Rovinski, B. and Benchimol, S. (1988). Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. *Oncogene*, 2, pp. 445-452.

62. Schiffman, M., Herrero, R., DeSalle, R., Hildesheim, A., Wacholder, S., Rodriguez, A.C., Bratti, M.C., Sherman, M.E., Morales, J., Guillen, D., Alfaro, M. and Hutchinson, M. (2005). The carcinogenicity of human papillomavirus types reflects viral evolution. *Virology*, 337(1), pp. 76–84.

63. Seier, J.V., Mdhluli, M., Dhansay, M.A., Loza, J. and Laubscher, R. (2002). A toxicity study of *Sutherlandia* leaf powder *(Sutherlandia frutescens* sub-species microphylla) consumption. Tygerberg (South Africa): *Medical Research Council.*

64. Shimizu, S., Narita, M. and Tsujimoto, Y. (1999).Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature*, 399, pp.483-487.

65. Sigal, A. and Rotter, V. (2000) Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Research*, 60, pp. 6788-6793.

66. Syntichaki, P*. and* Tavernarakis, N*. (*2002*).* Death by necrosis. Uncontrollable catastrophe, or is there order behind the chaos? *EMBO Reports,* 3*, pp.* 604–609*.*

67. Tatsuta, M. (1990). Inhibition by y-amino-n-butyric acid and Baclofen of gastric carcinogenesis induced by N-methyl-N1-nitro-Nnitrosguanidine in Wistar rats. *Cancer Research,* 50, pp. 4931-4934.

68. Thomson, S. (2002). Canavanine toxicity: Is Sutherlandia a healthy herb or potential poison? HIV Positive and AIDS Sufferers Beware:The Remedy may be Worse than the Alleged Disease.(http://www.gaiaresearch.co.za/) 05/18/04, Part 1.

69. Van Schalkwyk, S.L., Maree, J.E, Wright, S.C.D. (2008). Cervical cancer: the route from signs and symptoms to treatment in South Africa. *Reproductive Health Matters*, 16(32), pp. 9–17.

70. Van Wyk, B.E. and Albrecht, C. (2008). A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). *Journal of Ethnopharmacology,* 119, pp. 620-629.

71. Vogt, M., Butz, K., Dymalla, S., Semzow, J. and Hoppe-Seyler, F. (2006). Inhibition of Bax activity is crucial for the antiapoptotic function of the human papillomavirus E6 oncoprotein. *Oncogene*, 25(29), pp. 4009-15.

72. Walker, N.I., Harmon, B.V., Gobe, G.C. and Kerr, J.F. (1988) .Patterns of cell death. *Methods and Achievements in Experimental Pathology*, 13, pp.18–54.

73. Wang, S., Miura, M., Jung, Y.K., Zhu, H., Li, E. and Yuan, J. (1998). Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell*, 92, pp. 501–509.

74. Wei, M. C., Zong ,W. X., Cheng, E. H., Lindsten, T.,Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B. and Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, 292, pp. 727–730.

75. Wink, M. (1999). Functions in plants secondary metabolites and their exploration in biotechnology. Sheffield Academic Press, England, United Kingdom, pp. 188-273

76. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dotsch, V., Andrews, N.C., Caput, D., and McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Molecular Cell,* 2, pp**.** 305–316.

77. Yuan, H., Fu, F., Zhuo, J., Wang, W., Nishitani, J., An, D. S., Chen, I. S. and Liu, X. (2005). Human papillomavirus type 16 E6 and E7 oncoproteins upregulate c-IAP2 gene expression and confer resistance to apoptosis. *Oncogene*, 24, pp.5069–507810.

78. Zimmermann, K.C., Bonzon, C. and Green, D.R. (2001). The machinery of programmed cell death. *Pharmacology and Therapeutic,* 92, pp. 57–70.

79. Zimmermann, H., Richter, E., Reichle, C., Westphal, I., Geggier, P.,Rehn,U., Rogaschewski, S., Bleiss,W. and Fuhr,G. R.(2001). Mammalian cell traces morphology, molecular composition, artificial guidance and biotechnological relevance as a new type of ™bionanotube™. *Applied Physics*, 73, pp.11 26.

80. ZurHausen, H. (2002). Papillo- mavirusesandcancer:frombasic studies to clinical application. *Nature Reviews Cancer,* 2, pp.342–350.

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