



Faculty of Sciences

Department of Biological Sciences and Biotechnology

**Cytotoxicity and Gene Expression Mediated Antiproliferative Potential of
Selected Medicinal Plants of Eastern Botswana
on HeLa Cells**

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DEDICATION

To you my departed parents, mum, and dad, it has been decades without you but still I strive to make you proud in your absentia. I keep falling on the way, but I dust myself up and keep moving despite anything to reach a target you set for me.

To my two sons, Malte and Mathias, this is to say to you, we are carving our way up to greatness and this is the path worth taking, the road is not easy, but it is worth the struggle.

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LIST OF ABBREVIATIONS

AG- *Azanza garckeana*

AGF - *Azanza garckeana* Fruits

CC₅₀ – Half-maximal Cytotoxic Concentration

cDNA – Complimentary Deoxyribonucleic acid

CDK – Cyclin Dependant Kinase

CD95- Cluster of Differentiation 95

CYFRA 21-1 - Cytokeratin 19 Fragments

DMEM - Dulbecco's Modified Eagle's Medium

DNA - Deoxyribonucleic acid

EE- *Elephantorrhiza Elephantina*

EET - *Elephantorrhiza Elephantina* Tubers

EGFR - Epidermal Growth Factor Receptor

FBS - Fetal Bovine Serum

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

HSP90 - Heat Shock Protein-90 (

MACC1 - Metastasis Associated In Colon Cancer 1

MTT - 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NASP Gene - Nuclear Autoantigen Sperm Protein

NC – Negative Control

PBS – Phosphate Buffered Saline

PC – Positive Control

PO- *Portulaca Oleracea*

POLB- *Portulaca Oleracea* Leaves and Branches

RNA – Ribonucleic acid

ROS - Reactive Oxygen Species

RT-qPCR – Reverse Transcriptase quantitative Polymerase Chain Reaction

TNF- α - Tumour Necrosis Factor Alpha

TPC - Total Phenolic Content

TT- *Tribulus Terrestris*

TTLB - *Tribulus Terrestris* Leaves and Branches

VEGF - Vascular Endothelial Growth Factor

WS- *Withania Somnifera*

WSL - *Withania Somnifera* Leaves

WST-1 - Water Soluble Tetrazolium salt 1

ABSTRACT

Cancer is a global problem, which contribute to many deaths annually when compared to other non-communicable diseases. Amongst women in Botswana, cervical cancer has the highest incidence and mortality rate. Literature has shown that medicinal plants have potential cytotoxicity on cancer cells.

A literature survey with consultations of traditional healers coupled with identification and verification by Botswana National Herbarium led to the selection of five medicinal plants of Botswana for the investigation of their potential cytotoxicity on cancer cells. The identified plants were *Elephantorrhiza elephantina* (Burch.) Skeels, *Azanza garckeana* (F.Hoffm.) Exell & Hille, *Withania somnifera* (L.) Dunal, *Tribulus terrestris* L. and *Portulaca oleracea* L. whose identities were verified by the Botswana National Museum Herbarium. The plants were collected in the Central and Kweneng Districts of Botswana. The plants were crudely extracted with water, ethanol and methanol and the extracts were applied on cervical cancer cells (HeLa). MTT and WST-1 assays (cell viability assays) were carried out on the treated HeLa cells and morphological changes were then observed. During morphology analysis, the cells were treated with the lowest and highest concentrations of extracts used in serial dilutions in cell viability assays for a period of 24 hours. Gene expression analysis was performed on treated cells for cancer markers MACC1, VEGF, EGFR, CYFRA 21-1 and the tumor suppressor gene CD95; all normalized to GAPDH.

A. garckeana ethanol and methanol extracts had CC₅₀ values of 1.8 and 1.88 mg/mL respectively while aqueous extracts had cell viability not exceeding 50%. *P. oleracea* ethanol and aqueous extracts both had CC₅₀ values of 2.38 mg/mL while methanol extracts had 5.2 mg/mL. *T. terrestris* ethanol and aqueous extracts showed CC₅₀ values of 9.6 and 6.7 mg/mL respectively with methanol extracts cell viability of below 50%. *E. elephantina* had CC₅₀ values of 6.9, 4.6 and 5.6 mg/mL for ethanol, water and methanol extracts respectively. *W. somnifera* had CC₅₀ values of 42.0, 32.5 and 52.5 mg/mL for ethanol, aqueous and methanol extracts respectively. Morphologically, signs of detachment, cells becoming more spherical and dying were observed on all treatments of all extracts but were more prominent in the *P. oleracea* aqueous extracts for 1.7725 mg/mL and 28.36 mg/mL treatments of all the extraction solvents. Gene expression analysis revealed that *P. oleracea* aqueous extracts led to the most down regulation of EGFR, MACC1, VEGF and CYFRA21-1 and the most upregulation of CD95 with fold decreases of 0.44, 0.4, 0.35, 0.38 and a fold increase of 0.32 for these genes respectively. *T. terrestris* aqueous extracts led to fold decreases as follows; EGFR = 0.3, MACC1 = 0.29, VEGF = 0.3, CYFRA21-1 = 0.35 with a CD95 fold increase of 0.27. *A. garckeana* aqueous extracts displayed fold decreases as follows; EGFR = 0.3, MACC1 = 0.31, VEGF = 0.28, CYFRA21-1 = 0.29 and a CD95 fold increase of 0.25. *E. elephantina* aqueous extracts led to fold decreases as follows; EGFR = 0.15, MACC1 = 0.2, VEGF = 0.17, CYFRA21-1 = 0.2 and CD95 fold increase of 0.16. *W. somnifera* aqueous extracts led to fold decreases as follows; EGFR = 0.2, MACC1 = 0.2, VEGF = 0.1, CYFRA21-1 = 0.22 and a CD95 fold increase of 0.18. Cisplatin (positive control) gave a 0.8 fold decrease in the

expression of EGFR, MACC1, VEGF and CYFRA21-1 and a 0.77 fold increase in CD95 expression. In conclusion, findings of this study have shown the potential cytotoxicity on HeLa cells of selected plant extracts through their evidenced suppression of cancer marker expression as well as their induction of antitumour activity through the upregulation of CD95. Additionally, extracts from the selected plants appeared to suppress the proliferation of cancer cells through their supposed interruption of cell attachment in culture and induction of cell death. Findings of this study warrant future investigation of extracts of the plants that showed the greatest cytotoxic potential through the purification of these extracts and the identification of their individual phytochemicals. Furthermore, potential anticancer ingredients may be manufactured from these phytochemicals based on the synergistic effects of different combinations of these phytochemicals on cancer cells.

CHAPTER 1: INTRODUCTION

1.1 Background

Cancer cells are cells that have lost the ability to follow normal cell division control that the body exerts on all cells (Mattiuzzi and Lippi, 2019). Different types of cells in the body have different functions and all work together coherently, each serving its function for proper functioning and maintenance of the body. Essentially, cancer is when something goes wrong in the intricate control mechanism of cell division leading to the continuous growth of cells. These cells are together referred to as a tumour and may spread to other cells or organs forming malignant tumours (Wang *et al.*, 2021). Others, called benign tumours, which do not metastasize, get localized in one tissue (Psychogios *et al.*, 2021). Normal cells constantly receive signals to differentiate into other cells, divide, or die depending on the needs of the body (Hejmadi, 2010). Cancer cells evade these control signals, consequently resulting in uncontrollable cell proliferation and growth.

In mammals, the molecular mechanisms of controlling cell proliferation, differentiation and death (apoptosis) are similar. However, the development of cancer is due to alterations of these mechanisms at the molecular, biochemical and cellular levels (Hejmadi, 2010). These changes vary from one cell to another, thus leading to the formation of different types of cancers. It is easy for malignant cancer cells to move from one tissue to another because unlike normal cells, cancer cells have lost the cell adhesion molecules on their surface that holds cells together, so they can easily break away from other adjacent cells (Bergers and Fendt, 2021).

The ability of cancer cells to elude cell cycle signalling controls can be attributed to mutations in the cell genome (Chikowe *et al.*, 2021). DNA mutations result in the production of proteins that change the equilibrium between cells that are actively dividing and their dormancy state (Hejmadi, 2010). Proteins and RNAs control the activities and signalling pathways within the cells and their progenitors (stem cells). Therefore, they determine the types of cells that will be produced from stem cells, their functions, when cell division occurs and when cells will undergo apoptosis. Proteins are a consequence of gene expression, therefore genes control the growth and reproduction of cells in an intricate way.

1.1.1. Types of cancers

1.1.1.1 Cervical Cancer

Cervical cancer develops in the cervix and according to Ferlay *et al.*, (2021), it has the second highest incidence and mortality rates of all cancers in most regions globally, especially Southern Africa. This cancer originates in the squamous cells of the cervix at the junction where the ectocervix and endocervix meet with a combination of columnar epithelium of endocervix and squamous epithelium of ectocervix (Ludmir and Sehdev, 2000). It is in the squamocolumnar junction where cervical cancer develops due to dysplasia of cells in the region (Franco *et al.*, 2003). From a research perspective, cervical cancer has played a significant role because the first human cells to be successfully grown in the laboratory, called HeLa cells,

were isolated from cervical cancer cells derived from a woman called Henrietta Lacks after whom HeLa cells are named (Lucey *et al.*, 2009). HeLa cells are immortal because they can grow uncontrollably, be cultured for many generations and even overgrow other cells in the same culture (Gartler, 1967).

Symptoms of cervical cancer include abnormal vaginal bleeding, especially after sexual intercourse, pain when passing urine, vaginal discomfort and unpleasant discharge from the vagina (Mingo *et al.*, 2012). If the cancer has spread beyond the cervix, which occurs when the cancer is invasive and has affected the entire epithelial layer including the basal membrane, it affects nearby organs like the uterus, bladder and rectum; then, it can lead to symptoms like constipation and bloody urine (Gien and Covens, 2010). Cervical cancer can be diagnosed through a pap smear where cells collected from the cervix are examined for dysplasia and if the pap smear comes out positive for dysplasia, a biopsy can be conducted with the aid of a colposcopy to carry out further and specific confirmatory tests (Ratnam *et al.*, 2000). There are several clinical ways to alleviate cervical cancer including surgical removal of the endocervix, removal of the tumour site or totally removing the uterus and the lymph nodes associated with the uterus and the cervix (Franco *et al.*, 2001). However, at advanced stages, the only alleviation methods available for cervical cancer would be radiation and chemotherapy.

1.1.1.2 Breast Cancer

Breast cancer, also referred to as breast carcinoma, is the most common cancer in women with the highest incidence and mortality rates (Ferlay *et al.*, 2021). It rarely affect men. Breast cancer develops as a result of the loss of control of the cell cycle in the epithelial cells of the breast (Zhang *et al.*, 2022). The breast is a milk producing tissue with glandular tissues specifically responsible for milk production and it lies on top of the pectoral muscles in the chest, with three main parts (Tao *et al.*, 2015). The glandular tissue within it is the alveoli and its sole responsibility is the production of milk. Around the glandular tissue is the tissue made mostly of adipose tissue and contributing to the largest section of the breast called the stroma. The lymphatic vessels underlie the skin which covers the breast and drain the lymph into lymph nodes in the armpit (Ramsay *et al.*, 2005).

The cells in the glandular tissue are receptive and responsive to the release and actions of hormones like oestrogen, progesterone and prolactin, stimulating the alveoli cells to proliferate thus enlarging the alveoli (Ellis and Mahadevan, 2013). Due to fluctuations in the production and release of these hormones as a result of the menstrual cycle, alveoli cells undergo division and apoptosis every cycle. The risk of breast cancer development emanates from the fact that alveoli cells undergo rapid division every menstrual cycle and rapid cell division has higher chances of mutations which could lead to the formation of tumours (Satyanarayana and Kaldis, 2009). Therefore, the production and release of oestrogen and progesterone, which is a normal physiological phenomenon controlling the menstrual cycle, can pose a risk and increase chances of breast cancer development. In order to reduce the risk of breast cancer development, tumour suppressor genes like BRCA-1, BRCA-2 and TP53 are always there as guardians of the physiology to guard against the uncontrollable growth of cells (McPherson *et al.*, 2000).

However, mutations in these genes can lead to increased chances of breast cancer development as there will not be any mechanism to guard against cells with tumour forming abilities.

Symptoms of breast cancer include painless lumps in the breast or in the axillary area if the cancer has spread to the axillary lymph nodes (McPherson *et al.*, 2000). The breast can retract and the nipple may be itchy and release a discharge. The initial diagnostic procedures of breast cancer may be feeling a lump on the breast, mammography, and using imaging techniques which can be followed by a biopsy of the affected tissue for confirmatory purposes (Rojas and Stuckey, 2016). Treatment procedures for breast cancer are mostly dependent on the stage and progression of the cancer. Mastectomy, which is the total or partial removal of the breast and associated tissues and lymph nodes, can also be performed. Hormonal therapy can be employed to balance hormones, thus reducing the chances of uncontrolled cell division of the alveolar cells (Waks and Winer, 2019). Radiation and chemotherapy can also be used depending on the stage of the cancer.

1.1.1.3 Prostate Cancer

Prostate cancer is the carcinoma of the prostate gland naturally found in males only. It is the most occurring cancer with highest mortality globally (Sung *et al.*, 2021). Prostate cancer is usually malignant, which means that it can spread to other tissues besides the prostate gland and affect them (Boyle *et al.*, 2003). Prostate cancer mostly develop due to a genetic mutation in the luminal columnar cells lining the small lumen in the prostate resulting in the luminal cells losing control of the cell division mechanism (Timms and Hofkamp, 2011). Genetic mutations leading to prostate cancer occurs in the BRCA-1 and 2 genes, which also result in breast cancer development (Berg *et al.*, 2005). It is not easy to diagnose prostate cancer at the initial stages because it is asymptomatic until it grows towards the urethra where the patient can experience its effects. Therefore, the tumour can grow large before being recognized as it normally starts in the posterior zone of the prostate far away from the urethra (Timms and Hofkamp, 2011).

Prostate cancer can be easily diagnosed using digital rectal examination where a hard lump can be felt in the posterior zone of the prostate. Trans rectal ultrasound and biopsy can be used to confirm the existence of the tumour lump (Crawford, 2003). Like other cancers, treatment options for prostate cancer depend on the stage of the cancer during diagnosis and the level of metastasis. Treatment options include surgical removal of the tumour, chemotherapy, radiation and hormonal therapy (Costello, 2020).

1.1.1.4 Status of cancer in Botswana and globally

Botswana is an upper middle-income country in Southern Africa and like many countries in Africa, the country is faced with a lot of challenges in the health sector. A large portion of the population of Botswana is entirely dependent on the government for health care. Cancer, like many other diseases, poses a great threat to this small nation. There are only two government hospitals with oncology departments in Botswana. These are Princess Marina Hospital in

Gaborone and Nyangabgwe Referral Hospital in Francistown (Grover *et al.*, 2015). Because of sparse and limited health facilities, most people come to hospital with advanced stages of cancer resulting in late diagnosis either because of lack of cancer awareness or healthcare facilities (Brown *et al.*, 2018). This leads to increased chances of mortality. The longer the time taken for diagnosis and initiation of a management protocol, the higher the chances of metastasis at the time of conclusive diagnosis (Carpenter *et al.*, 2020). The mortality rate of patients presenting with cancer in Botswana is a worrisome factor which has the possibility of crippling the economy of the country since more resources are channelled into the fight against cancer.

Cervical cancer contributes a large ratio of cancer mortality in Botswana (Grover *et al.*, 2015). This is not only a problem in Botswana, but in many countries around the world. The Ministry of Health in Botswana established, in 1999, the Botswana National Cancer Registry (BNCR), whose mandate is currently limited to the registry of cancer patients and basic clinical prognosis (Slone *et al.*, 2018). Because of resource limitations and accessibility, BNCR cannot reach all corners of the country. Cancer patients are enrolled at Princess Marina Hospital and Gaborone Private Hospital in Gaborone, Botswana, in order to configure the epidemiology of cancer in Botswana and the patterns of care. There has been some efforts to help the Ministry of Health partner with international organisations on cancer research and education of medical personnel since the government recognises the importance of establishing sustainable facilities that can help alleviate cancer in Botswana (Suneja *et al.*, 2013). Figures 1-4 below depicts global cancer incidence and mortality rates of different cancers according to gender.

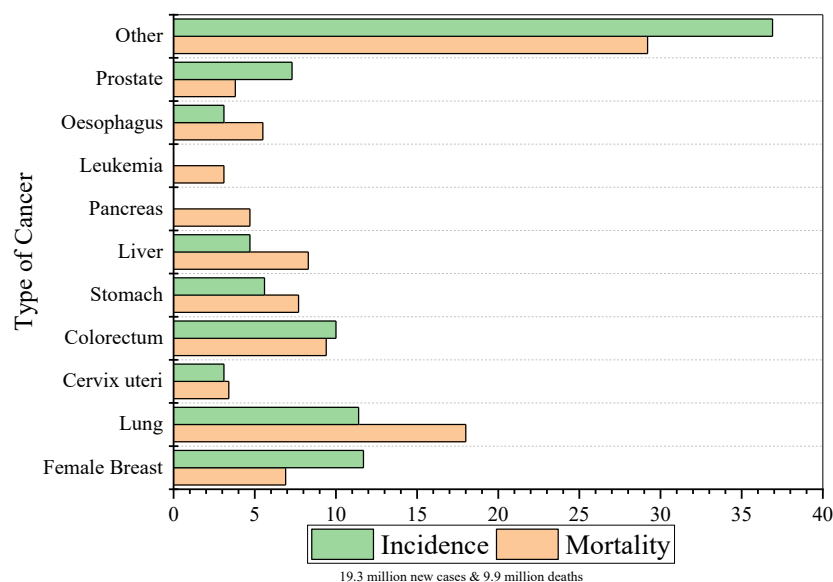


Figure 1: The incidence and mortality rates of cancers in both sexes worldwide. Lung cancer has the highest mortality rate while female breast cancer has the highest incidence rate globally (Adapted from Global Cancer Statistics, 2020).

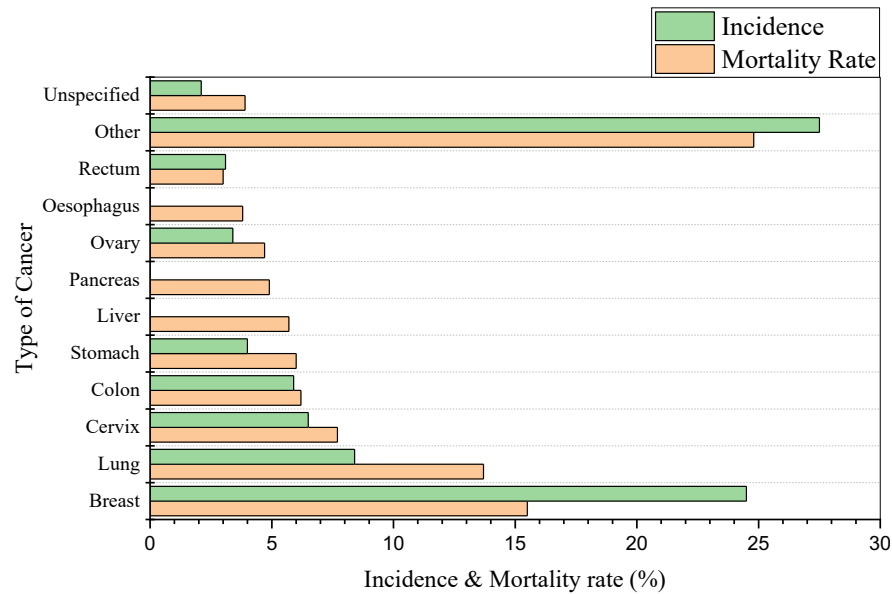


Figure 2: Cancer incidences and mortality rates in females worldwide. Breast cancer has both the highest incidence and mortality rates amongst females globally (Adapted from Global Cancer Statistics, 2020).



Figure 3: Cancer incidence and mortality rates in males across the world. Lung cancer has both the highest incidence and mortality rates amongst males globally (Adapted from Global Cancer Statistics, 2020).

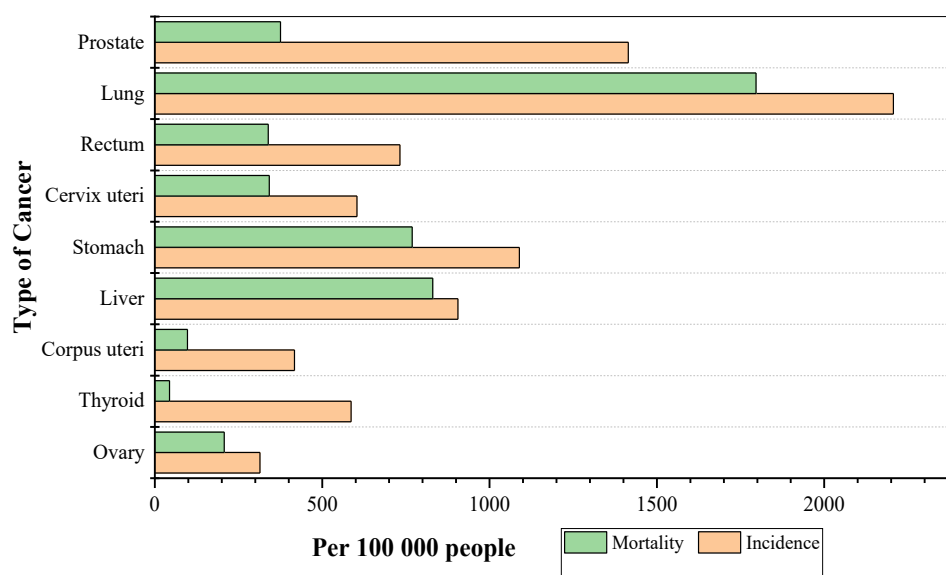


Figure 4: Age-standardized incidence and mortality rates of different cancers across the world. Per 100, 000 people across the world, lung cancer has both the highest incidence and mortality rates (Adapted from Global Cancer Statistics, 2020).

Lung cancer has the highest mortality rate of all the specified cancers while female breast cancer has the highest incidence of all the specified cancers (Figure 1). According to the Global Cancer Statistics Report of 2020, the most common types of cancers for both very high and low Human Development Index (HDI) in females are breast cancer, followed by lung cancer, then cervix uteri, all of which contribute to the high number of cancer deaths globally (Figure 2) (Sung *et al.*, 2021). In males, lung cancer has both the highest incidence and mortality rates when compared to other cancers. Although prostate cancer has the second highest incidence rate, prostate cancer mortality rates are lower than those of liver and stomach cancers (Figure 3) (Sung *et al.*, 2021). Lung cancer has the highest global cancer incidence and mortality rates (Figure 4).

1.2 Significance of Study

Cancer is slowly becoming a pandemic in Botswana and conventional cancer treatment and management methods are not always effective and can have undesirable side effects. Therefore there is a need for the development of easily accessible, effective and safer cancer management strategies.

1.3 Hypothesis

This study hypothesises that there may be some plants of Botswana that produce phytochemicals that can antagonize the growth and proliferation of cancer cells *in vitro*

To test this hypothesis, the following aim was set;

1.4 Aim

The aim of this study was to investigate 5 selected medicinal plants of Botswana for their potential cytotoxicity and antiproliferative effects on HeLa cells.

To achieve this aim, the following specific objectives were set;

1.5 Objectives

- To seek the knowledge of traditional healers of Botswana and conduct literature searches on plants with possible cytotoxicity on cancer cells
- To collect explants of identified plants from their localities and extract crude phytochemicals from them.
- To carry out cell viability assays on HeLa cells treated with crude plant extracts from the 5 selected plants of Botswana and HeLa cells treated with cisplatin.
- To observe and study morphological changes on untreated HeLa cells, HeLa cells treated with crude extracts of the 5 selected plants and HeLa cells treated with cisplatin.
- To carry out gene expression analysis on HeLa cells treated with crude extracts of the 5 selected plants, HeLa cells treated with cisplatin and untreated HeLa cells.
- To compare the gene expression profiles of cervical cancer markers in HeLa cells treated with crude extracts of the 5 selected plants, those treated with cisplatin and untreated HeLa cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Genes associated with cancer and cell cycle progression

A cell is normally quiescent, where it is inactive or in a dormant state. A quiescent cell has the ability to leave this state and enter the cell cycle under the controlled environment of proteins called CDKs and cyclins (Satyanarayana and Kaldis, 2009). There is a possibility of the cell encountering some errors during cell cycle progression (Grindedal *et al.*, 2009). Therefore, it is important to have some check points for any possible errors in the process. The first check point is at the start of the G1 phase of cell cycle progression which checks for errors in the DNA of the cell. At this check point, CDK 4 and 6 bind to cyclin D, making a protein called E2F which attaches to a retinoblastoma protein (Fisher, 1997). The release of E2F from the complex will cause the cell to progress to the synthesis phase of the cell cycle. CDK 2 and cyclin E form another complex at the end of the G1 phase which gives the cell a free pass to the synthesis phase (S phase).

For a cell to progress from the S phase to the G2 phase, CDK 1 and 2 complexed with cyclin A give the cell another go ahead. CDK 1 and cyclin B then allow the cell to progress to the M phase from the G2 phase (Limas and Cook, 2019). If CDKs and cyclins are low, the cell does not progress properly in the cell cycle, but if they are too high, cells continuously enter the cell cycle uncontrollably and this is how cancer occurs (Besson *et al.*, 2008). It can now be deduced that whatever increases the potential of having more CDKs and cyclins in the cell is the basis for the development of cancer. When the control mechanisms brought about by CDKs and cyclins complexes are bypassed, for whatever reason, the cell will undergo uncontrolled cell growth, producing many copies of itself, more than what the body requires, thus leading to the development of tumours.

Uncontrollable cell growth during cancer is caused by either or both of the two main changes that occur in the cell; the activation of oncogenes like the RAS gene and MYC gene or the inactivation of tumour suppressor genes like p53, APC and BRCA 1 and 2 (Lee *et al.*, 1985). RAS is a normal gene in the cell coding for the RAS protein, which sits next to the growth factor receptor. The growth factor stimulates the growth factor receptor which in turn activates the RAS protein. The activation of the RAS protein will lead to a cascade of phosphorylation reactions which eventually activate a transcription factor (Watanabe *et al.*, 1999). The transcription factor has an effect on DNA, transcribing the mRNA that code for cell growth proteins like CDKs and cyclins. Therefore, a mutation in the RAS gene can lead to the production of the RAS protein which is already activated, leading to the over expression of transcription factors, thus leading to the continual production of cell growth proteins, resulting in the cell always going through the cell cycle. The MYC gene normally codes for proteins required for cell growth, activity, survival and its ability to interact with many proteins enhances the potential to work as an oncoprotein (Lourenco *et al.*, 2021). Therefore, a mutation in this gene can make the cell to have these features being overexpressed, thus giving it more chance to persevere (Watanabe *et al.*, 1999). The overexpression and activation of oncogenes

can allow the cell to bypass all the check points in the cell cycle and allow the cell to divide uncontrollably.

Normally, tumour suppressor genes stop any abnormal cell from entering the cell cycle, therefore inhibiting further proliferation of the cell because it has a faulty genetic makeup. Due to DNA damage, the cell produces tumour suppressor genes like p53, which contributes to more than 50% of cancers if it is mutated (Huang *et al.*, 2019). The p53 gene codes for the p53 protein that will assume the function of a transcription factor (Li *et al.*, 1994). The p53 gene reads the DNA and when it recognises that there is damage in the DNA, it leads to a cascade of reactions which leads to the production of the p21 protein which leads to cell arrest. In that case, the cell cycle is arrested from the G1 to the S-phase by the action of p21 binding to the cyclin-CDK complex (Besson *et al.*, 2008). The cyclin-CDK complex is responsible for normal progression of the cell cycle from the G1 to the S-phase. The p21 gene arrests the cell cycle by inhibiting CDKs and cyclins which drive this process.

The p53 gene leads to the production of proteins responsible for cell repair so that when the cell cycle is arrested, the damaged DNA can be repaired by the cell repair mechanism or if the cell is not repaired, apoptosis is induced in that cell (Li *et al.*, 1994). Therefore, if there are mutations in tumour suppressor genes, leading to their inactivation, proteins leading to cell arrest, repair or apoptosis will not be produced resulting in uncontrollable cell growth and division (Li *et al.*, 1994). There are two copies of p53 alleles in each cell, one paternal and the other maternal. Mutations occurring in the p53 allele are recessive, indicating that for a gene to malfunction, both copies of the allele should be mutated and this is an unlikely scenario (Stegh, 2012). Nevertheless, if one of the alleles inherited was already mutated, then chances of cancer development would increase.

Homeostasis of the p53 gene has to be intricately regulated and any deviation from their normal levels, which is at low concentrations in normal and healthy cells (Zhang and Chen, 2019), would have a negative impact on the cell (Stegh, 2012). The upregulation or overexpression of p53 can lead to unnecessary apoptosis, while its down regulation can lead to oncogenes finding a window of opportunity to be expressed, thus leading to tumour development (Li *et al.*, 1994). Under normal conditions, where there is no DNA damage and p53 is not called into action, p53 becomes unstable on its own. Therefore, to stabilize it, another protein called MDM2 binds to p53, tagging it for ubiquitination through ubiquitin-mediated degradation. Only the p53 protein will be degraded while the MDM2 protein will be recycled (Brooks and Gu, 2006). When the action of the p53 protein is required, such as during DNA damage, both the p53 and MDM2 proteins will be phosphorylated and both proteins will not be able to bind to each other (Fuchs *et al.*, 1998). Therefore, the p53 protein will be available, its concentration will increase and it will induce cell cycle arrest at the G1 phase.

2.2 Genes associated with cervical cancer

Cervical cancer contributes to the many incidences of cancer in Botswana amongst women, and, for that reason, many patients in oncology wards in hospitals are victims of cervical cancer.

Cervical cancer has and still continues to affect many families and individuals in Botswana, especially those infected with HIV. It has the third highest incidence and mortality rates globally and according to Botswana Human Papillomavirus and Related Cancers Fact Sheet of 2022, approximately 55% of cervical cancer incidences result in mortality in Botswana.

2.2.1 Vascular Endothelial Growth Factor (VEGF)

The human vascular endothelial growth factor (VEGF) gene is located on chromosome 6 and has different subtypes such as VEGF-A which is mostly involved in pathological angiogenesis. VEGF-B, VEGF-C, VEGF-D and VEGF-E all have different functions in angiogenic pathways (Muratori *et al.*, 2018). VEGF-A promotes the proliferation and migration of endothelial cells as well as activating protease activity (Psoma *et al.*, 2020). VEGF-B promotes the existence of vascular cells by suppressing the expression of apoptotic genes like BH-3 (Muhl *et al.*, 2016). VEGF-C and VEGF-D are lymph angiogenesis-related growth factors while VEGF-E proliferates keratinocytes and stimulates epidermal regeneration (Siveen *et al.*, 2017). VEGF-F is a molecular marker for most cancers and is targeted by anti-cancer drugs which downregulate its expression. This downregulation eventually deprive tumour cells of blood supply, ultimately halting their proliferation (Roskoski Jr, 2017). Phytochemicals from indigenous plants that have been used as traditional medicines may target VEGF expression, down regulating it and ultimately inhibiting blood vessel connectivity and supply to tumours, therefore stopping tumour proliferation.

2.2.2 Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) gene is located on chromosome 7 and its overexpression has been implicated in many cancers and thus it is counted amongst the leading players in tumour development (Tan *et al.*, 2017). The overexpression of EGFR can lead to uncontrolled proliferation of tumour cells (Oh *et al.*, 2011). Therefore, the presence of EGFR in a tissue or organ is indicative of the presence of tumour cells. The EGFR protein is a transmembrane protein that is normally upregulated in malignant tumours resulting in increased tumour angiogenesis, metastasis and the death of normal cells (Kim *et al.*, 2005). EGFR expression can therefore be targeted in cancer alleviation protocols which down regulate its expression.

2.2.3 Metastasis-Associated In Colon Cancer 1 (MACC1)

Metastasis-associated in colon cancer 1 (MACC1) is a biomarker of metastasis and cancer cell migration (Link *et al.*, 2019). Because it characterizes cervical cancer, it is expressed in HeLa cells. It is associated with increased fluidity and increased tumour cell migration and spread to adjacent tissues by reducing cell to cell adhesion (Li *et al.*, 2017). Since the upregulation of MACC1 increases the chances of cancer spreading to other tissues and organs, its expression can be targeted by possible cancer alleviation protocols. If metastasis can be slowed down or even stopped, management of tumours would be eased at a clinical level.

2.2.4 Cytokeratin 19 Fragments (CYFRA 21-1)

Cytokeratins are structural proteins that usually characterizes epithelial cells (Yildiz *et al.*, 2022). Cytokeratin fragments vary, with CYFRA 21-1 being a cytokeratin 19 fragment. Since they are found in epithelial cells, it is fitting that they are also identified as biomarkers of epithelium originating cancers (Uenishi *et al.*, 2003). A tumour in the epithelium results in activated caspase-3 protease, which elevates cytokeratin deterioration through apoptosis, consequently eliciting cytokeratin fragments into circulation and when the fragments are quantified, CYFRA 21-1 concentration is elevated (Dohmoto *et al.*, 2001). Since CYFRA 21-1 is always found to be in the presence of various cancer cells like cervical carcinoma, oesophageal and breast cancer cells, it is thus a molecular marker for these cancers (Muley *et al.*, 2018) and can be used clinically by the observation of its expression patterns (Senga *et al.*, 1996) during the treatment and management of cancer symptoms.

2.2.5 Cluster of Differentiation 95 (CD95)

Cluster of differentiation 95 (CD95) is known to support cell death, which is a vital mechanism in physiological maintenance of homeostasis (Risso *et al.*, 2022). However, it has also been found to be involved in pathways that counteract apoptosis, resulting in cell proliferation, development and the spread of tumours (Seyrek and Lavrik, 2019). CD95 is activated by its ligand, CD95L for it to promote apoptosis. However, any changes or mutations in the CD95/CD95L cascade play a role in cancer progression by shutting down immune response by lymphocytes (Peter *et al.*, 2015). The non-apoptotic inducing property of CD95 has therefore been attributed to post-translational modifications of the protein. Induction of its apoptotic nature has the possibility of inhibiting tumour cell proliferation (Matou-Nasri *et al.*, 2017). CD95 expression has been detected in the cytoplasm of liver cancer cells and in the cytoplasm of other tumours, with close association to the metastasis of tumours (Hammam *et al.*, 2012). This suggests that CD95 can be classified with other molecular markers of cancer cells. The expression level of CD95 is usually low during cancer cell proliferation and this has the possibility to make tumour cells resistant to apoptosis (Chen *et al.*, 2010a). This suggests that the overexpression of this gene could increase the chances of apoptosis on cancer cells, thus leading to antiproliferative effects on the cancer. Despite the variable expression of CD95 in some tumours, its expression pattern can be evaluated by subjecting cancer cells to tumour inhibiting substances like phytochemicals derived from indigenous medicinal plants.

2.3 HeLa Cells in Research

HeLa cells are immortalized cells which have been propagated over time in laboratories globally, with the ability to divide endlessly and are easily cultured under laboratory conditions (Lucey *et al.*, 2009). Before the discovery and isolation of HeLa cells, laboratories and researchers had made several attempts to propagate human cells with no success as the cells could not be maintained in cultures for more than weeks (Nott, 2020). The ability of HeLa cells to be propagated indefinitely plays a crucial role in the continuity of research credibility of research finding thereof. They were isolated from a woman called Henrietta Lacks who had

and died from cervical cancer in the 1950s (Lyapun *et al.*, 2019). The name HeLa is derived from the first two letters of both her name and last name (Scannell, 2010; Gamble, 2014). The HeLa cell line provide an important approach in cancer research and scientific research as a whole because they are easy to work with. Therefore, they have been used by many scientists to study how cells in the human body function and have been recently used as models to develop vaccines for diseases like polio and other emerging ailments (Nott, 2020).

Over the years, continued cultivation of cell lines under laboratory conditions have proven to be the basis for such cell lines to accumulate mutations overtime, thus altering their genetic identity. However, according to Nott (2020), HeLa cells have been able to keep their genetic identity intact. Therefore, in view of how HeLa cells are used in biomedical research, some scientists have embarked on comparing the original genome of the HeLa cell line to the current HeLa cell lines used in laboratories. They have determined that due to many reorganizations of the cell line genome over many cultivations, the original genome of the cells isolated from Ms Lacks' cervical biopsy has been altered to the current genome in HeLa cells used in research laboratories (Mittelman and Wilson, 2013). According to Heng (2013), the donor's HeLa cell genome had 46 chromosomes synonymous with the natural human chromosome number, but most HeLa cells currently used in research have approximately 70 to 90 chromosomes. These alterations play a crucial role in the choice and use of HeLa cells as primary cell lines in research.

2.4 Emergence of natural products in cancer research

In recent years, a lot of interest has developed in the use of natural products for better, safe and reliable management of cancer (Gezici and Şekeroğlu, 2019). There has been great strides made by the scientific community in the application of natural products like plant secondary metabolites at clinical level and finding new opportunities in cancer therapy (Huang *et al.*, 2021). Phytochemicals from some medicinal plants have been isolated in the past and used *in vitro* in cytotoxicity assays on various cancer cell lines (Adhami *et al.*, 2018; Abdel-Hameed *et al.*, 2012) and *in vivo* in cytotoxicity experiments to study their effects on induced cancer (Degfie *et al.*, 2022). Natural products are considered as a fountain of biologically active metabolites with the possibility of being a source of treatment for many diseases which in the past were untreatable (Huang *et al.*, 2021). In a study by Ri *et al.*, (2021), traditional medicine and natural compounds were tested to check their ability to antagonize a complex of programmed cell death protein (PD-1) and its ligand PD-L1. They were shown to inhibit PD-1/PD-L1 complex and played a part in their interaction. The PD-1/PD-L1 complex is an important target for cancer immunotherapy because it can aid cancer cells to evade the body's immune system (Guo *et al.*, 2020).

Some researchers have embarked on using both natural products and synthetic means to create drugs that target certain cancers through the use of combinatorial chemistry (Newman and Cragg, 2020). In this case, they use natural phytochemicals as the basic structure on which new and specific structures are designed and referenced to. The deployment of bioactive compounds thus can be made effective through the identification of their targets and their targets can be

identified through the use of phenotypic screening. Calixto (2019) reported that 35% of the annual global medicine market, which is worth trillions of US Dollars, comes from natural products and plant products contributed a quarter of the natural products value. They also reported that natural products contributed to one third of the most profitable drugs in the world. It goes without saying that natural products have emerged as the future of medicine. They can either be used in their natural state, pure as they come, as raw materials for developing semi-synthetic drugs or as a basic skeleton on which new drugs are designed.

2.5 Medicinal plants in cancer alleviation

Recently, more interest has developed in the use of plants as sources of drugs and pharmacological components, some of which target cancer. Phytochemicals from plants have been found to target proteins involved in cancer development like ABC transporters, epidermal growth factor receptor, p53 and others (Mbaveng *et al.*, 2017). Benzophenones, isoflavonoids and isoquinolone alkaloids are some of the phytochemicals produced by plants with antiproliferative effects (Rowe *et al.*, 2013). Some alkaloids like vincristine and vinblastine, have been isolated from a plant called *Caranthus roseus* and combretastatines were isolated from *Combretum caffrums* and these are already used clinically in cancer management (Mbaveng *et al.*, 2017). According to Roy *et al.*, (2017), *Actaea racemosa* produces actein which has inhibitory effects on hepG2 liver cancer cells. *Ardisia crenata* produces ardisiacrispin which inhibits liver cancer cell proliferation. Extracts from *Bidens pilosa* have antitumor effects. Such studies go a long way in indicating the rich biodiversity and potential that plants may have in the alleviation of cancer.

2.5.1 Medicinal plants of Botswana with potential cancer cell cytotoxicity and anti-cancer properties

2.5.1.1 *Portulaca oleracea* L.

Portulaca oleracea (Tswana name; Selele) is a common succulent plant sometimes referred to as Purslane. It is normally considered as a weed and an invasive species of field crops and lawns (Kazemi, 2021). *Portulaca oleracea* belongs to the genus *Portulaca* in the family *Portulacaceae*, which is a small family consisting of 21 genera and 580 species (Zhou *et al.*, 2015). It occurs natively in America even though there are some species found in Arabia. *Portulaca oleracea* is abundant in most regions of Africa including Botswana (Uddin *et al.*, 2014). Purslane possesses high content of vitamin A which is a natural antioxidant. Antioxidants are well known for their activity against reactive oxygen species (ROS). Reactive oxygen species play a role in the development of cancer cells. Methanol extracts of *P. oleracea* have been shown to have high total phenolic content (TPC), thus, they are used to quantifying flavonoids and antioxidant activities of the plant (Lim and Quah, 2007). *Portulaca oleracea* also produces minerals such as phosphorus, calcium and magnesium (Srivastava *et al.*, 2021) and omega-3 fatty acids which are precursors of oxylipins. *P. oleracea* has been used in folk medicine for many years in many countries and exhibits a wide range of pharmacological effects like antibacterial, antiulcerogenic, anti-inflammatory, antioxidant and wound healing

properties (Zhou *et al.*, 2015). It has also been used to protect against cardiovascular diseases, cancer and other chronic diseases (Rahimi *et al.*, 2019a). Uddin *et al.* (2014) postulated that high levels of glutathione corresponded with elevated levels of antioxidant enzymes while in turn lowering the expression levels of malondialdehyde and nitric oxide in rats, thus showing antioxidant activity of *Portulaca oleracea*. *P. oleracea* has also been proven to contain vitamin C and beta-carotene which have antioxidant activity and therefore the ability to neutralize free radicals, and so the potential to prevent cardiovascular diseases and cancer related illnesses (Rifici and Khachadurian, 1993). Beta-carotene content is said to be the highest in *Portulaca oleracea* when compared to other related plants in the same genus. According to Simopoulos *et al.* (2005), Purslane contains high amounts of oxalate which exhibits antioxidant characteristics with its leaves reported to contain 671–869mg/100 g fresh weight of oxalate. Polysaccharides from the *Portulaca oleracea* have been shown to antagonise the accumulation of free radicals and enhance the immunity of rats with ovarian cancer (Zhou *et al.*, 2015). Sulphated extracts of a water-soluble polysaccharide called POP from *Portulaca oleracea* are believed to inhibit the growth of HeLa and HepG2 cells *in vitro* (Chen *et al.*, 2010b). This, therefore, suggests that when POP is sulphated, its cytotoxicity increases in tumour cells. *Portulaca oleracea* also produces cerebrosides, homoisoflavonoids and alkaloids which have shown cytotoxic activity towards cultures of human cancer cell lines like A-549 and HepG2 (Al-Sheddi *et al.*, 2015). According to Zheng *et al.* (2014), Portulacocerebroside A (PCA), a polysaccharide from *Portulaca oleracea*, can stimulate the death of the human liver cancer line HCCLM3 by activating the p38MAPK and the JNK-triggered mitochondrial death pathways, resulting in the developing tumour subsiding. The activation of the p38MAPK and JNK pathways maybe a target for therapeutic purposes. These pathways are activated in response to inflammatory and tumour stimuli, cytokines, UV radiation and osmotic stress (Zheng *et al.*, 2014).



Figure 5: A growing *Portulaca oleracea* plant. (Image adapted from the article; Comparative study of flavones content in different extracts obtained from *Portulaca oleracea*)

A growing *Portulaca oleracea* whole plant has reddish branches and stems. It can grow up to 30 cm tall and has alternate leaves, most of which are packed towards the ends of the branches.

The leaves are oval to elliptic in shape, attached to the branches by their bases with no stock. The leaves are very succulent

2.5.1.2 *Tribulus terrestris* L.

Tribulus terrestris (Tswana name; Tshetlho/Mosetlho) is a small, silky and hairy herb which is indigenous to tropical regions of Southern Europe, south and Western Asia, Africa and Australia. It is a member of the Zygophyllaceae family (Hashim *et al.*, 2014). *Tribulus terrestris* is regarded as a troublesome weed because of its thorny fruits which are a hazard to grazing animals and humans. The phytochemistry of *T. terrestris* has been well studied and documented by various researchers like Kucuk *et al.* (2017) and Su *et al.* (2009), with many being able to identify saponins, alkaloids, flavonoids, various nutrients, glycosides and phytosteroids which are of pharmacological significance (Abbas, 2022). The contents of such phytoconstituents vary depending on the explant used, with fruits and roots reported to contain the highest amounts (Sharma and Kumar, 2020).

T. terrestris has been used for thousands of years in folk medicine to treat different diseases like heart problems, skin disorders, kidney stones and diuretic conditions especially in China and India (Sharma and Kumar, 2020). According to Hashim *et al.* (2014), *T. terrestris* has been used to treat sexual disorders and as an aphrodisiac. It has also been used to treat cardiac diseases as its saponins are able to help dilate coronary arteries thus helping in blood circulation. It is also believed to have antimicrobial and anthelmintic activities. Bedir and Khan (2000) reported that spirostanol glycosides extracted from *T. terrestris* are able to inhibit the growth of SKMEL, KB, BT-549 and SKOV-3 cancer cell lines. Furthermore, extracts from this plant such as dioscin, prosapogenin A and protodiocin have shown further anti-cancer activities on different cancer cell lines and some have been shown to be cytotoxic to cell lines from leukemia, solid tumours, colon cancer, renal cancer, and melanoma cancer cell lines (Hu and Yao, 2002).



Figure 6: Fresh *Tribulus terrestris* plant. (Images adapted from Flora of Botswana database)

Tribulus terrestris whole plant is covered with long snowy hairs and can grow up to 60 cm tall. Most of its branches creep on the ground. It has compound leaves, with ovoid leaflets that are arranged equally on opposite sides of the central stalk in pairs of five to eight pairs.

2.5.1.3 *Elephantorrhiza elephantina* (Burch.) Skeels

Elephantorrhiza elephantina known as Elephant root (Tswana name: Mositsane) is a plant of African origin. Together with its relatives in the genus *Elephantorrhiza*, *E. elephantina* derives its name from the name elephant root, which implies that it has a large underground stem. It is a very important plant especially in Southern Africa because of its supposed medicinal abilities and its tuberous rhizome stem which is consumed as food (Maroyi, 2017a). *Elephantorrhiza elephantina* is commonly sold and used as herbal medicine in markets across Southern African countries like Zimbabwe, Botswana and South Africa.

Medicinal properties of *Elephantorrhiza elephantina* include antibacterial, antifungal, antiplasmodial and antioxidant activities (Olaokun *et al.*, 2020). The antioxidant properties of *Elephantorrhiza elephantina* have been harnessed by the use of the plant in herbal medicine in Lesotho to treat breast cancer (Maroyi, 2017b). Flavonoids, ethyl gallate and phenolic compounds are some of the phytochemicals that have been extracted from *Elephantorrhiza elephantina* (Mpofu *et al.*, 2014) and they are closely linked with the antioxidant activity of this plant. Ethyl gallate has been specifically shown to be a free radical scavenger (Maroyi, 2017a) which is an important feature in antagonizing the growth and development of cancer cells resulting mainly from free radicals.

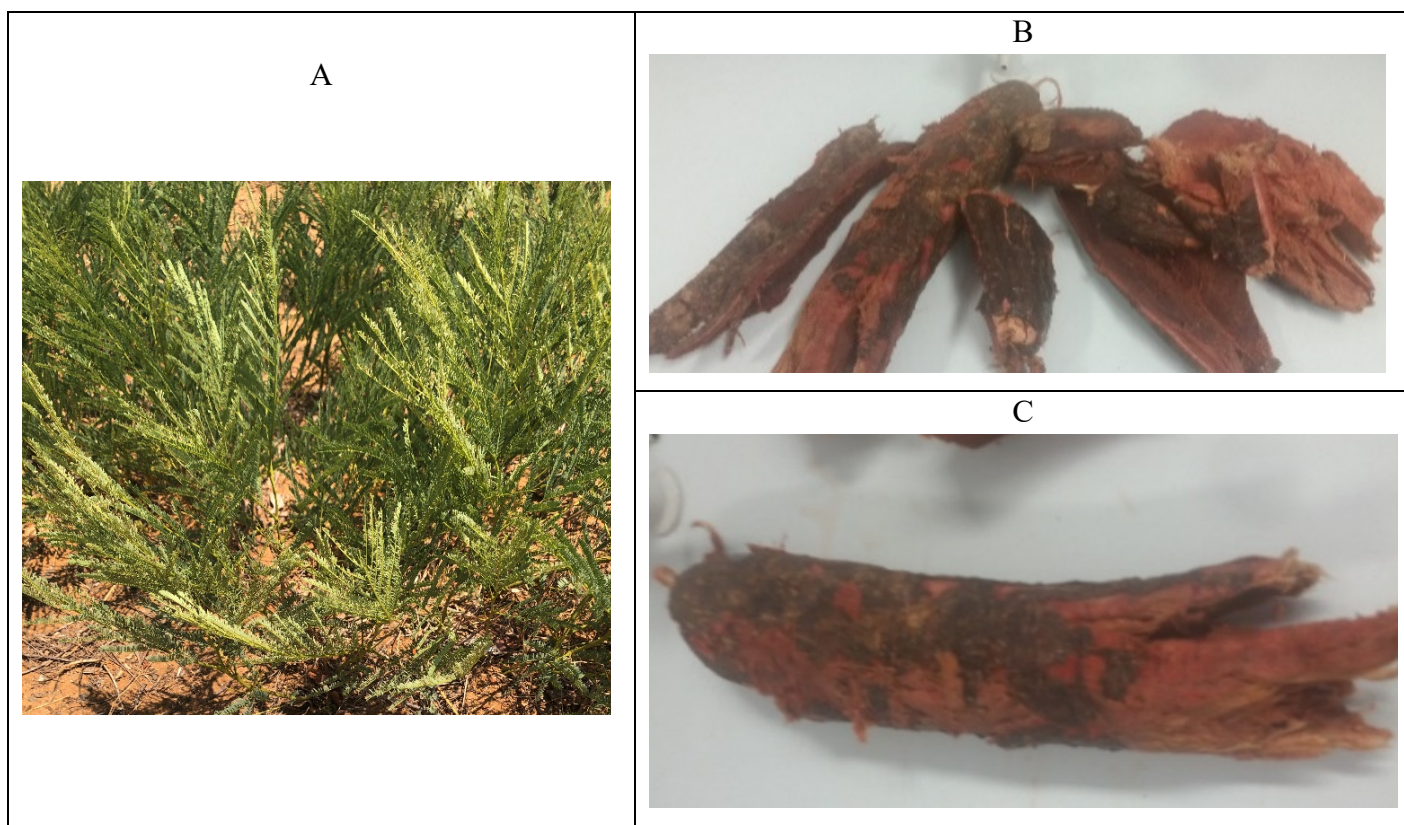


Figure 7: *Elephantorrhiza elephantina* plant in the wild (A) before it was harvested and its tubers (B and C) after harvesting.

Elephantorrhiza elephantina grows in the wild. Underneath the ground, it has a tuberous root network. The root network form extensions which result in the emergence of new plant populations as it keeps spreading and growing underground. The stems of this plant can grow up to 60 cm above the ground. They grow annually during rainy seasons and dry off and die during dry seasons. The leaves are arranged in a compound manner and are gathered around the high areas of the stem and branches.

2.5.1.4 *Azanza garckeana* (F.Hoffm.) Exell & Hillc

Azanza garckeana or Snot apple (Tswana name; Morojwa) is a semi-deciduous tree that is commonly found in East and Southern Africa. It can grow between 3-15 meters tall depending on the environment it grows in. *A. garckeana* is a member of the Malvaceae family with alternate, simple, round and 2-5 lobed leaves (Maroyi, 2017a). It can have several varying stems with hairy twigs when young which smoothens as it ages. The leaves of *A. garckeana* are hairy and can have up to five lobes. Its fruits are also hairy with a diameter of up to 4 cm and a woody spherical shape (Mojeremane and Tshwenyane, 2004).

In a study conducted by Michael et al. (2015), they evaluated phytochemicals from the seeds of *A. garckeana* and discovered that they have tannins, saponins, flavonoids, alkaloids,

phenols, glycosides and carotenoids. The benzopyrone ring structure, which is known for its antioxidant properties, can be found in flavonoids thus making the flavonoids to have antioxidant behaviour (Elshiekh and Ali, 2020). The antioxidant capacity of flavonoids gives them the ability to fight and remove free radicals in biological systems hence the ability to alleviate the development of tumorous cells (Michael *et al.*, 2015). The seeds of *A. garckeana* are said to be able to reduce the chances of cancer development as they have flavonoids, which are able to interfere with oestrogen synthase, an enzyme that produces oestrogen by inhibiting the oestrogen synthase (Okwu, 2005). A complex formed by Mansone G and β -Cyclodextrin, phytochemicals from *A. garckeana*, was found to exhibit high levels of cytotoxicity towards A549 lung cancer cells (Bioltif *et al.*, 2020).

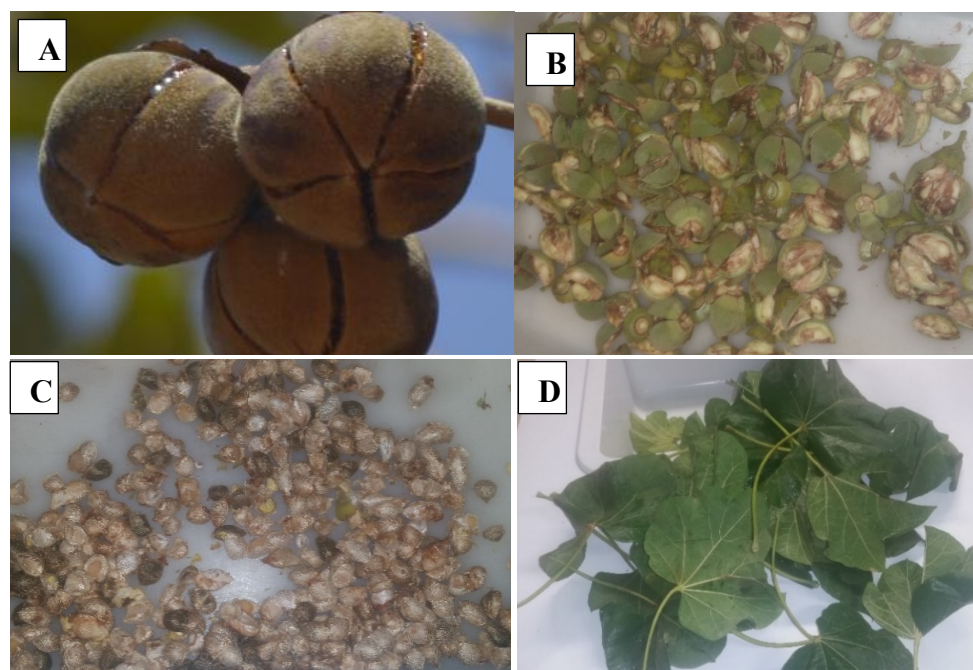


Figure 8: *Azanza garckeana* ripe fruits hanging from the branch (A), its unripe fruits after harvesting (B), its seeds separated from fruits (C) and its fresh leaves (D)

A. garckeana fruits can be spheroidal to ellipsoidal in shape, with subsections which make up to 5 sections. The leaves of *A. garckeana* are palmately shaped leaves, with heart-shaped bases which can have 3 to 5 lobes.

2.5.1.5 *Withania somnifera* (L.) Dunal

Withania somnifera, also called Winter cherry (Tswana name; Modikasope) is a small, bushy, evergreen shrub, which, on average, grows up to 2 m tall. Its bushy branches and stems cover the width of approximately 1 m. This plant is widely distributed across the world but is highly abundant in South Africa and Botswana (Welman, 2011). *W. somnifera* is a member of the Solanaceae family. It has a brownish stem and alternating leaves which are hairless on top with dense hairy features underneath (Gaurav *et al.*, 2015). It produces clusters of bright yellow or greenish flowers all year round; October to July in most regions of Southern Africa (Welman, 2011). The highest blossom is between March and July (Mirjalili *et al.*, 2009). *W. somnifera* has been found to prefer dry and warm regions with rocky landscape in order to keep its roots

cool (Gaurav *et al.*, 2015). In Botswana, it is abundant in the Tswapong region of the country (Setshogo and Mbereki, 2011).

There are many secondary metabolites present in *Withania somnifera* such as alkaloids and steroids. The anti-inflammatory, cytotoxic, antibiotic and anti-tumour activities of these compounds, mostly extracted from roots and leaves, have been reported in many studies (Welman, 2011), (Kim *et al.*, 2020), (Nema and Khare, 2020). Therefore, *Withania somnifera* plays a very crucial role in African traditional medicine. Its leaves have been used traditionally in Southern African countries like Botswana to treat septic wounds, abscess and inflammation (Welman, 2011). Abscesses and inflammation can be symptomatic of cancer as they can form as an extension from a tumour region (Hejmadi, 2010). Withanolides and withaferins are some of the active compounds present in *Withania somnifera* which have been found to be cytotoxic to tumour cells through their action on various pathways involved with cancer cells like the p53 pathway and the PTEN pathway (Rai *et al.*, 2016). *W. somnifera* aids in the alleviation of cancer by inhibiting pathways that lead to the metastasis of tumours and counteracting crucial mechanisms that cancer depends on like evading apoptosis, the ability to replicate continuously and cell proliferation (vel Szic *et al.*, 2014). It has been shown that root extracts of *W. somnifera* can inhibit vimentin, a protein normally found in regions of metastasis, thus, this suggests its counteractive effects on tumour formation in breast cancer (Yang *et al.*, 2013). Suppression of prostate cancer has been shown by the action of *W. somnifera* active compounds deactivating Cdc2, resulting in the subsequent apoptosis of prostate tumour cells (Roy *et al.*, 2013). *W. somnifera* has the ability to repair the damage caused by oxidative stress imposed by tumour cells therefore causing a reduction in inflammation which forms the basis of its anti-tumour activities (Rai *et al.*, 2016).

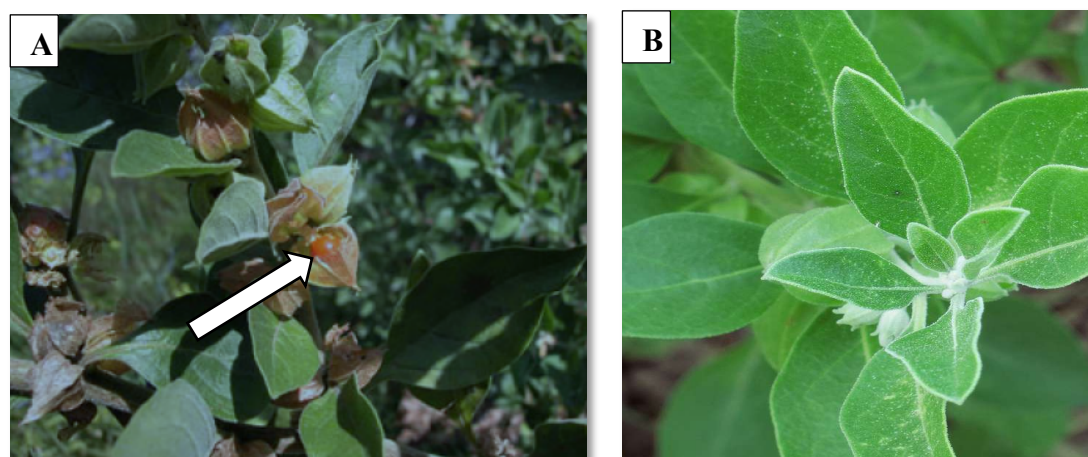


Figure 9: *Withania somnifera* orange fruits (indicated by the arrow) (A) and its leaves (B)

Withania somnifera has orange fruits and its leaves are in close proximity and usually pale green in colour. The leaves are arranged in an elliptical manner around the branches. The branches, which are covered with thick woolly hairs, emerge from the central stem in a spiral fashion.

CHAPTER 3: MATERIALS AND METHODS

3.1 Sampling

All experimental work in this study was conducted at the Botswana International University of Science and Technology in the Department of Biological Sciences and Biotechnology. The preparation of plant samples which included drying at room temperature on the laboratory bench, crushing samples into powder and packaging for storage was carried out in the Plant Biotechnology Research laboratory while the extraction of plant samples was carried out in the general laboratory. Prepared samples were stored at 4°C in the cold room located in the microbiology laboratory. Culturing of HeLa cells and all cell viability assays were carried out in the Cell and Tissue Culture laboratory. Plant samples were collected from different regions of Botswana. Some locations were recommended by traditional healers who were consulted during surveys. Palapye and Lecheng areas in the Central district and Kumakwane and Monwane villages in the Kweneng district of Botswana were the principal areas where samples were collected.

3.2 Rationale for use of survey questionnaire, study sites selection, identification, and collection of plant samples

Traditional healers were consulted on plants that have been and are used traditionally to alleviate some symptoms of cancer. A survey method was used as it was one of the best possible method that could be used to gather information from people who had little to no scientific knowledge, yet the knowledge they have, basic and primitive as it may seem, may form a basis for scientific research. Traditional healers do help in treating certain diseases and symptoms from their folkloric knowledge, but they cannot integrate and interpret such knowledge in modern scientific ways. The current research to some extent tried to integrate traditional knowledge into scientific research,. It is for these reasons that a survey method to gather basic information was used.

The questionnaire was oral survey and it was decided on from the start because there was always going to be a possibility of some of the target respondents not being literate, thus not being able to read and write. Therefore the questionnaire had to be administered orally so that it could be explained and interpreted to the respondents to ensure the information given would be relevant for the research. The consulted traditional healers were affiliated with the Botswana Dingaka Association, a governing body for traditional medicinal practitioners in Botswana. This was proven by show of their membership cards as subscribing members of the governing body of traditional healers. A consent was obtained in the form of oral consent. It is worth noting that the traditional healers who were approached were eager and willing to help and were made aware of consent and asked if they needed it in writing, but they had no problem and consented orally. Some who were not eager to take part were not coerced into taking part, but were rather left alone. They were also made aware that the information obtained from them would not in any way jeopardize them and their trade anyhow. They were also made aware that the results obtained from their help would be shared with them once the research is completed. Their full identity was kept confidential and there were no recordings except the anonymous written responses. The responses were all summarized and such data was the one presented,

not specific response of any healer. Any mention of any healer in other sections of the thesis was not associated with specific information they provided.

The consulted traditional healers were from Mmakanke settlements, Gamodubu village and the outskirts of Monwane settlement, all in the Kweneng district. Others were located in Mmathubudukwane village in the Kgatleng region while others were in Lecheng in the Central district of Botswana (Figure 10). The traditional healers were referred by fellow researchers and supervisors. Traditional healers are well known within their communities, therefore approaching them was not a problem as they were always accepting clients who knew about them through various channels. Identities of the traditional healers were kept confidential for protection of their privacy. The traditional practitioners were asked a series of questions using questionnaires, which outlined the symptoms of cancer and how they, the traditional healers, alleviate such symptoms using traditional medicinal plants. The information provided by the traditional healers advised the selection of plant collection sites. Some of the plants were found growing in the vicinity of their localities, making it easy to collect the plant samples. The initial identification of the plants was done using a taxonomy book by Setshogo (2005). The identities of collected plant species were verified by the Botswana National Museum and Monuments Herbarium Unit. The plants and explants were given collector codes as follows: 001BRAG.F-BIUST (*Azanza garckeana* fruits); 001BRAG.L-BIUST (*Azanza garckeana* leaves); 002BREE.P-BIUST (*Elephantorrhiza elephantina* plant); 003BRPO.P-BIUST (*Portulaca oleracea* plant); 004BRTT.P-BIUST (*Tribulus terrestris* plant) and 005BRWS.P-BIUST (*Withania somnifera* plant). Five plants and plant explants were used in this study. These were; *Portulaca oleracea* leaves and branches, *Tribulus terrestris* leaves and branches collected at the following coordinates: 24°39'17.1"S; 25°42'31.3"E. *Elephantorrhiza elephantina* tuberous roots (24°19'05.1"S; 25°12'59.0"E) collected at Monwane village and *Azanza garckeana* fruits (22°35'40.7"S; 27°07'26.2"E) were also used in this study as were *Withania somnifera* leaves (22°39'32.3"S; 27°14'13.1"E) which were collected at Lecheng village.

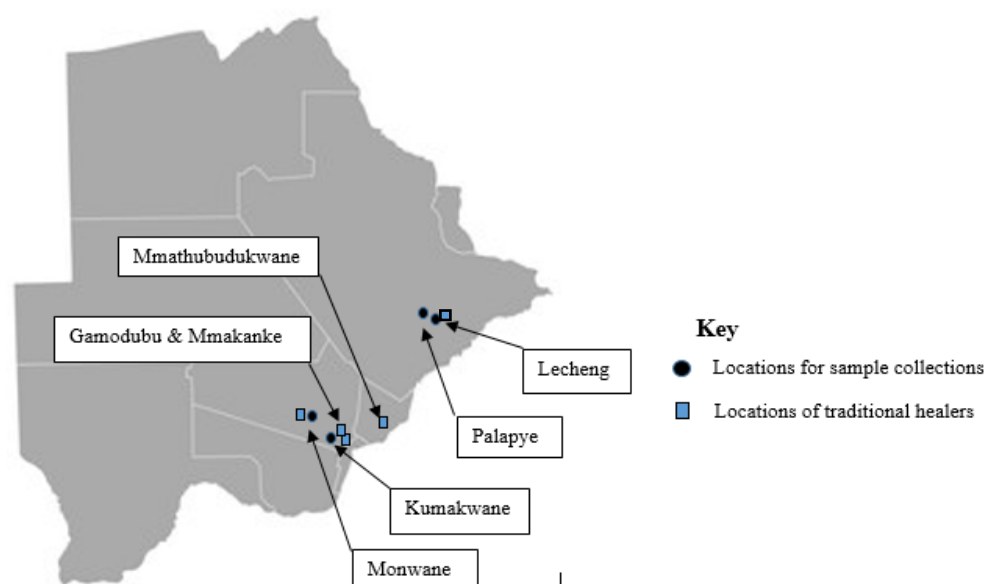


Figure 10: Map showing locations of traditional healers and sample collection locations.

3.3 Extraction of phytochemicals

Water, ethanol and methanol, which are all polar solvents, were used to extract phytochemicals from collected plant samples. Sonication was selected for the extraction of crude phytochemicals because it has been used successfully in the past (Palai and Shekhawat, 2022; Suprayitno and Yanuhar, 2019) and is quicker as the extraction process for each sample takes about 2 hours when compared to other methods like maceration. Sonication uses heat, which was monitored not to exceed 40°C because it could potentially destroy heat sensitive phytochemicals. The use of heat and ultrasound in breaking open the cells during extraction increases the chances of extracting higher quantities of phytochemicals from the explants. Moderate heat improves the extraction efficiency and aid the dissolution of phytochemicals in the solvents and improves solvent viscosity (Zabot *et al.*, 2021). Water, ethanol and methanol are all polar solvents and were the only ones used in the extraction as literature has shown that they were frequently used for cytotoxicity assays of plant species being investigated and other plant species. Water as well is commonly used by traditional healers in extraction of crude metabolites they administer to their patients.

A research permit was not acquired because all the plants used in the research are not endangered hence not protected by "National Plant Protection Organisation" (NPPO) of Botswana. As can be noted on the amounts of specimen used (10g for each extraction), the plants were not collected in large amounts which would have warranted the acquisition of a research permit.

3.3.1 Sonication, filtration and drying

Ten grams (10 g) of each plant sample was transferred into a 250 ml conical flask and 150 ml of either water, ethanol or methanol was added to the flask. The procedure was repeated for all plant samples being *Elephantorrhiza elephantina* tubers, *Azanza garckeana* fruits, *Withania somnifera* leaves, *Tribulus terrestris* leaves and branches and *Portulaca oleracea* leaves and branches. For the sake of eventually comparing the plant species amongst themselves, the same amount in mass of explants were extracted. Phytochemicals from the plant samples were crudely extracted through sonication using a Scientech-Ultrasonic cleaner sonicator (Cape Town, South-Africa) for 1 hour at 40 kHz and 40°C following the manufacturer's instructions. The contents of the conical flask were then filtered using a 150 mm diameter Whatman filter paper (Merck Sigma Aldrich, USA) and funnelled into pre-weighed and pre-labelled beakers according to the plant species and the solvent used for extraction. The filtration process was carried out in the CHC Lab International fume hood (Madrid, Spain). The plant sample residues left on filter papers after filtration were scraped back into the original conical flasks which had some of sample residues and this time 100 ml of the same solvent was added to the contents of the flask and sonicated for another 1 hour. Contents were then filtered again using fresh 150 mm diameter Whatman filter papers into same beakers.

The solvents evaporated at room temperature in their respective beakers in the CHC Lab International fume hood. Drying of the water extracts was aided by placing beakers containing extracts on a BT Lab Systems hot plate (USA) set at 50°C. The reason for aiding the evaporation of water from the water extracts is because, unlike methanol and ethanol, water

has a higher boiling point and would therefore evaporate slower than ethanol and methanol from the extracts. The longer the extracts are in a solvent during drying, the more chances of contamination from the surrounding air as the evaporation environment was not maintained sterile.

3.3.2 Resuspension with growth medium

After the drying of extracts in beakers, the beakers were weighed again and their masses recorded. The differences in the masses of beakers without the filtrates and with filtrates were calculated in order to get the actual mass of filtrates (Yield). The filtrates were suspended in high glucose DMEM (Dulbecco's Modified Eagle's Medium) (Merck Sigma Aldrich, USA) which comprises of 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate (Table D1). The stock solution concentrations were determined based on the mass of filtrates. *Elephantorrhiza elephantina* tubers stock solution concentration for water, ethanol and methanol was 64.16mg/mL while *Azanza garckeana* fruits stock solution concentration for water, ethanol and methanol was 12.88 mg/mL. *Withania somnifera* leaves stock solution concentration for water, ethanol and methanol was 48mg/mL and *Tribulus terrestris* leaves and branches stock solution concentration for water, ethanol and methanol was 27.62mg/ml while *Portulaca oleracea* stock solution concentration for water, ethanol and methanol was 28.36 mg/mL. In order to get enough volumes for all the planned assays, all filtrates were dissolved/re-suspended in 25ml of the growth media (DMEM). Resuspension was carried out by dissolving extracts in the determined volume of media and storing them in glass vials. The plant extracts were filter sterilized with 0.25 µm and 0.4 µm (Merck Millipore LTD Millex-HA, Germany) filter sterilizers. The filtered plant extracts were then transferred into sterile storage vials which were wrapped with aluminium foil and kept in the dark at 4°C while HeLa cell culture was undertaken. Then, the 0.4 µm filter was used first for each plant extract followed by the 0.25 µm filter. The difference in sizes of the filters determine the size of particles they can filter out, thus removing possible contaminants from contaminating the extracts and eventually the cell cultures.

3.4 Culture of HeLa cells

HeLa cells in 1 mL cryopreservation vials were retrieved from liquid nitrogen storage tank where they had been stored at passage 3 and provided by fellow researchers and quickly thawed in a 37°C water bath. The cells were cultured in DMEM supplemented with; 10% Foetal Bovine Serum (FBS) which aids the attachment of cells, and gives the cells the necessary hormones and nutrients. DMEM also contained 0.2% sodium bicarbonate which supply carbonate ions needed for metabolic functions of the cells and penicillin and streptomycin (PenStrep) to reduce microbial growth in culture. Cells were grown in a 5% CO₂ incubator (Thermo Fisher Scientific, Finland) at 37°C. The cells were cultured in T-25 flasks and passaged and split 3 times until they reached 90% confluency. After the cells had attached to the cell culture plates, cell culture media was aseptically aspirated in the biosafety cabinet and the cells were washed two times with Phosphate buffer solution (PBS) (Merck Sigma-Aldrich). The cells were then detached using 0.25% Trypsin EDTA (Sigma-Aldrich Life Science). Two (2ml) of trypsin was added to the cells in the T-25 flask and incubated for two minutes.

Detachment of the cells was confirmed under an inverted microscope then 8ml of DMEM was added to the flask to inactivate trypsin. The cell suspension was then aspirated from the flask and transferred to a fresh 15 ml tube and centrifuged at 200xg for 5 minutes at 4°C in a Thermo Fisher Scientific SL-16R centrifuge, USA. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1 ml of DMEM growth medium and then gently vortexed using the Lasec-Laboratory Solution Provider (South Africa) vortexer to disperse the cells and break the pellet. The cells were counted using a hemacytometer (Thermo Fisher Scientific, USA) to determine the number of viable cells in the 1 ml cell suspension. The trypan blue exclusion assay was used in this sense to ensure cells being passaged for subsequent experiments were in higher percentage. The cells were maintained by changing their growth medium every 3 to days and were passaged after attaining at least 70% confluency.

3.5 HeLa cell morphology analysis

Cells were cultured in 6-well plates at concentration of 5×10^6 cells/mL, allowed to attach to bottom of plates and reached at least 95% confluency. Analysis of the morphology of HeLa cells was observed at 0 hours and 24 hours after treatment with the ethanol, methanol and aqueous extracts of *Azanza garckeana* fruits, *Elephantorrhiza elephantina* tubers, *Portulaca oleraceae* leaves and branches, *Tribulus terrestris* leaves and branches and *Withania somnifera* leaves using the Leica DMIL- LED inverted light microscope. The procedure was repeated three times (technical repeats) for the three extracts (ethanol, methanol and water) of *Azanza garckeana* fruits, *Elephantorrhiza elephantina* tubers, *Portulaca oleraceae*, *Tribulus terrestris* leaves and *Withania somnifera* leaves.

3.6 Treatment of HeLa cells with extracts, positive control, and negative control

The stock concentrations of the different explants were 64.16 mg/mL for *Elephantorrhiza elephantina* water, ethanol and methanol extracts, 12.88 mg/mL for *Azanza garckeana* water, ethanol and methanol extracts, 48 mg/mL for *Withania somnifera* water, ethanol and methanol extracts, 27.62 mg/ml for *Tribulus terrestris* water, ethanol and methanol extracts and 28.36 mg/mL *Portulaca oleracea* for water, ethanol and methanol extracts. The stock concentrations were serially diluted five times at a ratio of 1:2. The different concentrations of plant extracts were applied on HeLa cells at 95% confluency and then incubated for 24 hours. To ensure that the HeLa cells were at 95% or more confluency, when the cells were passaged for the fifth time (P5), cells were cultured at a higher concentration of 5×10^6 cells/mL. The stock solutions were prepared from the suspensions of the plant extracts, which had varying concentrations. The stock solutions were such that the total volume of each specific extract was 25 ml because this volume was enough for all the undertaken experiments such as cell viability assays, cell morphology analysis and gene expression analysis. Since each plant was extracted with water, ethanol and methanol, the working concentrations were all standardized. The process was monitored and observed on a 12 hourly interval from the time of application of plant extracts and images were taken at time of application (0 hours) and after 24 hours. Stock solution concentrations were determining using the formula below.

$$\text{Concentration} = \frac{\text{Mass of Crude extracts}}{25\text{mL}}$$

Where: Concentration is in mg/mL and

Mass of crude extracts is in mg

Cisplatin was used as a positive control in this study since it is a well-known anticancer agent. The stock concentration of cisplatin was prepared using nuclease free water and was 100 µg/mL. It was serially diluted down six times at a ratio of 1:2. Phosphate Buffered Saline (PBS) was used as a negative control due to its physiological stability.

3.7 HeLa cell proliferation and viability assays

HeLa cells were cultured in cell culture plates in DMEM supplemented with 10% FBS and 0.1 mg/ml of PenStrep at 37°C in 5% CO₂. Proliferation assays are used to analyse the quantity of live cells through cleaving the tetrazolium salts which had been added to the culture medium. The tetrazolium salts are broken by enzymes within the cell, resulting in formazan crystals. When the number of viable cells increase, it results in the proportional increase of the overall activity of mitochondrial dehydrogenases in the sample, subsequently leading to increase in the amount of formazan dye formed. The resultant formazan dye is then quantified using a microplate spectrophotometer.

To measure cell viability and proliferation, Water Soluble Tetrazolium salt (WST-1) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays (Roche) were used following protocols as described by the manufacturer's instructions. WST-1 is used for the non-radioactive, spectrophotometric quantification of cell proliferation, cell growth and cell viability using 96-well-plates. It can be used to quantify the cytotoxicity of compounds in anti-cancer research and drug compounds. MTT assay quantifies metabolic activities in the cell to check for proliferation, cytotoxicity and viability of the cells. It is dependent on the yellow tetrazolium salt being reduced by metabolically active cells to formazan crystals which are purple in colour. The purple formazan crystals are insoluble but are solubilized by dimethyl sulfoxide (DMSO) and the absorbance of the resulting solution is measured using a 96-well plate spectrophotometer. If the number of metabolically active cells are high, then the solution will be darker. HeLa cells were grown in DMEM and plated into a 96-well plate at a density of 1×10^4 cells/well for 24 hours at 37°C in a 5% CO₂ incubator to allow for their adherence. After incubation, the cells were treated with serially diluted concentrations of the plant extracts and incubated again for 24 hours at 37°C in a CO₂ incubator. Each extract was serially diluted five times at ratio of 1:2 and the resultant concentrations were used in the cell proliferation assays. The highest and lowest concentrations were as follows for each plant species: *Azanza garckeana*; 12.88 mg/mL and 0.805 mg/mL, *Elephantorrhiza elephantina*; 64.16 mg/mL and 4.01 mg/mL, *Portulaca oleraceae*; 28.36 mg/mL and 1.7725 mg/mL, *Tribulus terrestris*; 28.62 mg/mL and 1.726 mg/mL and *Withania somnifera*; 48 mg/mL and 3 mg/mL. The concentrations of plant species varied because the level of extraction mass differed with each plant, but the same mass (10 g) was extracted from each plant. After 24 hours of incubation of the cells in 96-well plates with plant extracts, culture media was removed and replaced with

100µl of fresh media plus 10µl of cell proliferation reagent WST-1 per well. For the MTT assays, 0.5mg/mL concentration was used; aspirating the culture media and replacing it with 50µl of fresh media plus 50µl of MTT solution. The plate was then incubated for 4 hours at 37°C and 5% CO₂ in an incubator for WST 1 and 3 hours for MTT assay in the same conditions. The plate was shaken for 1 minute in the Thermo Scientific Multiskan microplate reader (USA) and the intensity of the dissolved formazan crystals were quantified using a plate reader at 440 nm (ELISA). The reference wavelength was set at 600 nm. For WST-1 assay, the formazan crystals were water soluble, therefore did not need DMSO (dimethyl sulfoxide) to be dissolved. For the MTT assay, 100 µl of DMSO for solubilizing formazan crystals was added to each well and 550 nm wavelength was used in measurements with 680 nm wavelength as the reference wavelength. The final concentration of the MTT assay was 0.5 mg/ml.

Percentage of viable cells was then calculated using the following formula:

$$\% \text{ Cell viability} = \frac{OD \text{ of Treated cells}}{OD \text{ of Control cells}} \times 100$$

The % cell viability derived from the calculations above was then plotted against the specific concentrations to determine the CC₅₀ (half-maximal cytotoxic concentration) values for each plant extract treatment. Linear Regression Analysis was then used to calculate and determine the CC₅₀ values for each extract cytotoxicity. The assays were carried out in triplicates (technical repeats) and the experiments were repeated three times for each of the WST-1 and MTT assays.

3.8 Gene Expression Analysis

To analyse the expression cancer markers (Table 1), HeLa cells were cultured in T75 cell culture flasks in DMEM. The cells were allowed to reach 100% confluency. Five of the flasks were treated with the aqueous extracts of *Elephantorrhiza elephantina* tubers, *Azanza garckeana* fruits, *Withania somnifera* leaves, *Tribulus terrestris* leaves and branches and *Portulaca oleracea* leaves and branches. The aqueous extracts were used because of all the five plant extracts, water showed the best inhibition of cell morphology and cell proliferation assays with either the lowest or among the lowest CC₅₀ values. One flask of HeLa cells was treated with cisplatin (positive control) (CC₅₀ concentration of 36.5 µg/mL) and the other flask was treated with PBS, a negative control. The treatments were applied on day 7 after culturing of HeLa cells when the cells in all flasks had reached 100% confluency.

For each of the plant extracts, the one with the best inhibitory effects on morphology and cell viability was selected for treating HeLa cells for subsequent gene expression analysis. Therefore, for each of the five plant species, only one extract was selected, which were the aqueous extracts of *Azanza garckeana* fruits, *Elephantorrhiza elephantina* tubers, *Portulaca oleraceae* leaves and branches, *Tribulus terrestris* leaves and branches and *Withania somnifera* leaves.

3.8.1 RNA Extraction and cDNA Synthesis

HeLa cells were cultured at concentration of 5×10^6 cells/mL in cell culture plates (Thermo Scientific™ Nunc™, USA) and allowed to attach and reach 100% confluency. DMEM was aseptically aspirated and each plate of HeLa cells was treated with 5 ml of sterile CC_{50} concentrations of the aqueous extracts of the five plant extracts. Each of the five plant extracts used in this study had been extracted with water, ethanol and methanol but the water extracts of all the five plant extracts showed the best inhibition of cell morphology and cell proliferation with the lowest CC_{50} values. They were therefore selected for total RNA extraction and subsequent gene expression analysis. One of the HeLa cell culture plates was treated with about 5 ml of the CC_{50} concentration for cisplatin which was the positive control and one plate treated with about 5 ml of PBS, which was the negative control. The 7 HeLa cell culture plates were incubated for 24 hours. The 5 mL of treatment applied in each of the 7 flasks (5 aqueous plants extracts, 1 cisplatin and 1 PBS) was enough to cover the surface of the bottom of the flasks.

Total RNA was extracted using the Zymo Research, Quick-RNA™ Miniprep Plus Kit (Catalog No. R1058, USA) from HeLa cells in the 7 culture plates following the manufacturer's instructions. The total RNA was then quantified using a Nanodrop (ThermoFisher Scientific, USA) at 260 nm wavelength. Then, 2 µg of the total RNA was used to synthesize complementary DNA (cDNA) using the BioLabs_{Inc.} ProtoScript 11 First Strand cDNA Synthesis Kit (New England) according to the manufacturer's instructions. A Nanodrop spectrophotometer was used to measure the quality and quantity of the extracted RNA of HeLa cells treated with plant extracts and with the two controls (positive and negative), together with the synthesized cDNA, which was ultimately used in running the qPCR.

3.8.2 RT-qPCR

After the synthesis of complementary DNA (cDNA), quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) was performed using the Luna Universal qPCR Master Mix (New England BioLabs_{Inc.}, USA) according to the manufacturer's instructions in the Applied Biosystems 7500 Fast cyclers (Life Technologies, Singapore). Primer sequences used in this study were acquired from published literature (Table 1). The published primer sequences were used and ordered from Inqaba Biotechnology (South Africa). The qPCR was performed under the following conditions; initial denaturation at 95 °C for 60 seconds and 1 cycle, denaturation at 95 °C for 15 seconds and extension at 60 °C for 30 seconds both at 45 cycles. The melt curve was run from 60 – 95 °C. The level of expression of the target genes was normalized to the housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (Nazet *et al.*, 2019).

The list of primers in table 1 shows the specific primer sequences of genes whose expression was analysed in this study. The primer sequences were adapted from published literature and the primers were ordered from Inqaba Biotechnology, South Africa. The targeted cervical cancer molecular markers in this study were: Vascular Endothelial Growth Factor-A (VEGF), Epidermal Growth Factor Receptor (EGFR), Metastasis-Associated in Colon Cancer -1

(MACC1), Cluster of Differentiation 95 (CD95), Cytokeratin Fragment 19 (CYRA 21-1) and (Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house keeping gene which was used as a standard reference gene.

Table 1: Primer sequences adapted from NCBI database for molecular markers of cervical cancer and the house keeping gene

Gene	Forward	Reverse
VEGF-A primer pairs (Psoma <i>et al.</i> , 2020)	5'--AGGATGGGGCTGACTAGGTAA-3'	5'CCCCCTTTTCCTCCAACCTCTC-3'
	5'-GCCCATTCCTCTTTAGCCA-3	5'-AGTGAGGTTACGTGCGGACAG-3'
EGFR primer pairs (Tan <i>et al.</i> , 2017; Qi <i>et al.</i> , 2016)	5'-GACGGGCAACGGCGTATTCTCAG-3'	5'- CCCACCTTGCCTTTGTCTCCTGTC-3'
	5'-GCGTCTCTTGCCGGAATGT-3'	5'-CTTGGCTCACCTCCAGAAG-3'
MACC1 primer pairs (Li <i>et al.</i> , 2019; Tong <i>et al.</i> , 2019)	5'-TTCTTTTGATTCTCCGGTGA-3'	5'-ACT CTGATGGGCATGTGCTG-3
	5'-GTCATGTGGCTGTGGGAGAA-3'	5'-TTTCCAACAACGGGCTCACA-3'
CYFRA 21-1 primer pairs (Marrakchi <i>et al.</i> , 2008)	5'AAGCTAACCATGCAGAACCTCAAC GACCGC-3'	5'-TTATTGGAGGTCAGGAGAAGAGCC-3'
	5'-TCCCGCGACTACAGCCACTACTACAC GACC-3'	5'-CGCGACTTGATGTCCATGAGCCGCTGG TAC-3'
CD95 primer pairs (Wang <i>et al.</i> , 2015b ; (Müschén <i>et al.</i> , 1999)	5'-cactcgagCTTTCACCTTCGGAGGATTGC-3'	5'gtgaattctGACCAAGCTTTGGATTTCATT TC-3'
	5'-ATGGAAGTCTTTGATCTCTGG-3'	5'-AGATTCCTCAAATTGATCAGAG-3'
GAPDH primer pairs (Nazet <i>et al.</i> , 2019 ; Tong <i>et al.</i> , 2019)	5'-GCCCTCAACGACCACTTTG-3'	5'-CCACCACCCTGTTGCTGTAG-3'
	5'-ACAACCTTGGTATCGTGGAAGG-3'	5'-GCCATCACGCCACAGTTTC-3'

3.9 Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) by OriginPro 2018 statistical software and Microsoft Excel 2016. Values are presented as means $\pm$ standard deviation (SD). Statistical significance of difference among means was estimated at $P < 0.05$ according to one-way ANOVA and Tukey-Kramer's multiple range test. For gene expression analysis, data obtained were calculated as described by Yuan *et al* (2006). The data obtained were expressed as the ratio of the reference gene to the target gene using the standard formula: $\Delta Ct = Ct$ (test gene) $- Ct$ (GAPDH). To determine the relative expression levels, of the genes the following formula was used: $\Delta\Delta Ct = \Delta Ct$ (treated) $- \Delta Ct$ (calibrator). Therefore, the expression levels were presented as n-fold differences relative to the calibrator. Values obtained were used to plot the expression levels of apoptotic and angiogenic genes using the relative quantification ($2^{-\Delta\Delta Ct}$) (Yuan *et al.*, 2006). The cytotoxicity and gene expression assays were all done in triplicates (technical repeats).

CHAPTER 4: RESULTS

4.1 HeLa cell morphology

HeLa cells were cultured to accumulate a high number of cells for subsequent use in experiments. HeLa cells were cultured in complete DMEM and images were captured on Day 4 of culture at x20 (Figure 11a) and x40 (Figure 11b) magnifications. The cells were approximately 80% confluent. The cells had attached well to culture plates and displayed normal HeLa cell morphology and good adaptation to culture conditions.

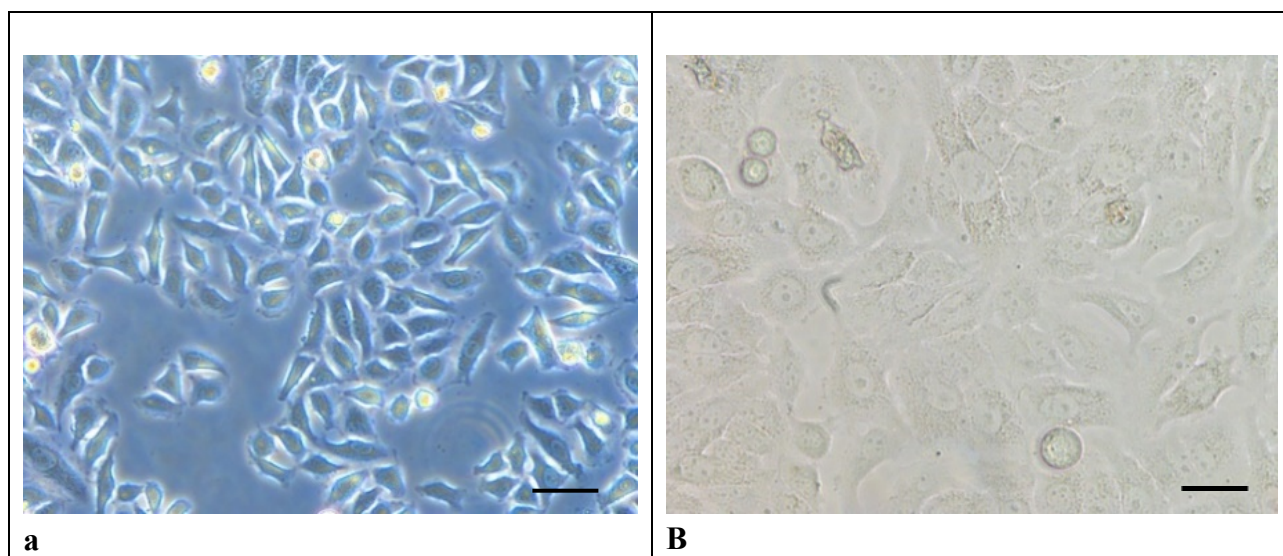


Figure 11: HeLa cells in culture at passage 3 (P3) at x20 magnification, scale bars =10 μm (a) and x40 magnification, scale bars =10 μm (b).

Cells were then treated with both the negative (PBS) and the positive (cisplatin) controls to determine their behaviour in these control substances. PBS was used as a negative control because it is a non-cytotoxic balanced salt solution which can be used in cell culture to wash cells before any passaging. Therefore, under normal conditions, it has no negative effect on cells. Cells cultured in PBS attached well in culture and displayed nearly 100% confluency as well as normal HeLa cell morphology (Figure 12a). Cells treated with cisplatin were detached from cell culture plates and were roundish in shape, dark in colour and were floating about in culture media (Figure 12b).

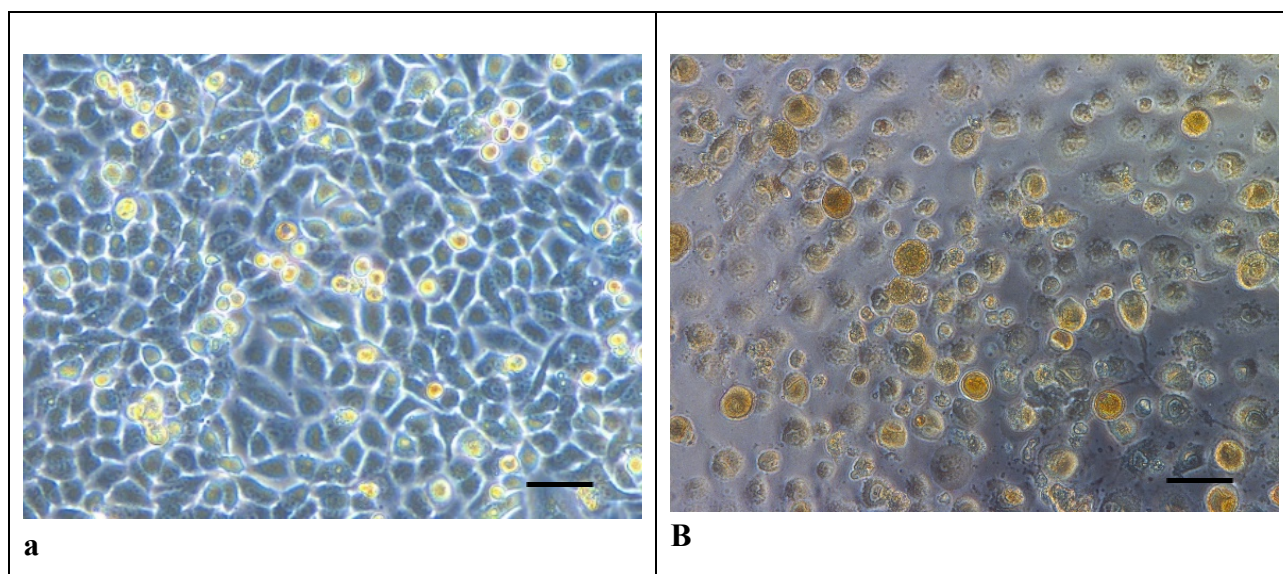


Figure 12: Control treatments of HeLa cells (P3). HeLa cells treated with PBS (negative control) (a) and HeLa cells treated with Cisplatin = 50 µg/mL (positive control) (b)

HeLa cells were plated at a concentration of 1×10^5 cells/mL and allowed to attach to the culture plates for 24 hours. They were then treated with 1.7725 mg/mL and 28.36 mg/mL concentrations of *Portulaca Oleracea* leaves and branches (POLB) ethanol, methanol and aqueous extracts. The treated cells showed no changes in morphology 0 hours post treatment with POLB extracts (Figures 13a, 13d and 13g). The cells were sparse and attached well to cell culture plates. Twenty-four (24) hours post treatment with 1.7725 mg/mL of POLB ethanol extracts, the lowest concentration, some cells began to detach from the culture plate and appeared darker (blue and red arrows) (Figure 13b). The red arrow shows an attached cell that looks lighter in colour. After 24 hours of treatment with 28.36 mg/mL of POLB ethanol extracts, the highest concentration, most of the HeLa cells appeared darker (blue circle) (Figure 13c) with uneven shapes. The cells looked blebbed and had detached and were floating in media. After 24 hours of treatment with 1.7725 mg/mL and 28.36 mg/mL of POLB methanol extracts respectively (Figures 13e and 13f), HeLa cells appeared spherical and darker and had detached from culture plates (blue arrows). After 24 hours of treatment with 1.7725 mg/mL and 28.36 mg/mL of POLB aqueous extracts respectively (Figures 13h and 13i), most of the cells appeared spherical with darker outlines (blue arrows). They had also detached from culture plates and had lost their shape, appearing blebbed with lost turgidity.

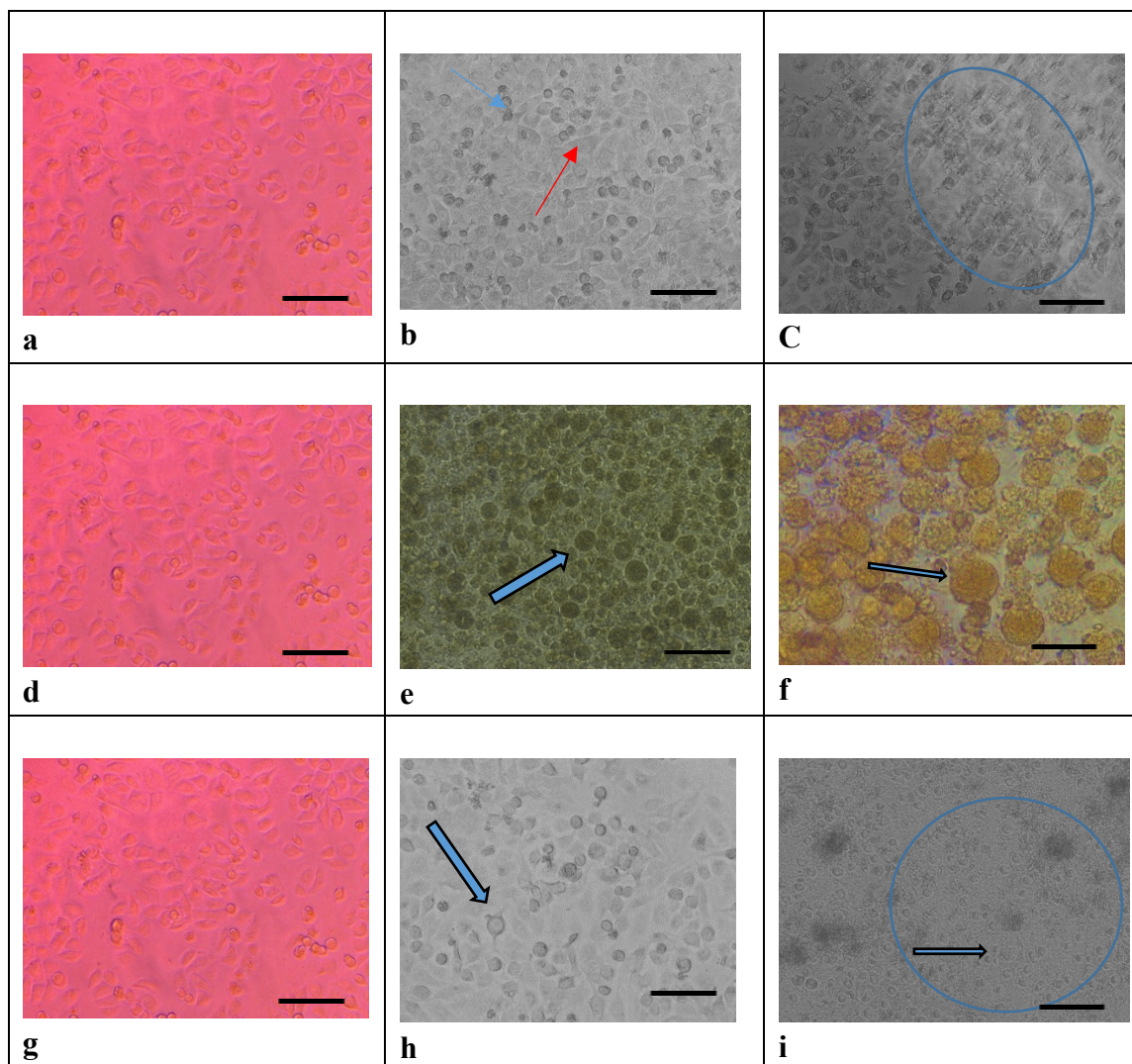


Figure 13: Morphology of HeLa cells immediately after treatment (0 hours) (a, d, g) and 24 hours after treatment (b, c, e, f, h, i) with different concentrations of extracts of *Portulaca Oleracea* leaves and branches (POLB). Cells were plated at a concentration of 1×10^5 cells/mL and allowed to attach to culture plates over a period of 24 hours and then treated with POLB ethanol, methanol, and aqueous extracts for another 24 hours: a, b, c = POLB ethanol extracts; d, e, f = POLB methanol extracts; g, h, i = POLB aqueous extracts. Images were captured at x100 magnification Scale bars =20 μ m

After 0 hours post treatment with *Azanza garckeana* fruits (AGF) ethanol, methanol, and aqueous extracts respectively, there were no changes in HeLa cell morphology (Figures 14a, 14d and 14g). The cells were globular (blue arrows), sparse and attached to the cell culture plates. After 24 hours of treatment with 0.805 mg/mL of AGF ethanol extracts, the lowest concentration, the cells appeared to have detached from the cell culture plate (black circle) but were still globular in shape but darker in colour (Figure 14b). Twenty-four hours after treatment with 12.88 mg/mL of AGF ethanol extracts, the highest concentration, some of the cells appeared to be alive and attached but media discoloration was observed (Figure 14c). HeLa cells treated for 24 hours with 0.805 mg/mL and 12.88 mg/mL of AGF methanol extracts were spherical in shape, darker in colour and detached from culture plates (black circles) (Figures 14e

and 14f). After 24 hours of treatment in 0.805 mg/mL and 12.88 mg/mL of AGF aqueous extracts, HeLa cells appeared spherical and detached from culture plates (blue arrows) (Figures 14h and 14i).

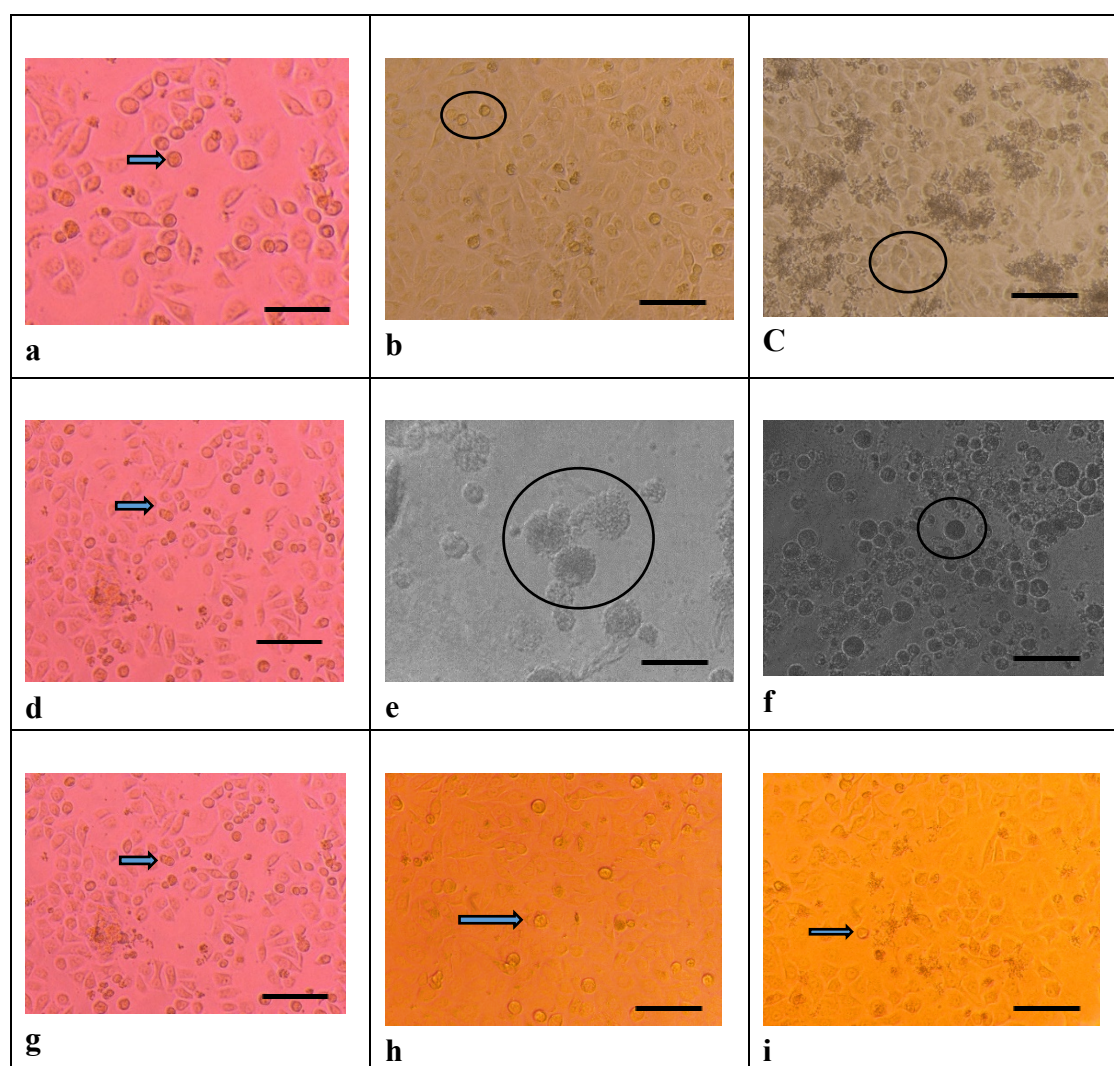


Figure 14: Morphology of HeLa cells immediately after treatment (0 hours) (a, d, g) and 24 hours after treatment (b, c, e, f, h, i) with different concentrations of extracts of *Azanza garckeana* fruits (AGF). Cells were plated at a concentration of 1×10^5 cells/mL and allowed to attach to culture plates over a period of 24 hours and then treated with AGF ethanol, methanol, and aqueous extracts for another 24 hours: a, b, c = AGF ethanol extracts; d, e, f = AGF methanol extracts; g, h, i = AGF aqueous extracts. Images were captured at x100 magnification. Scale bars = 20 μ m

Zero hours after treatment with *Elephantorrhiza elephantina* tubers (EET) ethanol, methanol and aqueous extracts, HeLa cells displayed normal morphology (Figures 15a, 15d and 15g). The cells were spread out and attached to cell culture plates. After 24 hours of treatment with 4.01 mg/mL (the lowest concentration) and 64.16 mg/mL (the highest concentration) of EET ethanol extracts respectively (Figures 15b and 15c), 4.01 mg/mL and 64.16 mg/mL of EET methanol extracts respectively (Figures 15e and 15f) and 4.01 mg/mL and 64.16 mg/mL of

EET aqueous extracts respectively (Figures 15h and 15i), cells were detached from cell culture plates and globular in shape (black circles).

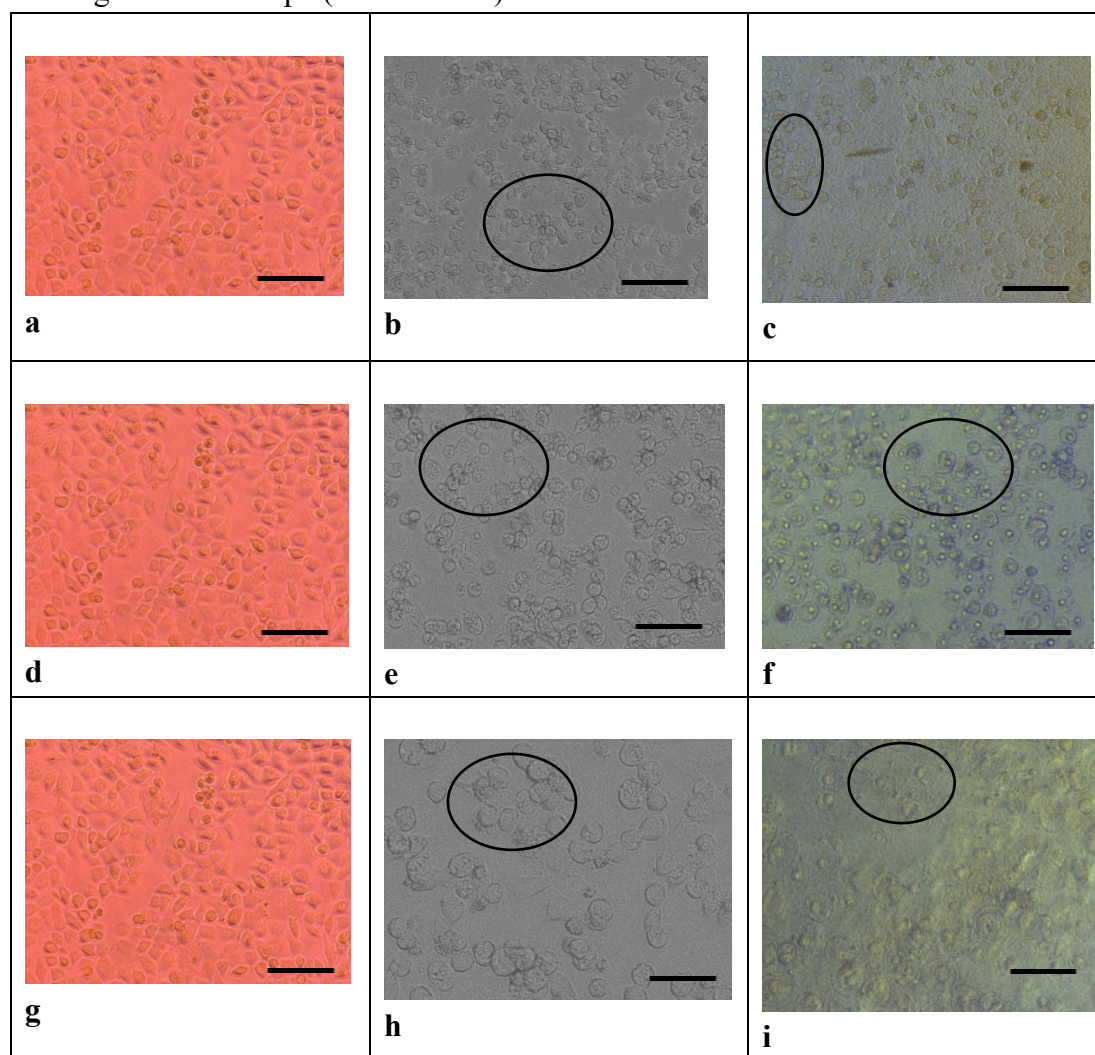


Figure 15: Morphology of HeLa cells immediately after treatment (0 hours) (a, d, g) and 24 hours after treatment (b, c, e, f, h, i) with different concentrations of extracts of *Elephantorrhiza elephantina* tubers (EET). Cells were plated at a concentration of 1×10^5 cells/mL and allowed to attach to culture plates over a period of 24 hours and then treated with EET ethanol, methanol, and aqueous extracts for another 24 hours: a, b, c = EET ethanol extracts; d, e, f = EET methanol extracts; g, h, i = EET aqueous extracts. Images were captured at x100 magnification. Scale bars = 20 μ m

After 0 hours of treatment with *Tribulus terrestris* leaves and branches (TTLB) ethanol, methanol, and aqueous extracts respectively, HeLa cells maintained normal morphology (Figures 16a, 16d and 16g) and were spread out and attached to cell culture plates. After 24 hours of treatment with 1.726 mg/mL (the lowest concentration) and 27.62 mg/mL (the highest concentration) of TTLB ethanol extracts respectively (Figures 16b and 16c), 1.726 mg/mL and 27.62 mg/mL of TTLB methanol extracts respectively (Figures 16e and 16f) and 1.726 mg/mL and 27.62 mg/mL of TTLB aqueous extracts respectively. (Figures 16h and 16i), HeLa cells were detached from cell culture plates (black circles and red arrows) and appeared globular in shape and darker in colour. Additionally, there were signs of cell death as indicated by the

shrinkage and blebbing of the cells. These observations were made for most of the cells except for cells treated with 1.726 mg/mL of TTLB methanol extracts (Figure 16e) where the cells appeared normal in culture and attached to cell culture plates.

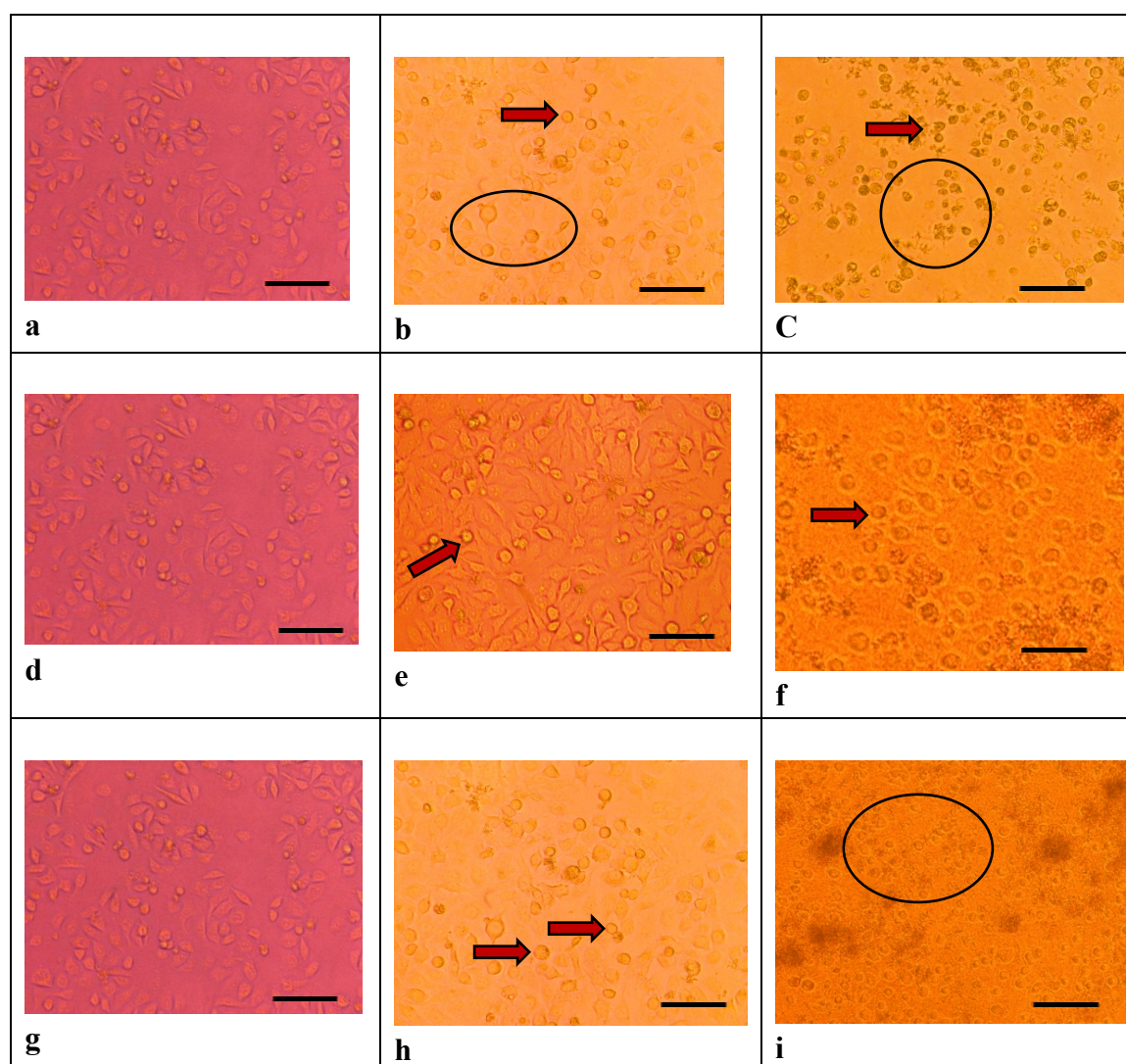


Figure 16 Morphology of HeLa cells immediately after treatment (0 hours) (a, d, g) and 24 hours after treatment (b, c, e, f, h, i) with different concentrations of extracts of *Tribulus terrestris* leaves and branches (TTLB). Cells were plated at a concentration of 1×10^5 cells/mL and allowed to attach to culture plates over a period of 24 hours and then treated with TTLB ethanol, methanol, and aqueous extracts for another 24 hours: a, b, c = TTLB ethanol extracts; d, e, f = TTLB methanol extracts; g, h, i = TTLB aqueous extracts. Images were captured at x100 magnification. Scale bars = 20 μ m

HeLa cells at 0 hours of treatment with *Withania somnifera* leaves (WSL) ethanol, methanol and aqueous extracts respectively appeared normal, were spread out and attached to culture plates (Figures 17 a, 17d and 17g). Figures 17b and 17c show cells after 24 hours of treatment with 3.0 mg/mL and 48 mg/mL of *W. somnifera* ethanol extracts respectively. Figures 17e and 17f show HeLa cells treated with 3.0 mg/mL and 48 mg/mL of *W. somnifera* methanol extracts respectively while Figures 17h and 17i show HeLa cells treated with 3.0 mg/mL and 48 mg/mL

of *W. somnifera* aqueous extracts respectively. HeLa cells treated with WSL ethanol extracts detached from culture plates and appeared globular in shape and darker in colour (red arrows) (Figures 17b and 17c). HeLa cells treated with WSL methanol extracts appeared globular in shape (black circles) (Figures 17e and 17f) while cells treated with WSL aqueous extracts were shrunk and blebbed (black circle and red arrow) (Figures 17h and 17i).

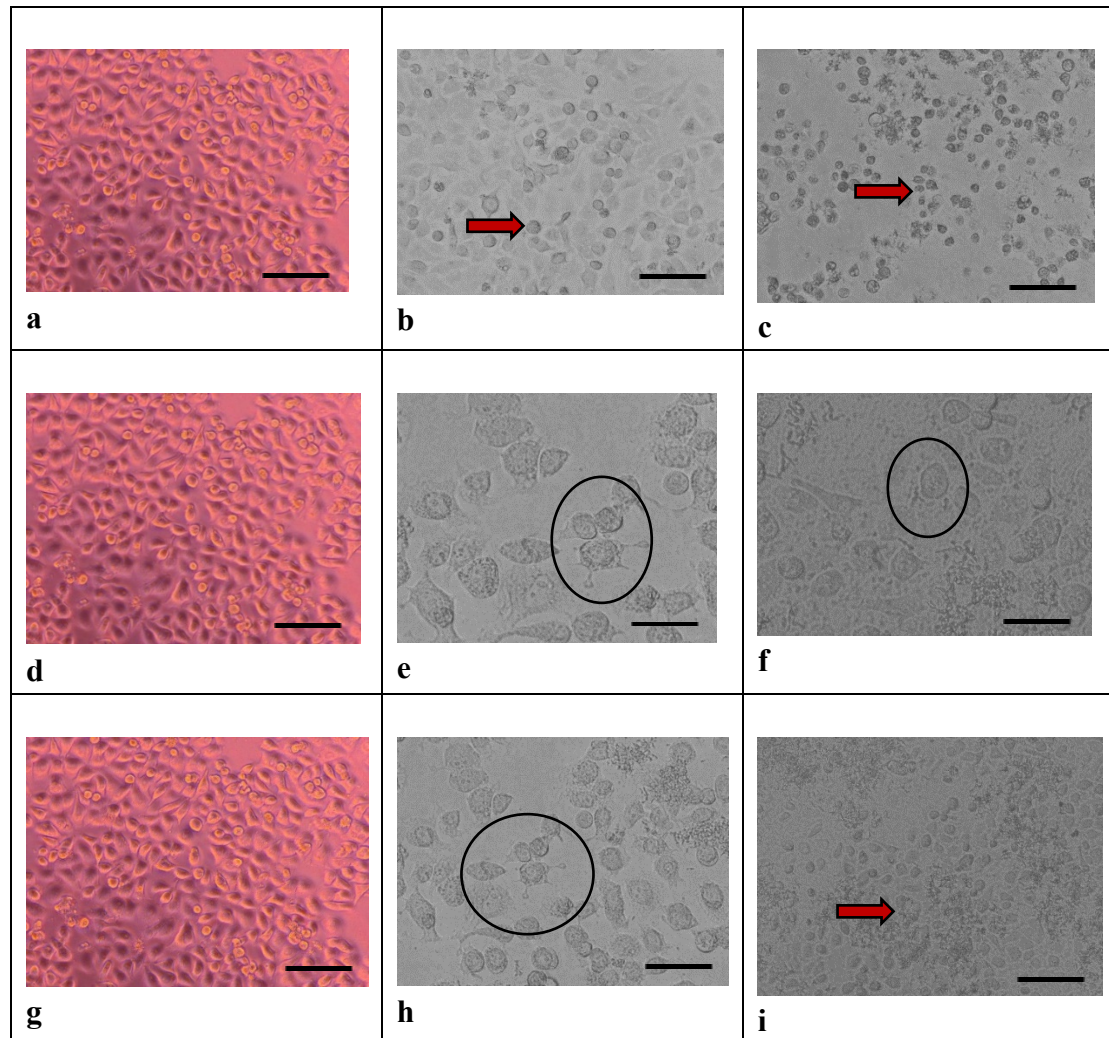


Figure 17: Morphology of HeLa cells immediately after treatment (0 hours) (a, d, g) and 24 hours after treatment (b, c, e, f, h, i) with different concentrations of extracts of *Withania somnifera* leaves (WSL). Cells were plated at a concentration of 1×10^5 cells/mL and allowed to attach to culture plates over a period of 24 hours and then treated with WSL ethanol, methanol, and aqueous extracts for another 24 hours: a, b, c = WSL ethanol extracts; d, e, f = WSL methanol extracts; g, h, i = WSL aqueous extracts. Images were captured at x100 magnification. Scale bars = 20 μ m

4.2 Cell Viability Assays

MTT and WST-1 assays were used to determine the viability of HeLa cells after treatment with aqueous, ethanol and methanol extracts from *Elephantorrhiza elephantina* tubers, *Azanza garckeana* fruits, *Withania somnifera* leaves, *Tribulus terrestris* leaves and branches and *Portulaca oleracea* leaves and branches. Cell viability was also determined in HeLa cells treated with PBS (negative control) and those treated with cisplatin (positive control).

As the concentration of cisplatin increased, HeLa cell % viability decreased (Figure 18), indicating increasing antiproliferative effects on the HeLa cells. The CC_{50} for cisplatin was found to be $36.5 \mu\text{g/mL} \pm 3.7$. The highest concentration of serial dilutions of $100 \mu\text{g/mL}$ allowed 46.6 % viability of HeLa cells while the lowest concentration of $3.125 \mu\text{g/mL}$ allowed 79.1% viability of HeLa cells. The results show that the antiproliferative effects of cisplatin on HeLa cells decreased with decreasing concentration of cisplatin.

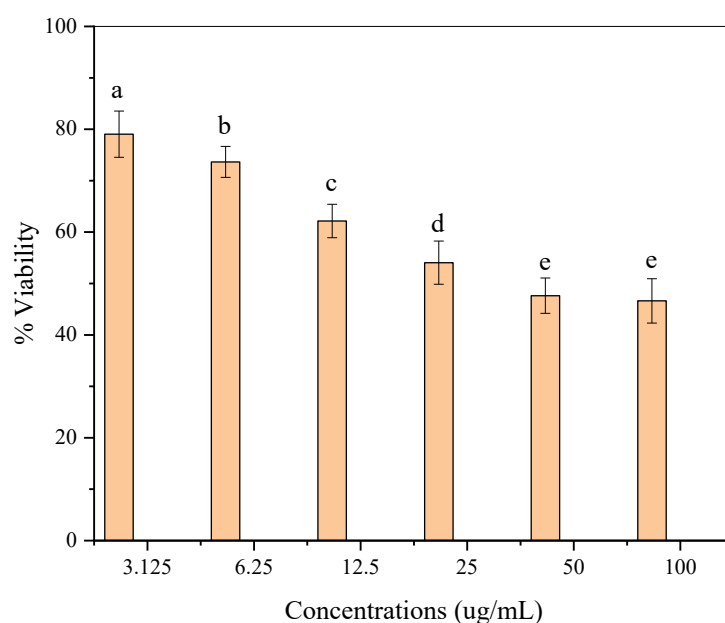


Figure 18: Cytotoxic effects of cisplatin on HeLa cells (P4) after 24 hours of treatment with a six step, 1: 2 serial dilution of cisplatin. HeLa cells had been cultured for 21 days and passaged 3 times prior to seeding on 96-well plates. Results are presented as mean ($n = 3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$)

HeLa cells treated with *Portulaca oleracea* leaves and branches methanol extracts showed 6.04% more survival rate at 28.36 mg/mL concentration than both aqueous and ethanol extracts (Figure 19). The highest survival rate difference between the three solvents was 32.13% observed at a concentration of 1.7725 mg/mL . Aqueous and ethanol extracts treatments displayed the lowest % viability of HeLa cells at a concentration of 28.36 mg/mL with no statistical differences between them and with the same CC_{50} values of 2.38 mg/mL . At a concentration of 1.7725 mg/mL , both water and ethanol extracts displayed 54.36% HeLa cell

viability (Figure 19). Across all concentrations, there were no statistical differences between the percentage viabilities displayed by *Portulaca oleracea* leaves and branches water and ethanol extracts. However, water and ethanol extracts % viabilities were significantly different from methanol extracts % viabilities across all concentrations. The viability assays were carried out based on serial dilutions of the plant extracts and the means were compared within each serial concentration but not between concentrations. Therefore each concentration was assigned its letter subscript and the means were separated by the numbers following the letters depending on whether the difference is significant or not.

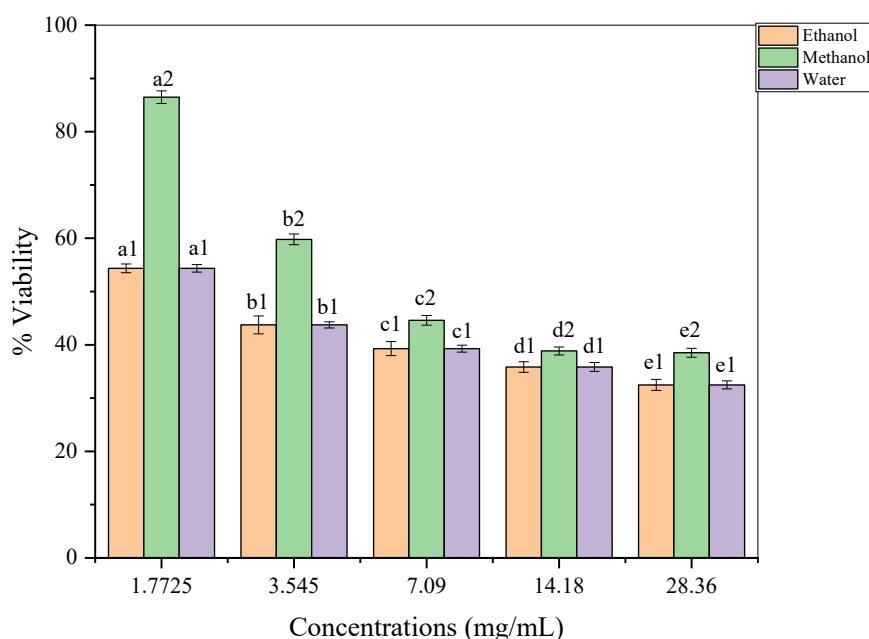


Figure 19: Cytotoxic effects on HeLa cells (P4) after 24 hours of treatment with a five step, 1: 2 serial dilution of *Portulaca oleracea* leaves and branches extracts. HeLa cells had been cultured for 21 days and passaged 3 times prior to seeding on 96-well plates. Results are presented as mean ($n = 3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$).

The mean separation letters indicate that the means were significantly different when the letters are assigned different numbers. However, if the letters have same numbers, it indicates that the means were not significantly different from each other.

Azanza garckeana water extracts showed the highest cytotoxicity on HeLa cells than both ethanol and methanol extracts across all concentrations (Figure 20). The highest % viability across all concentrations was 55.4% for *Azanza garckeana* methanol extracts at a concentration of 0.805 mg/mL while the least % viability was 35.5% for water extracts at a concentration of 12.88 mg/mL (Figure 20). Ethanol and methanol extracts treatments displayed 1.8 ± 0.84 and 1.88 ± 0.88 CC₅₀ values respectively. The CC₅₀ value of water extracts was not determined, indicating that the CC₅₀ concentration was less than the lowest concentration of 0.805 mg/mL used in the assay. None of the *A. garckeana* water extracts concentrations allowed HeLa cells to grow beyond 50% viability. Since the cell viability assay was carried out based on serial

dilutions, the means were compared within each serial concentration but not between concentrations. Therefore each concentration was assigned its letter subscript and the means were separated by the numbers following the letters depending on whether the difference is significant or not.

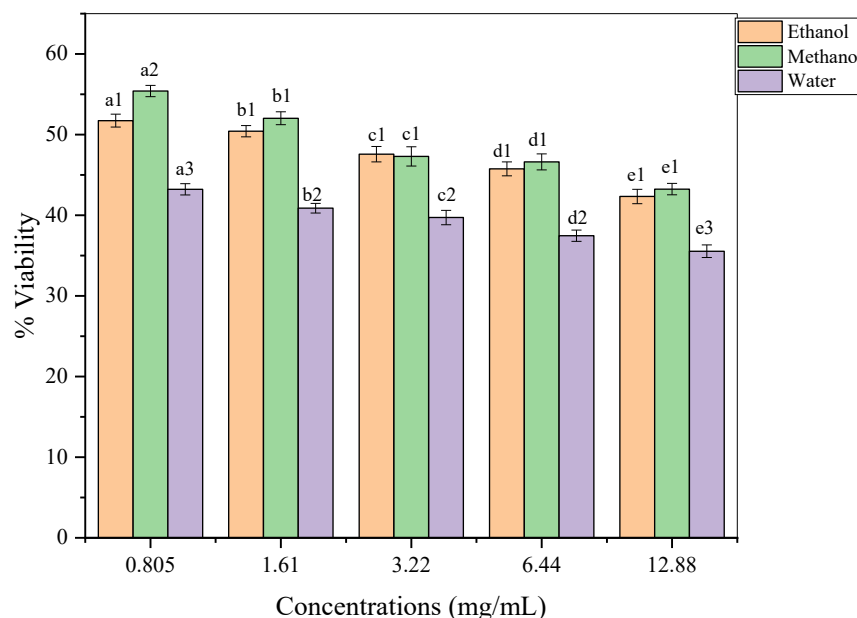


Figure 20: Cytotoxic effects on HeLa cells (P4) after 24 hours of treatment with a five step, 1: 2 serial dilution of *Azanza garckeana* fruits extracts. HeLa cells had been cultured for 21 days and passaged 3 times prior to seeding on 96-well plates. Results are presented as mean ($n = 3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$).

The mean separation letters indicate that the means were significantly different when the letters are assigned different numbers. However, if the letters have same numbers, it indicates that the means were not significantly different from each other. For 1.61, 3.22, 6.44 and 12.88 mg/mL *Azanza garckeana* fruits extracts concentrations, the means of the effects of ethanol and methanol extracts on cell survival rate were not significantly different but were both significantly different from the effects exerted by water extracts. At 0.805 mg/mL concentration, the means for the three extracts were significantly different from each other.

Elephantorrhiza elephantina tubers ethanol extracts displayed the highest HeLa cell % viabilities of 41.4, 44.1, 45.7, 49.1 and 53.6% for concentrations 64.16, 32.08, 16.04, 8.02 and 4.01 mg/mL respectively (Figure 21). These were followed by methanol extracts with % viabilities of 39.8, 42.3, 43.4, 46.3 and 51.7% at 64.16, 32.08, 16.04, 8.02 and 4.01 mg/mL concentrations respectively. *Elephantorrhiza elephantina* tubers aqueous extracts displayed the lowest % viabilities of 35.2, 39.0, 42.3, 45.6 and 50.9% at 64.16, 32.08, 16.04, 8.02 and 4.01 mg/mL concentrations respectively (Figure 21). The following CC_{50} values were observed for the different extracts; ethanol 6.9 ± 1.9 , methanol 5.6 ± 2.6 and aqueous 4.6 ± 1.2 mg/mL. The highest % viability of HeLa cells was recorded at 53.6% exerted by ethanol extracts at a concentration of 4.01 mg/mL while the least viability was recorded at 35.2% at a concentration

of 64.16 mg/mL exerted by aqueous extracts. Therefore, aqueous extracts of *Elephantorrhiza elephantina* tubers were the most cytotoxic on HeLa cells, followed by methanol and ethanol extracts respectively.

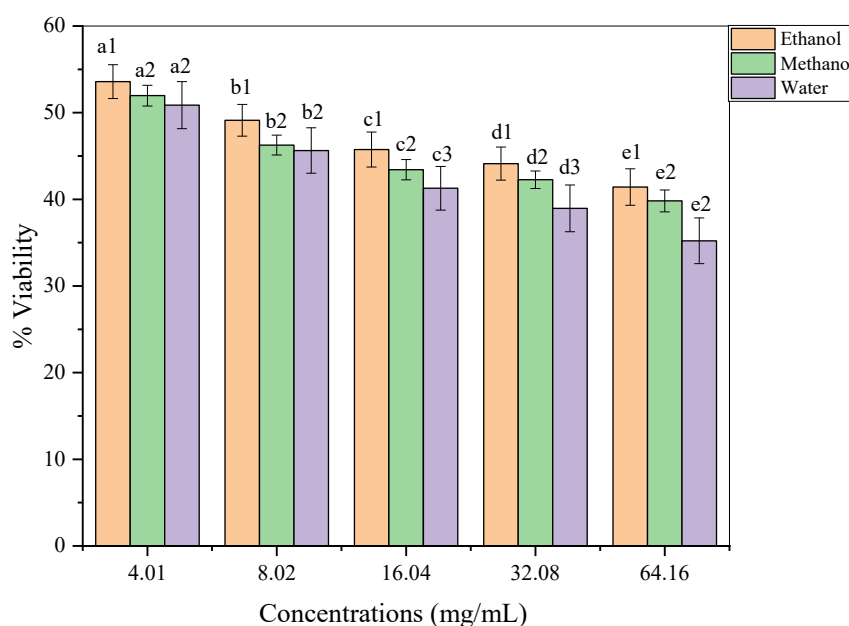


Figure 21: Cytotoxic effects on HeLa cells (P4) after 24 hours of treatment with a five step, 1: 2 serial dilution of *Elephantorrhiza elephantina* tubers extracts. HeLa cells had been cultured for 21 days and passaged 3 times prior to seeding on 96-well plates. Results are presented as mean ($n = 3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$).

The mean separation letters indicate that the means were significantly different when the letters are assigned different numbers. However, if the letters have same numbers, it indicates that the means were not significantly different from each other. For concentrations 32.08 and 16.04 mg/mL, the means for *E. elephantina* aqueous, methanol and ethanol extracts were significantly different from each other. However, the means for water and methanol extracts at concentrations 8.02 and 4.01 mg/mL were not significantly different from each other but they were both significantly different from ethanol extracts.

Tribulus terrestris leaves and branches methanol extracts showed the highest HeLa cell % viabilities of 55.6, 58.4, 58.7, 59.5 and 60.6% at 27.62, 13.81, 6.905, 3.4525 and 1.726 mg/mL concentrations respectively followed by ethanol extracts with 45.8, 48.5, 51.9, 57.5 and 59.9 % viabilities at similar concentrations. *T. terrestris* aqueous extracts of recorded the lowest HeLa cell % viabilities across all concentrations with 43.8, 46.9, 49.8, 55.7 and 57.5% (Figure 22). The highest concentration of *Tribulus terrestris* leaves and branches methanol extracts could not exhibit 50% inhibition on HeLa cell proliferation. Aqueous extracts of *T. terrestris* showed the highest cytotoxicity on HeLa cells followed by ethanol extracts with CC_{50} values of 6.7 ± 2.3 and 9.6 ± 2.1 mg/mL respectively.

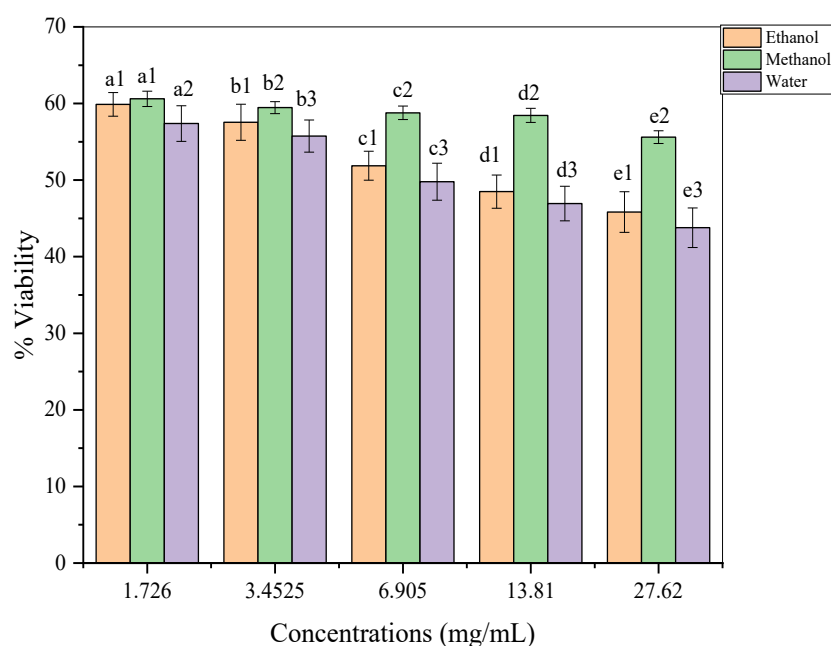


Figure 22: Cytotoxic effects on HeLa cells (P4) after 24 hours of treatment with a five step, 1: 2 serial dilution of *Tribulus terrestris* leaves and branches extracts. HeLa cells had been cultured for 21 days and passaged 3 times prior to seeding on the 96-well plates. Results are presented as mean ($n = 3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$).

The mean separation letters indicate that the means were significantly different when the letters are assigned different numbers. However, if the letters have same numbers, it indicates that the means were not significantly different from each other. There was a significant difference between the means for *Tribulus terrestris* leaves and branches ethanol, methanol and aqueous extracts across all concentrations except for 1.726 mg/mL where ethanol and methanol extracts means were not significantly different from each other but were both significantly different from the mean of the aqueous extract.

Withania somnifera leaves methanol extracts displayed the highest HeLa cell survival rate with 53.0, 77.7, 90.5, 101.5 and 105.0 % viabilities at 48.0, 24.0, 12.0, 6.0 and 3.0 mg/mL concentrations respectively (Figure 23). For 6.0 and 3.0 mg/mL concentrations, there was no inhibition to proliferation of HeLa cells as observed by cell viabilities of more than 100% (Figure 23). Aqueous extracts of *W. somnifera* leaves exerted the highest inhibition on HeLa cell proliferation with % viabilities of 45.8, 53.4, 67.7, 83.7 and 89.9% at 48.0, 24.0, 12.0, 6.0 and 3.0 mg/mL concentrations respectively.

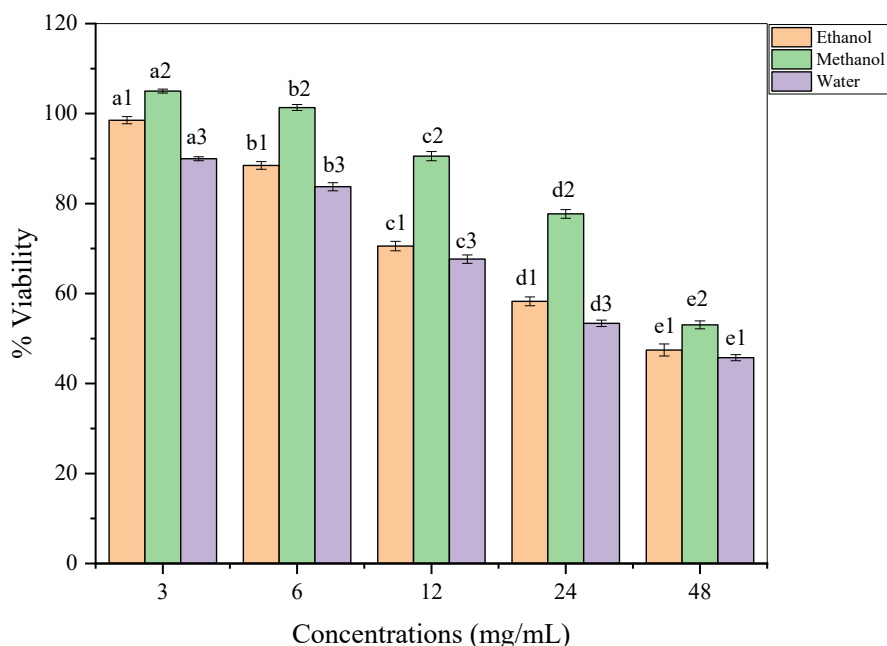


Figure 23: Cytotoxic effects on HeLa cells (P4) after 24 hours of treatment with a five step, 1: 2 serial dilution of *Withania somnifera* leaves extracts. HeLa cells had been cultured for 21 days and passaged 3 times prior to seeding on 96-well plates. Results are presented as mean ($n = 3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$).

The mean separation letters indicate that the means were significantly different when the letters are assigned different numbers. However, if the letters have same numbers, it indicates that the means were not significantly different from each other. *Withania somnifera* leaves ethanol and aqueous extracts displayed no significant difference between them at a concentration of 48 mg/mL but were both significantly different the methanol extracts. For concentrations 3.0, 6.0, 12.0, and 24.0 mg/mL, there were significant differences between the means of % viabilities of the three extraction solvents. Aqueous extracts showed the highest cytotoxicity across all concentrations followed by ethanol and methanol extracts respectively.

The CC_{50} values obtained for all the plant extracts were plotted and compared against each other. The CC_{50} value of *Azanza garckeana* fruits (AG) water extracts was below the least concentration used (Figure 24) while ethanol and methanol extracts CC_{50} values for AG were 1.8 and 1.88 mg/mL respectively. *Portulaca oleracea* leaves and branches (PO) methanol extracts recorded a CC_{50} value of 5.2 mg/mL while ethanol and aqueous extracts CC_{50} values for PO were both 2.38 mg/mL (Figure 24). The CC_{50} value of *Tribulus terrestris* leaves and branches (TT) methanol extracts was more than the highest concentration used in the assay, while aqueous extracts of TT gave the lowest CC_{50} value of 6.7 mg/mL followed by ethanol extracts with a CC_{50} value of 9.6 mg/mL. The CC_{50} values of *Withania somnifera* leaves (WS) were the highest amongst the five plant species across the three extraction solvents, with WS methanol extracts yielding the highest CC_{50} value of 52.2 mg/mL, followed by WT ethanol extracts with 42.0 mg/mL and aqueous extracts with 32.5 mg/mL. The highest CC_{50} value for *Elephantorrhiza elephantina* tubers extracts (EE) was yielded by ethanol extracts at 6.9 mg/mL, followed by EE methanol extracts at 5.6 mg/mL and EE aqueous extracts at 4.6

mg/mL. In the assays of the five plant extracts used, aqueous extracts yielded the lowest CC₅₀ values when compared to ethanol and methanol extracts except for PO where the CC₅₀ value of aqueous extracts was equal to that of ethanol extracts.

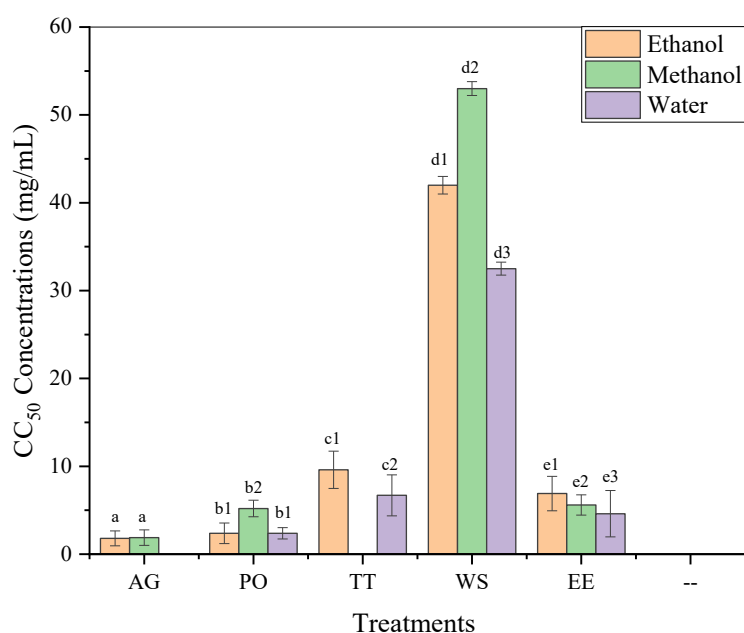


Figure 24: CC₅₀ concentrations of extracts obtained after treating HeLa cells extracts from five different plant species. Results are presented as mean (n = 3) ± standard deviation (SD). Bars with the same letters (a, b, c, d, e or f) are not significantly different (p < 0.05) and those with different letters are significantly different (p > 0.05). AG = *Azanza garckeana* fruits extracts, PO = *Portulaca oleracea* leaves and branches extracts, TT = *Tribulus terrestris* leaves and branches extracts, WS = *Withania somnifera* leaves extracts and EE = *Elephantorrhiza elephantina* tuber extracts.

4.3 Gene expression analysis by RT-qPCR

Gene expression analysis was carried out using quantitative reverse transcriptase polymerase chain reaction RT-qPCR with complementary DNA (cDNA) that was synthesized from the total RNA extracted from HeLa cells that had been treated with aqueous extracts of *Elephantorrhiza elephantina* tubers, *Azanza garckeana* fruits, *Withania somnifera* leaves, *Tribulus terrestris* leaves and branches and *Portulaca oleracea* leaves and branches. Aqueous extracts yielded the lowest CC₅₀ values when compared to ethanol and methanol extracts in all but one plant extract. Therefore, because aqueous extracts from all the five plant species consistently showed higher cytotoxicity on HeLa cells when compared to methanol and ethanol extracts, they were used for gene expression analysis. RT-qPCR was also undertaken for HeLa cells that had been treated with cisplatin (positive control) and PBS (negative control). The post-treatment expression levels of cervical cancer markers (Table 1) were normalized to the house keeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). These markers were Vascular Endothelial Growth Factor-A (VEGF), Epidermal Growth Factor Receptor (EGFR), Metastasis-Associated in Colon Cancer -1 (MACC1), Cluster of Differentiation 95

(CD95), and Cytokeratin Fragment 19 (CYRA 21-1). RT-qPCR was performed using the Luna Universal qPCR Master Mix (NEW ENGLAND BioLabs Inc, USA) according to the manufacturer's instructions in the Applied Biosystems 7500 Fast cycler (Life Technologies, Singapore).

There was significant ($p > 0.05$) up regulation of CD95 in HeLa cells after treatment with aqueous extracts of *Azanza garckeana* fruits (AG), *Elephantorrhiza elephantina* tubers (EE), *Portulaca oleraceae* leaves and branches, (PO) *Tribulus terrestris* leaves and branches (TT) and *Withania somnifera* leaves (WS) (Figure 25). Cisplatin (positive control) yielded the highest up regulation of CD95 with a fold increase of 0.77 relative to the negative control (NC). Cisplatin was followed by PO with a fold increase of 0.32, then TT and AG with fold increases of 0.27 and 0.25 respectively with no significant difference between them ($p < 0.05$) (Figure 25). EE and WS showed the least up regulation of CD95 compared to other treatments with no significant difference between them ($p < 0.05$) with 0.16 and 0.18 fold increases respectively.

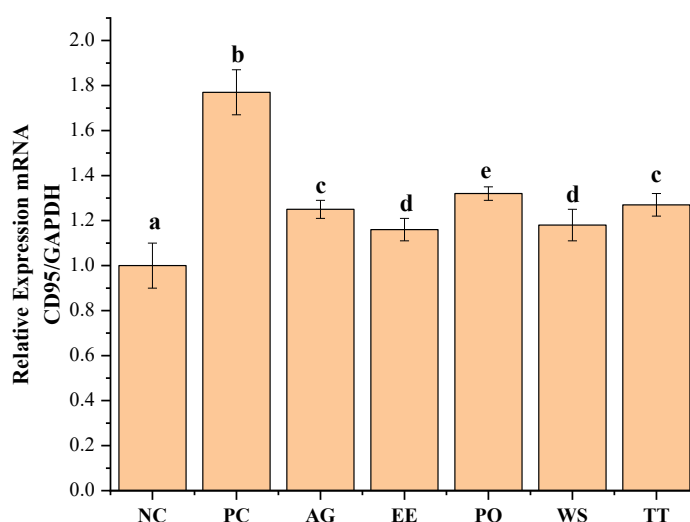


Figure 25: The relative expression of CD95 in different treatments with different plant extracts in HeLa cells. Results are presented as mean ($n=3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$). GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase, PC = Positive Control (Cisplatin), NC = Negative Control (Phosphate Buffered Saline), AG = *Azanza garckeana*, EE = *Elephantorrhiza elephantina*, PO = *Portulaca oleracea*, WS = *Withania somnifera* and TT = *Tribulus terrestris*.

There was significant ($p > 0.05$) down regulation of CYFRA 21-1 after treatment with aqueous extracts of *Azanza garckeana* fruits (AG), *Elephantorrhiza elephantina* tubers (EE), *Portulaca oleraceae* leaves and branches (PO), *Tribulus terrestris* leaves and branches (TT) and *Withania somnifera* leaves (WS) (Figure 26). Cisplatin (positive control) yielded the highest down regulation of CYFRA 21-1 with a fold decrease of 0.8 followed by PO and TT with fold decreases of 0.38 and 0.35 respectively and with no significant difference between them ($p < 0.05$) (Figure 26). AG displayed a fold decrease of 0.29. EE and WS showed the lowest down regulation of CYFRA 21-1 with 0.20 and 0.22 fold decreases respectively and with no significant difference between them ($p < 0.05$).

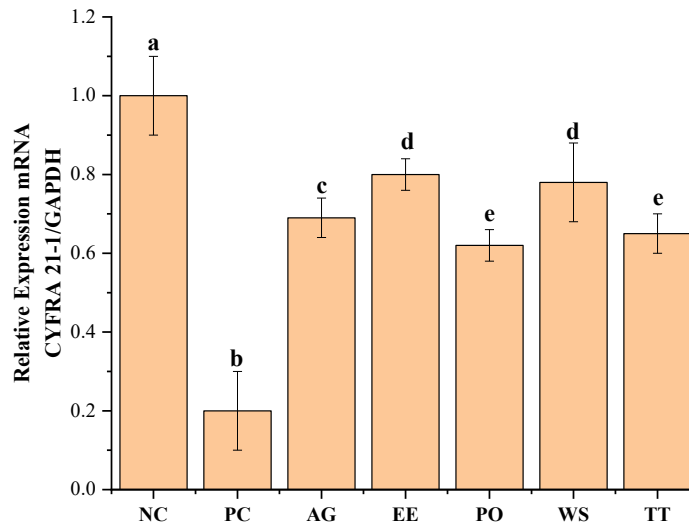


Figure 26: The relative expression of CYFRA 21-1 in different treatments with different plant extracts in HeLa cells. Results are presented as mean ($n=3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$). GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase, PC = Positive Control (Cisplatin), NC = Negative Control (Phosphate Buffered Saline), AG = *Azanza garckeana*, EE = *Elephantorrhiza elephantina*, PO = *Portulaca oleracea*, WS = *Withania somnifera* and TT = *Tribulus terrestris*.

EGFR was significantly ($p > 0.05$) down regulated after treatment with aqueous extracts of *Azanza garckeana* fruits (AG), *Elephantorrhiza elephantina* tubers (EE), *Portulaca oleracea* leaves and branches (PO), *Tribulus terrestris* leaves and branches (TT) and *Withania somnifera* leaves (WS) (Figure 27). There was no significant difference between the expression levels of EGFR for AG, PO and TT. Cisplatin (positive control [PC]) yielded the highest inhibition of EGFR expression with a fold decrease of 0.8 followed by PO with 0.44, then TT with 0.30 and AG with 0.30 with no significant difference ($p < 0.05$) between PO, TT and AG (Figure 27). There was no significant difference ($p < 0.05$) between EE and WS which effected the least inhibition, with 0.15 and 0.20 fold decreases respectively.

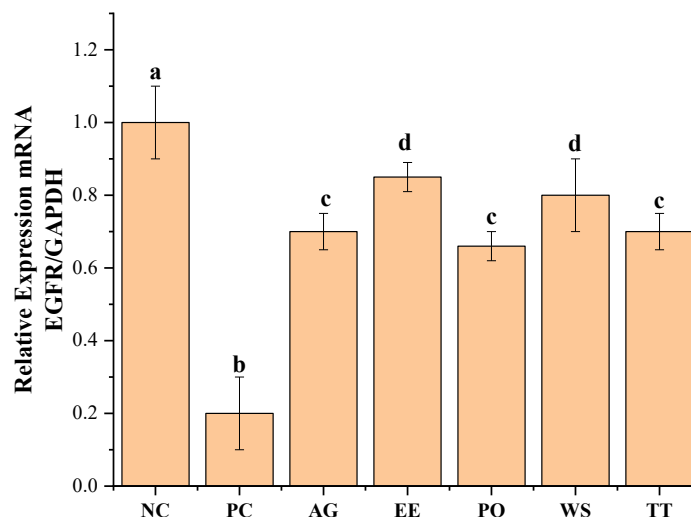


Figure 27: The relative expression of EGFR in different treatments with different plant extracts in HeLa cells. Results are presented as mean (n=3) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, or d) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$). GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase, PC = Positive Control (Cisplatin), NC = Negative Control (Phosphate Buffered Saline), AG = *Azanza garckeana*, EE = *Elephantorrhiza elephantina*, PO = *Portulaca oleracea*, WS = *Withania somnifera* and TT = *Tribulus terrestris*.

The expression of MACC1 was significantly ($p > 0.05$) down regulated after treatment of HeLa cells with aqueous extracts of *Azanza garckeana* fruits (AG), *Elephantorrhiza elephantina* tubers (EE), *Portulaca oleracea* leaves and branches (PO), *Tribulus terrestris* leaves and branches (TT) and *Withania somnifera* leaves (WS) (Figure 28). Cisplatin (positive control) yielded the highest inhibition with a fold decrease of 0.80 followed by PO with a 0.40 fold decrease, then AG and TT with fold decreases of 0.31 and 0.29 respectively and with no significant difference ($p < 0.05$) between them (Figure 28). Both with 0.2 fold decreases, EE and WS effected the least down regulation of MACC1.

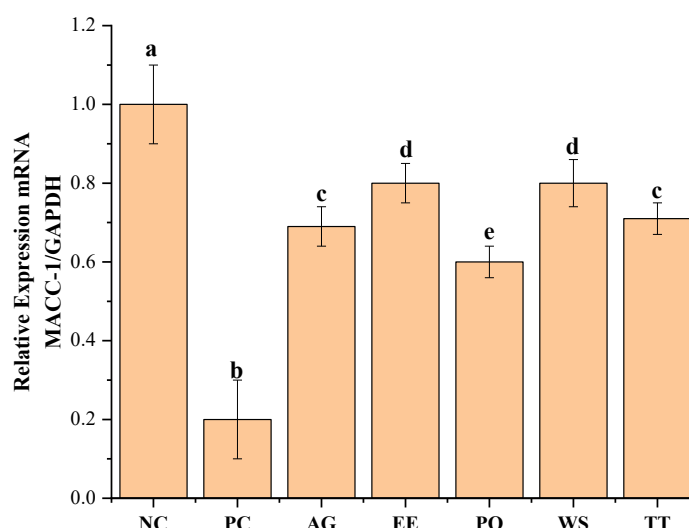


Figure 28: The relative expression of MACC1 in different treatments with different plant extracts in HeLa cells. Results are presented as mean ($n=3$) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d or e) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$). GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase, PC = Positive Control (Cisplatin), NC = Negative Control (Phosphate Buffered Saline), AG = *Azanza garckeana*, EE = *Elephantorrhiza elephantina*, PO = *Portulaca oleracea*, WS = *Withania somnifera* and TT = *Tribulus terrestris*.

The expression of VEGF gene was significantly ($p > 0.05$) down regulated after treatment of HeLa cells with aqueous extracts of *Azanza garckeana* fruits (AG), *Elephantorrhiza elephantina* tubers (EE), *Portulaca oleraceae* leaves and branches (PO), *Tribulus terrestris* leaves and branches (TT) and *Withania somnifera* leaves (WS) (Figure 29). Cisplatin (positive control) yielded the highest inhibition with a fold decrease of 0.80 followed by PO with a 0.35 fold decrease, then AG and TT with fold decreases of 0.28 and 0.30 respectively with no significant difference ($p < 0.05$) between them (Figure 29). EE and WS yielded the least down regulation of 0.17 and 0.1 fold decreases respectively.

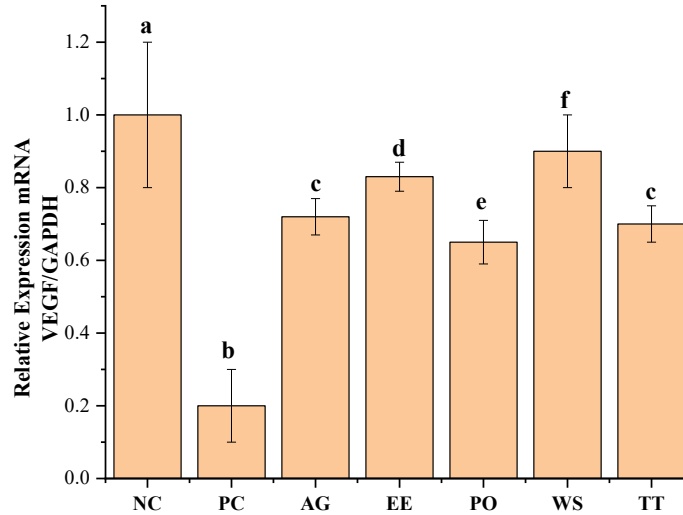


Figure 29: The relative expression of VEGF in different treatments with different plant extracts in HeLa cells. The results presented as mean (n=3) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d, e or f) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$). GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase, PC = Positive Control (Cisplatin), NC = Negative Control (Phosphate Buffered Saline), AG = *Azanza garckeana*, EE = *Elephantorrhiza elephantina*, PO = *Portulaca oleracea*, WS = *Withania somnifera* and TT = *Tribulus terrestris*.

The relative expression levels of the five genes; CD95, CYFRA 21-1, EGFR, MACC1 and VEGF normalized to GAPDH were compared after HeLa cells were treated with Cisplatin (positive control [PC]), PBS (negative control [NC]) and aqueous extracts of the five plant extracts; AG, EE, PO, WS and TT. Cells treated with cisplatin showed the lowest expression levels of CYFRA 21-1, EGFR, MACC1 and VEGF as well as the highest expression of CD95 while PBS (negative control) had no effect on the fold expression levels of these genes (Figure 30). Amongst the plant extracts, cells treated with PO had the lowest relative expression of CYFRA 21-1, EGFR, MACC1 and VEGF and the highest fold increase of CD95, followed by TT then AG extracts. WS extracts showed a relatively lower effect on the relative expression of the genes than the PO, TT and AG extracts (Figure 39). Cells treated with EE showed the highest fold increase in the expression of all genes except for CD95 and VEGF. There was variability in fold increases of each gene depending on the plant extract, with no specific pattern on the level of expression of each gene.

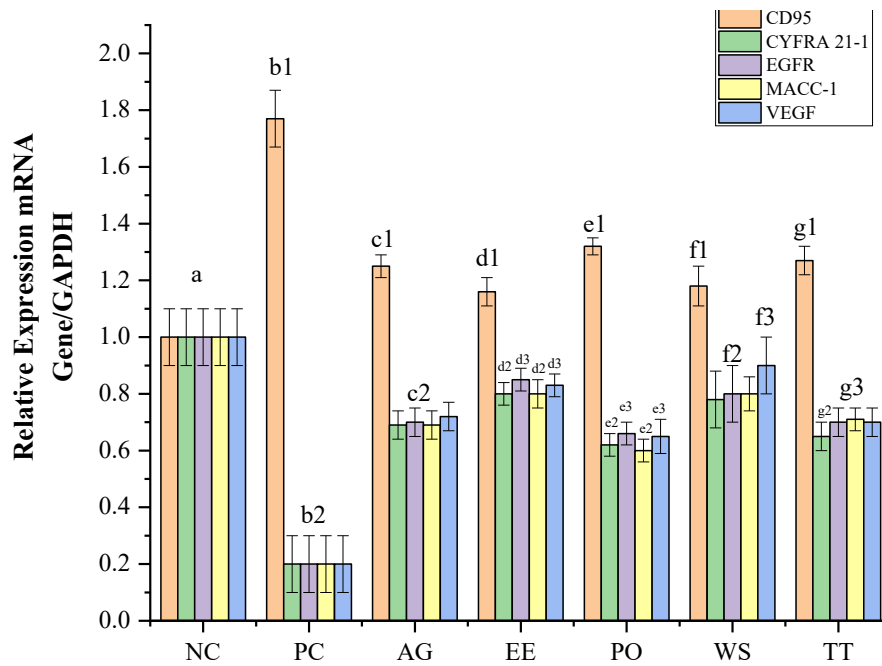


Figure 30: The relative expression of CD95, CYFRA 21-1, EGFR, MACC1 and VEGF normalised to GAPDH. Results are presented as mean (n=3) $\pm$ standard deviation (SD). Bars with the same letters (a, b, c, d, e, f or g) are not significantly different ($p < 0.05$) and those with different letters are significantly different ($p > 0.05$). GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase, PC = Positive Control (Cisplatin), NC = Negative Control (Phosphate Buffered Saline), AG = *Azanza garckeana*, EE = *Elephantorrhiza elephantina*, PO = *Portulaca oleracea*, WS = *Withania somnifera* and TT = *Tribulus terrestris*.

CHAPTER 5: DISCUSSION

Five traditional healers were interviewed and consented to this study, three of whom are active members of Botswana Dingaka Association and the other two had their membership expired, pending annual renewal but all of them were card carrying members. Botswana Dingaka Association is an oversight group, listed in the Botswana Gazette, which controls the scope and reach of traditional healers in Botswana. Traditional healers in Botswana fall under two categories, herbalists and divine healers (Ulin, 1975), with the latter known for throwing bones as part of their ritual of healing their patients. Only one of the five traditional healers was able to read and write English even though he was not very fluent while the rest only understood when the questionnaire was read to them in Setswana. Some names of the medicines that traditional healers use could not be directly translated to English. There was another challenge encountered which was the use of different local names for the same plants by the traditional healers. The data collected from survey questionnaires with traditional healers was summarised in appendix A and B, tables A1 and B1 respectively. From the collected data, five plants which appeared the most in the questionnaires as can be noted in appendix B, table B1 were selected for further investigation in this study. The selected plants were collected, pressed and sent to the Botswana National Museum and Monuments Herbarium for verification of their identities.

Morphological changes were observed on the HeLa cells after treatment with *Azanza garckeana* fruits, *Elephantorrhiza elephanina* tubers, *Portulaca oleraceae* leaves and branches, *Tribulus terrestris* leaves and branches and *Withania somnifera* leaves that had been extracted by water, ethanol, and methanol at different and standardized concentrations. Results showed that generally, phytochemicals in the extracts had cytotoxic effects on HeLa cells in culture and possibly activated apoptotic pathways. Apoptotic pathways are activated as a consequence of molecular pathways of the cells being altered, leading to changes in normal biochemical and metabolic pathways and ultimately cell death (Saraste and Pulkki, 2000). When cell death occurs, the morphology of cells and their attachment on cell culture plates changes. HeLa cells normally grow in monolayers and spread out when attaching to cell culture plates. They keep dividing until they reach 100% confluency. Cell death is usually associated with a cell and its contents shrinking physically and detaching from the cell culture plates (Wen *et al.*, 1997). These morphological changes may be a result of key structural proteins being cleaved due to certain caspases being upregulated and thus proteolytically degrading structural proteins; leading to changes in the shape of the cells (Wang *et al.*, 1998). Caspases, which cleave structural proteins in a cell, seem to target microfilaments in the cytoskeleton which are responsible for maintaining the integrity of the cell shape (Brown *et al.*, 1997).

Concentrations of the extracts of the five selected plants were standardized. The different solvents used during extraction (ethanol, methanol, and water) recovered phytochemicals at varying concentrations. Therefore, stock concentrations were standardized or diluted. It must also be noted that, unlike in most cancer research studies which use $\mu\text{g/mL}$, as per the American Cancer Institute, concentrations of plant extracts used in this study were in mg/mL . According to the American Cancer Institute, for a crude extract to be accorded viable anticancer agent status, it needs to have an $\text{IC}_{50} < 20 \mu\text{g/mL}$ (Boik, 2001, Abdel-Hameed *et al.*, 2012). The reason

for using the concentration of extracts in mg/mL was to mimic the standards used in traditional medicine as the healers use the plants extract in their crude and concentrated state.

Physical and visible apoptotic features like shrinking and blebbing of the cells were observed in HeLa cells after treatment with different concentrations of *Portulaca oleracea* leaves and branches water, ethanol and methanol extracts respectively (Figure 13). This may indicate potential toxicity of the extracts on HeLa cells which can be facilitated by bioactive compounds such as POL-P3b, which has been shown to inhibit cervical cancer cell growth both *in vivo* and *in vitro* (Zhao *et al.*, 2017). However, the expression and activation of proteins such as TLR4 in HeLa cells can be indicative of cancer metastasis and anti-apoptotic features, resulting in successful progression of the tumour. Therefore, any compound with the ability to counteract the activity of proteins such as TLR4 would prove vital as a target in cancer therapy (Wang *et al.*, 2015a).

Despite the fact that *Portulaca oleracea* extracts were used in their crude form in this study without isolating specific phytochemical constituents, their effects were consistent with the effects of POL-P3b, which has been shown to act against HeLa cell proliferation via TLR4 and its signalling pathways (Zhao *et al.*, 2017). *Portulaca oleracea*, through its active metabolites, may inhibit tumour progression by the action of POL-P3b on the TLR4/NF- κ B pathway, thus decreasing the production of cytokine/chemokine and VEGF, hence inducing apoptosis of HeLa cells (Zhao *et al.*, 2017). The loss of normal cell attachment, shape and structure are consistent with findings of a study by Al-Sheddi *et al.* (2015), where seed oil extracts from *Portulaca oleracea* were shown to have apoptotic effects on human liver and lung cancer cell lines HeG2 and A-549 respectively.

There was a significant general decrease in HeLa cell viability from the highest to the lowest concentration and this decline was directly proportional to increases in concentrations of *Portulaca oleracea* extracts (Figures 19). At high concentrations, the content of phytochemicals which antagonize proliferation of cancer cells like antioxidants, phenols and alkaloids are high, which may explain why the viability of HeLa cells was high at low concentrations of extracts and low at high concentrations. These observations are consistent with a previous study where viability assays were carried out to quantify cytotoxicity of standardized extracts of poplar type propolis (Campoccia *et al.*, 2021). The cytotoxicity of *Portulaca oleracea* water and ethanol extracts on HeLa cells was high when compared to that of methanol extracts. Both *Portulaca oleracea* water and ethanol extracts displayed CC₅₀ values of 2.38 mg/mL while methanol showed a CC₅₀ value of 5.2 mg/mL (Figure 24). The lower the CC₅₀ value of an extract, the safer it is to use such an extract in treatment protocols because it would take lower concentrations to inhibit 50% proliferation of diseased cells (Campoccia *et al.*, 2021). When compared to methanol and ethanol, water is the most polar extraction solvent (Xu *et al.*, 2019), and therefore has a tendency to extract higher concentrations of phytochemicals than methanol and ethanol. MTT assay revealed that *Portulaca oleracea* had similar cytotoxic effects on the HeLa cells to those observed on HepG2 and A-549 cancer cell lines during cytotoxicity evaluation using MTT assay (Al-Sheddi *et al.*, 2015). Polysaccharides of *Portulaca oleracea* were used individually and conjugated with

other substances to evaluate their cytotoxicity on different cancer cell lines, including HeLa cells, resulting in noticeable and significant levels of cytotoxicity (Han *et al.*, 2021).

Cytotoxic effects of *Portulaca oleracea* on A-549 and HCT116 cell lines were reported to have a concentration-dependent inhibition of proliferation on these cancer cell lines (Erdoğan, 2021). It has also been reported that when normal cells are exposed to ethanol extracts of *Portulaca oleracea*, the same way that cancer cell lines were exposed to in this study, no cytotoxicity is observed, suggesting that these extracts may be a safer alternative (Rahimi *et al.*, 2019b). Portulacerebroside A, a secondary metabolite produced by *Portulaca oleracea*, has been shown to have control of the mitochondrial death pathway that consequently leads to the death of the mitochondria and increases the number of cells programmed to undergo apoptosis in human hepatic cancer cells via the upregulation of p38MAPK and JNK proteins (Erdoğan, 2021, Rahimi *et al.*, 2019a). The same secondary metabolite may target cervical cancer cells and induce cell death, which would ultimately reduce their proliferation and tumor progression. It has also been shown that *Portulaca oleracea* polysaccharides scavenge free radicals like superoxide anions and the activity of these free radicals can result in tumor growth, thus scavenging them would strengthen the immune system and antagonize tumor proliferation and other factors that support tumor growth thereof (Shen *et al.*, 2013). Colony forming assays were used to evaluate the effect of *Portulaca oleracea* extracts on colony formation by human colorectal and lung cancer cell lines, HCT116 and A549 respectively. The results indicated inhibitory effects by *P. oleracea* extracts when compared to the control (Franken *et al.*, 2006). Results of this study are in unison with the findings of other studies that have reported that *Portulaca oleracea* possess bioactive compounds which have anticancer potential like polysaccharides, alkaloids, cerebrosides and flavonoids (Alipour *et al.*, 2022).

Loss of turgidity, shrinkage and blebbing were observed in HeLa cells that were treated with *Azanza garckeana* fruits (Figure 14). Cells detached from the cell culture plates and lost their normal appearance. After 24 hours of treatment with *A. garckeana* ethanol, methanol and water extracts, the cells appeared dark in colour, an observation that has been previously reported (Saraste and Pulkki, 2000). Morphological changes were more severe at 12.88 mg/mL concentration, a suggestion that the higher the concentration, the more severe the effects of the extracts on HeLa cell morphology. *Azanza garckeana* produces flavonoids and phenolic compounds which have antioxidant properties (Elshiekh and Ali, 2020) that can inhibit the proliferation of HeLa cells. Therefore, the presence of antioxidant phytochemicals may be responsible for the morphological changes observed in HeLa cells that were treated with extracts of *Azanza garckeana* (Michael *et al.*, 2015, Azanza, 2016).

The proliferation of HeLa cells was significantly inhibited after treatment with *Azanza garckeana* aqueous, methanolic and ethanolic extracts, with the aqueous extracts showing the lowest percentage cell viability, indicating high cytotoxicity. *A. garckeana* ethanol and methanol extracts displayed CC₅₀ values of 1.88 mg/mL and 1.8 mg/mL respectively. The proliferation of HeLa cells after treatment with aqueous extracts of *A. garckeana* did not exceed 50%, disallowing the acquisition of the CC₅₀ value. This indicates that the lowest concentration of extracts that was used during cell viability assays was too toxic for HeLa cells to proliferate up to 50% viability. This suggests that lower concentrations of *Azanza Garckeana* fruits extracts

would be required in cancer cells cytotoxicity (Smith *et al.*, 2002). These results suggest possible antiproliferative effects of *Azanza garckeana* phytochemicals on HeLa cells as has been previously reported (Azanza, 2016).

Aqueous extracts of *Azanza garckeana* fruits were more cytotoxic to HeLa cells than methanol and ethanol extracts. Water, as an extraction solvent, has higher polarity than methanol and ethanol (Katritzky *et al.*, 2004), and, therefore, has the ability to extract higher content of phytochemicals like alkaloids, phenols, flavonoids and terpenoids. The higher the phytochemical content in an extract, the likely more effective the extract would be in inhibiting the proliferation of cancer cells because of their antioxidant properties, which is demonstrated when phytochemicals are deployed in cancer prevention assays (Mondal *et al.*, 2019).

This study has shown that ethanol, methanol and aqueous extracts of *Elephantorrhiza elephantina* tubers all had morphological effects on the HeLa cells in culture for 24 hours. These included changes in shape, adherence to culture plates and appearance of the cells when compared to untreated cells (Figure 15). These alterations in the morphology of HeLa cells in response to treatment with aqueous, methanol and ethanol extracts of *Elephantorrhiza elephantina* tubers indicate that the phytochemicals extracted from *Elephantorrhiza elephantina* had antiproliferative effects on the HeLa cells. The cytotoxicity and antioxidant properties of the roots extracts of *Elephantorrhiza elephantina* suggest that the plant has the potential to inhibit growth of cervical cancer cells (Olaokun *et al.*, 2020).

There was significant cytotoxicity of *Elephantorrhiza elephantina* tubers ethanol, methanol and water extracts on HeLa cells with aqueous extracts inducing the greatest cytotoxicity on the cells when compared to ethanol and methanol extracts. Ethanol extracts were the least cytotoxic to HeLa cells based on cell viability assays (Figure 21). These results are in line with a study where ethanol, acetone and water extracts of *Elephantorrhiza elephantina* were shown to have noticeable levels of cytotoxicity on H4IIE liver cells (Olaokun *et al.*, 2020). Aqueous extracts of *Elephantorrhiza elephantina* were the most toxic to HeLa cells with a CC₅₀ value of 4.6 mg/mL, followed by methanol extracts with CC₅₀ value of 5.6 mg/mL and ethanol extracts with a CC₅₀ value of 6.9 mg/mL. A higher CC₅₀ value means that an extract has to be used at higher concentrations in order to have a cytotoxic effect on cells, which compromises the safety physiologically.

Crude ethanol, methanol and water extracts from *Tribulus terrestris* leaves and branches had a significant effect on the morphology of HeLa cells. Treatment with the highest concentrations of *Tribulus terrestris* extracts proved to be cytotoxic to the HeLa cells due to observed changes in the shape of cells (Figure 16). Before treatment with the plant extracts, HeLa cells had attached to the cell culture plates and displayed their normal proliferative pattern of spreading out on the cell culture plate in a monolayer. At the end of 24 hours of treatment with *T. terrestris* extracts, some cells detached from cell culture plates and started shrinking, blebbing and changing shape. Alterations in the morphology of the cells after treatment with *Tribulus terrestris* leaves and branches extracts suggest antiproliferative effects of the extracts. Methanolic extracts of *Tribulus terrestris* were found to be cytotoxic to MCF-7 breast cancer cells but no toxicity was observed on non-cancerous cells by the same extracts (Patel *et al.*,

2019). Therefore, the results of this study are in line with previous research on *Tribulus terrestris* extracts.

A significant level of inhibition was observed on the proliferation of HeLa cells after treatment with *Tribulus terrestris* leaves and branches aqueous, methanol and ethanol extracts. HeLa cell viability reduced with increasing concentration of extracts, signifying increasing cytotoxicity (Figure 22). These results are in agreement a study where the fruits and aerial parts of *Tribulus terrestris* were extracted and tested on HeLa cells and SKOV-3 cells (Kucuk *et al.*, 2017). The lowest concentration of the aerial parts of the plant was found to exert the least cytotoxicity on the cells. In another study, methanol extracts of *Tribulus terrestris* were found to be cytotoxic to MCF-7 cells only and not toxic to non-cancerous cells (Patel *et al.*, 2019; Abbas *et al.*, 2022). It has also been previously shown that ethanolic extracts of *Tribulus terrestris* induces significant antiproliferative effects on several cancer cell lines like prostate, lung and liver cancers (Rashed *et al.*, 2022).

When treated with 48 mg/mL and 3 mg/mL of *Withania somnifera* leaves ethanol, methanol and water extracts, HeLa cells detached from cell culture plates and changed shape (Figure 17). This was indicative of the antiproliferative or cytotoxic effects of the extracts on the normal behaviour of HeLa cells (Palliyaguru *et al.*, 2016). *W. somnifera* extracts were shown to antagonize tumour proliferation in ovarian cancer cells by increasing the potential of tumour cell death as a result of DNA damage (Shikder *et al.*, 2020). *Withania somnifera* has bioactive compounds like withaferin-A and Withanolides which have been shown to have antiproliferative properties to most cancer cells (Sivasankarapillai *et al.*, 2020). The antiproliferative effects of these bioactive compounds lead to apoptosis of cancer cells and changes in the shape and morphology of the cells. Withaferin-A has been shown to down regulate breast cancer proliferation (Shikder *et al.*, 2020) and suppress growth factors of the cancer cells (Yin *et al.*, 2020, Yu *et al.*, 2010). Withanolides induced cell cycle arrest, leading to the death of breast cancer cells (Wang *et al.*, 2012).

Withania somnifera leaves water extracts were shown to lead to the least cell viability and therefore the most cytotoxicity (Figure23). Methanol resulted in the least cytotoxicity on HeLa cells. Recorded CC₅₀ values were 42 mg/mL, 32.5 mg/mL and 53 mg/mL for ethanol, water and methanol in that order. These results are in line with a study by Nile *et al.* (2019), who reported that Withaferin A, a bioactive compound found in *Withania somnifera*, was able to exhibit 78% inhibition of HeLa cell proliferation when compared to 90% inhibition by Berberine, a renowned clinically used anticancer compound. The extracts of *W. Somnifera* did not exhibit cytotoxicity when a normal cell line, MDCK, was exposed to them (Srivastava *et al.*, 2016). In extracts of all the three extraction solvents used in this study, cell viability decreased as concentration increased. Withanolide, a bioactive compound found in *Withania somnifera*, was extracted from the roots of this plant and shown to have antiproliferative effects against a number of cancer cells with less side effects (Maheswari *et al.*, 2020). The ability to inhibit cancer cell proliferation with less side effects gives *Withania somnifera* potential for use as an alternative in possible cytotoxicity of cancer when compared to modern medications which sometimes result in adverse side effects on patients (Thurston and Pysz, 2021).

There was an up regulation of CD95 in HeLa cells treated with different extracts of *Azanza garckeana* (AG), *Elephantorrhiza elephantina* (EE), *Portulaca oleracea* (PO), WS- *Withania somnifera* (WS) and *Tribulus terrestris* (TT) (Figures 25 and 30). HeLa cells treated with cisplatin (Positive Control), which is a drug used in cancer treatment (Becit and Aydin, 2020), showed the highest up regulation of CD95 gene with a 0.77 increase in fold change while HeLa cells treated with PBS (Negative Control) did not exhibit any change in expression levels of CD95. This is because PBS is a normal physiological solution (Fan *et al.*, 2022) which is used in cell culture and therefore does not have any effect on cells growing in normal culture. PO extracts exhibited the highest up regulation of CD95 amongst all plant extracts with a fold increase of 0.32. Because the CD95/CD95L complex is responsible for recruiting proteins which supports apoptosis, the up regulation of CD95 lead to more cancer cell death (Risso *et al.*, 2022), as observed in this study. The presence of oleracin A and B in the PO extracts could have led to the high up regulation of CD95.

CD95 is normally down regulated in cancer cells because it has the ability to increase apoptosis (Bullani *et al.*, 2002) by acting as a death receptor together with its ligand CD95L (Peter, 2015). When triggered by the CD95 ligand, CD95 then engages proteins like caspase-8, which support programmed cell death, generating a complex responsible for cell death. Therefore, for cancer to flourish, extrinsic and intrinsic apoptotic pathways are shut down in cancer cells. An increase in the expression of CD95 protein and its ligands can induce the activation of apoptotic pathways in cancer cells, leading to cytotoxicity of cancer cells, thus the antiproliferation of the tumours. The plants used in this study all led to the up regulation of CD95 in HeLa cells, suggesting the potential cytotoxic and antiproliferative properties of extracts from these plants. According to Müschen *et al.*, (2000), the expression of CD95 in malignant breast cancer cells deteriorated, but its ligand; CD95L, was expressed normally when MCF-7 breast cancer cells were initially resistant to cell death induced by CD95L. When CD95 expression was up regulated, MCF-7 cells became susceptible to CD95-L induced apoptosis. When comparing CD95 with the other cancer markers used in this research, CD95 is a tumour suppressor gene while the other four genes (CYFRA 21-1, EGFR, MACC1 and VEGF) are hall mark markers of cancer. This is elaborated by the fact that CD95 is expressed in low quantities in cancer cells because its high expression would inhibit cancer cell proliferation (Bullani *et al.*, 2002). The other markers (CYFRA 21-1, EGFR, MACC1 and VEGF) are all overexpressed in cancer cells.

A significant down regulation of CYFRA 21-1, EGFR, MACC1 and VEGF in HeLa cells treated with different plant extracts was observed. The aqueous extracts of *Portulaca oleracea* leaves exhibited the highest level of down regulation of all these genes (Figures 26-30). For *Portulaca oleracea*, these results may be attributed to the phytochemicals produced by the plant like cerebrosides and alkaloids which have been shown to inhibit the growth of cancer cells (Al-Sheddi *et al.*, 2015). Other phytochemicals produced by *Portulaca oleracea* include phenolic compounds, flavonoids, omega-3 fatty acids, vitamin A, vitamin C, oxalate and β -carotene which are non-enzymatic natural antioxidants (Asadi-Samani *et al.*, 2019, Lim and Quah, 2007).

Antioxidants are known for their free radical scavenging activity that can lead to DNA damage, mutations and ultimately cancer development. The down regulation of CYFRA 21-1, EGFR, MACC1 and VEGF, which are hallmark genes normally expressed in HeLa cells, suggest that phytochemicals from *Portulaca oleracea* may have interfered with pathways that lead to the expression of these genes. *Portulaca oleracea* also produce *Portulaca oleracea* polysaccharide (POP) and Portulacacerebroside which have been shown to be toxic to HeLa cells (Chen *et al.*, 2010b) and could have contributed to changes in the expression of these hallmark genes.

After *Portulaca oleracea*, aqueous extracts of *Tribulus terrestris* led to the most down regulation of CYFRA 21-1, EGFR, MACC1, VEGF (Figures 26-30) and the up regulation of CD95 (Figure 25). Some of the phytochemicals that are produced by *T. terrestris* include the saponins, alkaloids (Basaiyye *et al.*, 2018a), flavonoids, spirostanol glycosides, dioscin, protodioscin and prosapogenin, all of which play a role in the inhibition of cancer cell growth (Angelova *et al.*, 2013). Flavonoids interact with various targets in the cell and inhibit proliferation of cancer cells thus activating cell cycle arrest mechanisms and apoptosis, eventually counteracting metastasis of tumours and angiogenesis (Aggarwal *et al.*, 2020). The *T. terrestris* aqueous extracts induction of down regulation of CYFRA 21-1, EGFR, MACC1, VEGF and the up regulation on CD95 expression may indicate that extracts from *T. terrestris* interferes with molecular mechanisms that control the normal expression of these genes in cancer when there is no counteracting factor. Alkaloids are known for their ability to inhibit cancer cell proliferation by inhibiting enzymes responsible for metastasis and proliferation such as Bcl-2, caspase-3 and AKR1C3 (Mondal *et al.*, 2019), which are normally expressed in cancer cells. Previously, DNA fragmentation of HeLa cells and the activation of intrinsic pathways of apoptosis were observed when MCF-7 breast cancer cells were treated with methanol extracts of *T. terrestris* (Patel *et al.*, 2019). Aqueous extracts of *T. terrestris* have also been reported to cause cell cycle arrest and apoptosis (Kim *et al.*, 2011). Therefore, treatment with *T. terrestris* extracts may lead to the up regulation of genes that support apoptosis like caspase 3, Bax and p53 and the down regulation of overexpressed markers found in the region of metastasis like Bcl-2. It has been reported that extracts of *T. terrestris* activate both extrinsic and intrinsic pathways of apoptosis on cancer cells by up regulating Fas-associated death domain (FADD), Bcl-2 associated X-protein (Bax), caspase-3 and caspase-8, all of which support apoptosis (Angelova *et al.*, 2013, Basaiyye *et al.*, 2018b). Additionally, the up regulation of p53 also antagonizes tumour proliferation. In another related study, *T. terrestris* aqueous extracts were used to activate cell arrest and cell death in human liver cancer cells. It was shown that the extracts induced the accumulation of the G0/G1 phase of cell cycle progression in the liver cancer cells as well as inducing apoptosis by down regulating NF- κ B signalling (Kim, 2011).

Elephantorrhiza elephantina extracts led to the down regulation of CYFRA 21-1, EGFR, MACC1 and VEGF (Figures 26-30) as well as the up regulation of CD95 (Figure 25). *Elephantorrhiza elephantina* produces bioactive compounds like flavonoids, ethyl gallate and phenolic compounds, all of which display antioxidant abilities (Maroyi, 2017b). Flavonoids have the ability to reduce tumour size (Rajabi *et al.*, 2021) and their biological activity leads to the activation of caspase-3, which is responsible for DNA fragmentation and cytoskeleton

protein degradation, causing apoptosis (Jiang *et al.*, 2020). Caspase-3 has recently been used to check the effectiveness of cancer drugs by quantifying its expression. It has been determined that the more it is expressed during cancer treatment, the more effective the drug is against cancer cells (Jiang *et al.*, 2020). Because the Bcl-2 family of proteins is responsible for suppressing apoptosis, it is generally found elevated around cancer cells but is decreased when exposed to flavonoids (Warren *et al.*, 2019). The presence of flavonoids in aqueous extracts of *Elephantorrhiza elephantina* may have activated apoptosis on the treated HeLa cells therefore decreasing the relative expression of CYFRA 21-1, EGFR, MACC1 and VEGF and elevating CD95 expression.

Azanza garckeana aqueous extract led to an increase in the expression of CD95 (Figure 25) and decreases in the expression of CYFRA 21-1, EGFR, MACC1 and VEGF (Figures 26-30). This could be attributed to the presence of tannins and saponins (Neychev *et al.*, 2007), flavonoids, alkaloids, phenols and carotenoids (Michael *et al.*, 2015) in *Azanza garckeana* extracts, all of which have antioxidant properties. Apoptotic pathways could have been activated in HeLa cells after treatment with *Azanza garckeana* aqueous extracts, leading to cell death (Jiang *et al.*, 2020, Shree *et al.*, 2019).

Withania somnifera aqueous extracts also resulted in the up regulation of CD95 (Figure 25) and the down regulation of CYFRA 21-1, EGFR, MACC1 and VEGF (Figures 26-30) which could be attributed to the fact that *Withania somnifera* produces bioactive phytochemicals such as alkaloids, steroids, withanolides and withaferins (Nile *et al.*, 2019), all of which have anticancer properties. Withanolides and withaferins inhibit the p53 and PTEN pathways, thus inhibiting metastasis of cancer (Shikder *et al.*, 2020, Rai *et al.*, 2016). Like flavonoids, steroids also lead to the activation of caspase-3 and a decrease of BCL-2, both of which result in apoptosis of cancer cells. In this study, HeLa cells may have undergone apoptosis after being treated with *Withania somnifera* leaves aqueous extracts, thus leading to fold decreases in the expression of CYFRA 21-1, EGFR, MACC1 and VEGF and a fold increase in the expression of CD95. Withaferin A activates pathways that lead to the induction of apoptosis, growth arrest and the inhibition of oncogenes like STAT3 and vimentin, which is a protein found in the region of metastasis (Hahm *et al.*, 2020).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study has shown to a considerable extent that selected medicinal plants of Botswana have the potential to reduce cancer cell proliferation due to the cytotoxicity of their extracts on HeLa cells by leading to the reduced expression of cancer markers and the increased expression of a tumor suppressor gene (CD95). The aqueous, methanol and ethanol extracts of *Azanza garckeana* fruits, *Elephantorrhiza elephantina* tubers, *Portulaca oleracea* leaves and branches, *Withania somnifera* leaves and *Tribulus terrestris* leaves and branches all exerted alterations in morphological features of HeLa cells in culture. Signs of apoptosis like loss of shape and structure, shrinkage, detachment from cell culture plates and loss of turgidity were observed after the treatment of HeLa cells with the plant extracts. Cell viability assays showed the inhibition of proliferation of HeLa cell by plants extracts evidenced by decreased HeLa cell viability with increasing concentrations of extracts. Aqueous extracts from all the five plant species consistently showed higher cytotoxicity on HeLa cells when compared to methanol and ethanol extracts. This may be because water has higher polarity and, therefore, the potential to extract high phytochemical content. The CC₅₀ values of *Azanza garckeana* and *Portulaca oleracea* were the least of all the plant extracts. During gene expression analysis, the aqueous extracts of *Portulaca oleracea* showed the highest down regulation of CYFRA 21-1, EGFR, MACC1, VEGF and the highest up regulation of CD95. *Portulaca oleracea* was followed by *Tribulus terrestris* and *Azanza garckeana*, then *Elephantorrhiza elephantina* and *Withania somnifera* respectively. The plant extracts generally showed inhibition of the expression of CYFRA 21-1, EGFR, MACC1, VEGF and the up regulation of CD95. Therefore, in conclusion, extracts from these five plants were cytotoxic to HeLa cells, so they may possess anticancer properties. Amongst these plant species, *Portulaca oleracea* was the most potent in reducing HeLa cell proliferation followed by *Tribulus terrestris* and *Azanza garckeana* then *Elephantorrhiza elephantina* and *Withania somnifera* in that order.

6.2 RECOMMENDATIONS AND LIMITATIONS

- The budget for this study was very limited, making the acquisition of certain reagents challenging.
- This study used crude extracts from the plants. In future studies, characterization of the extracts could be performed by GC-MS and HP-LC to identify active biocompounds
- Extraction methods should be optimized for each plant species and relevant explant.
- Run gel electrophoresis for PCR products and record every data despite it not be used as part of the results.
- The aqueous extracts of the five plant species should be considered for further investigation of their cytotoxicity to other types of cancer cells.
- It is also recommended that a non-tumour cell line be studied alongside HeLa cells to determine the safety and cytotoxicity level on normal physiological cells.

APPENDICES

Survey questionnaires

Survey questionnaires were undertaken on consenting traditional healers on how they use traditional medicine to alleviate some symptoms which are synonymous with symptoms of cancer. Various symptoms were presented orally to traditional healers and they responded orally how they alleviate these symptoms when presented by their patients. The information was tabulated and a list of all the plant species used by each traditional healer is presented in Table A1. From this list of plants in Table A1, five plants that appeared more frequently were selected (Table B1: Highlighted in yellow). These plants were identified and collected from their natural habitats. The plants were *Elephantorrhiza elephantina*, *Portulaca oleracea*, *Withania somnifera*, *Tribulus terrestris* and *Azanza garckeana*.

Appendix A

Table A1: Traditional healers Questionnaires compilation in selection of medicinal plants used traditionally in alleviation of cancer symptoms

Disease/Symptoms	Plant used (Local name)	Scientific name of plant used	Preparation	Explant used	Plant distribution in Botswana	Common outcome
Vomiting/nausea	Kgalemela – a synergy of (mositsane+ modikaseope)	<i>Hirpicium bechuanense</i> <i>Withania somnifera</i> <i>Elephantorrhiza elephantina</i>	Boiled with water	Roots	Most of southern part of Botswana Rainy season	Difference observed from 2 days of treatment
	Selele	<i>Portulaca oleracea</i>	Cooked with food. Dried, pounded and boiled with water	Leaves, Stems	Most parts of Botswana during rainy season	Symptoms start to subside after few days
	Modikaseope	<i>Withania somnifera</i>	Boiled in milk	Roots	Tswapong area and most of Central district near places hills or rocky areas	Few days to observe the difference
	Tshetlho	<i>Tribulus terrestris</i>	Dried and boiled with water or taken in tea	Leaves, roots, and fruits	All over the country during rainy season	1 week and one can start feeling the difference
	Mositsane	<i>Elephantorrhiza elephantina</i>	Boiled in water	Dried roots/tubers	Southern Botswana (Kumakwane area, Monwane lands)	A day is enough to see the difference
Sexually Transmitted infections (eg: HPV- Human papilloma virus, genital warts)	Mositsane +Thetele	<i>Elephantorrhiza elephantina</i>	Boil in water	Tubers	Southern Botswana (Kumakwane area, Monwane lands)	1 week and difference observed

	Mmaba	<i>Securidaca longepedunculata</i>	Taken in soft porridge or boiled with water. Vagina steamed for candida	Root bark	Southern Botswana	Few days
	Pepere	<i>Schinus molle</i>	Bark and leaves boiled in water	Leaves, bark, fruit, resin	All over the country in homesteads	Few days
	Setlepetlepe	<i>Amaranthus spinosus</i>	Leaves cooked and eaten as food. Roots boiled with water	Leaves and roots	All of Botswana during rainy season around old livestock kraals	1 week and difference observed
	Morojwa	<i>Azanza garckeana</i>	Chewed when ripe like a gum	Fruits	Mostly homesteads around the country	The more you take the quicker you feel the effect
	Tshetlho	<i>Tribulus terrestris</i>	Dried and boiled with water or taken in tea	Leaves, roots, and fruits	All over the country during rainy season	1 week and one can start feeling the difference
	Moretologa	<i>Ximenia Americana & Ximenia caffra</i>	Fruits eaten ripe. Roots and root bark dried and boiled in water	Fruits, roots, root bark	All over the country, but not many of them	The more taken, the quicker the difference observed
Abnormal bleeding between periods	Letlhole	<i>Hydnora johannis</i>	Boiled in water whole or powdered	Bulb	Northern Botswana	Taken continuously during excessive bleeding while on periods until symptoms subside.
	Mpiletse-Betsho	<i>Helichrysum odoratissimum</i>	Roots and bark dried and boiled in water or milk	Roots and bark	North and northwest regions	Taken twice a day while on periods
	Modikaseope	<i>Withania somnifera</i>	Boiled in milk	Roots	Tswapong area and most of Central district near places hills or rocky areas	Twice a day for 15 days
	Masigomabe	<i>Plumbago zeylanica</i>	Boiled in milk or water and add a bit of sugar	Roots and root bark	Northern and Southeast Botswana in proximity of rivers	Taken twice a day until symptoms subside
	Setlepetlepe	<i>Amaranthus spinosus</i>	Leaves cooked and eaten as food.	Leaves and roots	All of Botswana during rainy season	1 week and difference observed

			Roots boiled with water		around old livestock kraals	
	Mositsane	<i>Elephantorrhiza elephantina</i>	Boiled in water	Dried roots/tubers	Southern Botswana (Kumakwane area, Monwane lands)	1 week and difference observed
	Morojwa	<i>Azanza garckeana</i>	Chewed when ripe like a gum	Fruits	Mostly homesteads around the country	The more you take the quicker you feel the effect
Unusual vaginal discharge	Selele	<i>Portulaca oleracea</i>	Cooked with food. Dried, pounded and boiled with water	Leaves, Stems	Most parts of Botswana during rainy season	Symptoms start to subside after few days
	Modikaseope	<i>Withania somonifera</i>	Boiled in milk.	Roots	North, Southwest and southeast	Twice a day for 15 days
	Masigomabe	<i>Plumbago zeylanica</i>	Boiled in milk or water and add a bit of sugar	Roots and root bark	Northern and Southeast Botswana in proximity of rivers	Taken twice a day until symptoms subside
	Setlepetlepe	<i>Amaranthus spinosus</i>	Leaves cooked and eaten as food. Roots boiled with water	Leaves and roots	All of Botswana during rainy season around old livestock kraals	1 week and difference observed
	Morojwa	<i>Azanza garckeana</i>	Chewed when ripe like a gum	Fruits	Mostly homesteads around the country	The more you take the quicker you feel the effect
Resistant and recurring infections (eg: HPV, HIV, Bacterial infections like <i>Staphylococcus</i> and <i>Helicobacter pylori</i>)	Mositsane +Thetele	<i>Elephantorrhiza elephantina</i>	Boiled in water	Dried roots/tubers	Southern Botswana (Kumakwane area, Monwane lands)	1 week and difference observed
	Selele	<i>Portulaca oleracea</i>	Cooked with food. Dried, pounded and boiled with water	Leaves, Stems	Most parts of Botswana during rainy season	Symptoms start to subside after few days

	Morojwa	<i>Azanza garckeana</i>	Chewed when ripe like a gum	Fruits	Mostly homesteads around the country	The more you take the quicker you feel the effect
	Tshetlho	<i>Tribulus terrestris</i>	Dried and boiled with water or taken in tea	Leaves, roots, and fruits	All over the country during rainy season	1 week and one can start feeling the difference
Persistent fatigue	Modikaseope	<i>Withania somnifera</i>	Boiled in milk	Roots	Tswapong area and most of Central district near places hills or rocky areas	Twice a day for 15 days
	Morojwa	<i>Azanza garckeana</i>	Chewed when ripe like a gum	Fruits	Northern distribution but found in homesteads on southern part of Botswana	The more you take the quicker you feel the effect
	Tshetlho	<i>Tribulus terrestris</i>	Dried and boiled with water or taken in tea	Leaves, roots, and fruits	All over the country during rainy season	1 week and one can start feeling the difference
Nagging cough or hoarseness	Mmaba	<i>Securidaca longepedunculata</i>	Taken in soft porridge or boiled with water	Root bark	Southern Botswana	Few days
	Mositsane	<i>Elephantorrhiza elephantina</i>	Root decoction boiled in water and taken orally	Tubers	Southern Botswana (Kumakwane area, Monwane lands)	1 week and difference observed
	Morojwa	<i>Azanza garckeana</i>	Chewed when ripe like a gum	Fruits	Mostly homesteads around the country	The more you take the quicker you feel the effect
	Mpiletse-betsho	<i>Helichrysum odoratissimum</i>	Roots and bark dried and boiled in water or milk	Roots and bark	North and northwest regions	Taken twice a day while on periods
Change in bowel	Modikaseope	<i>Withania somnifera</i>	Boiled in milk.	Roots	North, Southwest and southeast	Twice a day for 15 days

	Mmaba	<i>Securidaca longepedunculata</i>	Taken in soft porridge or boiled with water	Root bark	Southern Botswana	Few days
	Mogabala	<i>Coccinia sessifolia</i>	Fruit eaten raw or boiled. Roots, stem and leaves dried and boiled in water.	Fruit, roots, stem, and leaves	All over the country during rainy season	The more you take the quicker the symptoms subside
	Selele	<i>Portulaca oleracea</i>	Prepared as salad or even dried and taken with water	Whole plant	Most parts of Botswana during rainy season	Symptoms start to subside after few days
	Mositsane	<i>Elephantorrhiza elephantina</i>	Root decoction boiled in water and taken orally	Tubers	Southern Botswana(Kumakwane area, Monwane lands)	1 week and difference observed
	Hedge	<i>Dodonea viscosa</i>	Dried and pounded roots and leaves boiled in water	Leaves and roots	All over the country used as fence around homesteads	Taken as long as possible until a difference is observed

Table B1 compiles the most frequently used plants by the traditional healers. The first and second columns show the local and scientific names of the plants respectively. Column 4, displays the frequency of use of these plants by the traditional healers. Blue stars in the fourth column indicate the top five appearing plants in the questionnaires which were ultimately selected for this study. *Elephantorrhiza elephantina* appeared more often than the other plants followed by *Azanza garckeana* and *Withania somnifera* then *Portulaca oleracea* and *Azanza garckeana*.

Appendix B

Table B1: Plants that were mentioned the most during traditional healer questionnaires

Local name	Botanical name	Family	Frequency in questionnaire	Selected plant species	Supporting literature
Mositsane	<i>Elephantorrhiza elephantina</i> (Burch.) Skeels	Fabaceae	7	★	(Mpofu, 2014), (Olaokun <i>et al.</i> , 2020), (Saheed, 2017)
Selele	<i>Portulaca oleracea</i> L.	Portulacaceae	4	★	(Alipour <i>et al.</i> , 2022), (Farshori <i>et al.</i> , 2014)
Mogabala	<i>Coccinia sessifolia</i>		1		(Holstein, 2015), (Cai <i>et al.</i> , 2023)
Mmaba	<i>Securidaca longepedunculata</i> Fresen.	Polygalaceae	3		(Ngulde <i>et al.</i> , 2019), (Olaoye <i>et al.</i> , 2021)
Modikaseope	<i>Withania somonifera</i> (L.) Dunal	Solanaceae	6	★	(Ahmade <i>et al.</i> , 2017), (Kim <i>et al.</i> , 2020), (Raguraman <i>et al.</i> , 2016)
Tshetlho	<i>Tribulus terrestris</i> L.	Zygophyllaceae	5	★	(Abbas <i>et al.</i> , 2022), (Angelova <i>et al.</i> , 2013), (Hashim <i>et al.</i> , 2014)
Morojwa	<i>Azanza garckeana</i> (F.Hoffm.) Exell & Hille.	Malvaceae	6	★	(Azanza, 2016), (Elshiekh <i>et al.</i> , 2020), (Yasmin <i>et al.</i> , 2020)
Mpiletse-betsho	<i>Helichrysum odoratissimum</i> Sweet	Asteraceae	2		(Nkemzi <i>et al.</i> , 2022), (Hemaiswarya <i>et al.</i> , 2022)
Masigomabe	<i>Plumbago zeylanica</i> L.	Plumbagibaceae	2		(Kapare <i>et al.</i> , 2020), (Yadav <i>et al.</i> , 2019)
Setlepetlepe	<i>Amaranthus spinosus</i> L.	Amarathaceae	3		(House <i>et al.</i> , 2020), (Al-Tamimi <i>et al.</i> , 2021)
Moretologa	<i>Ximenia Americana</i> L. & <i>Ximenia caffra</i> Sond.	Olacaceae	1		(Sharief <i>et al.</i> , 2022), (Gomes <i>et al.</i> , 2019)
Pepere	<i>Schinus molle</i> L.	Anacardiaceae	1		(Hailan <i>et al.</i> , 2022), (Osman <i>et al.</i> , 2021)
Hedge	<i>Dodonaea viscosa</i> Jacq.	Sapindaceae	1		(Anandan <i>et al.</i> , 2019), (Al-Musawi & Al-Saadi, 2021)
Letlhole	<i>Hydnora johannis</i> Becc.	Aristolochiaceae	1		(Degfie <i>et al.</i> , 2022)

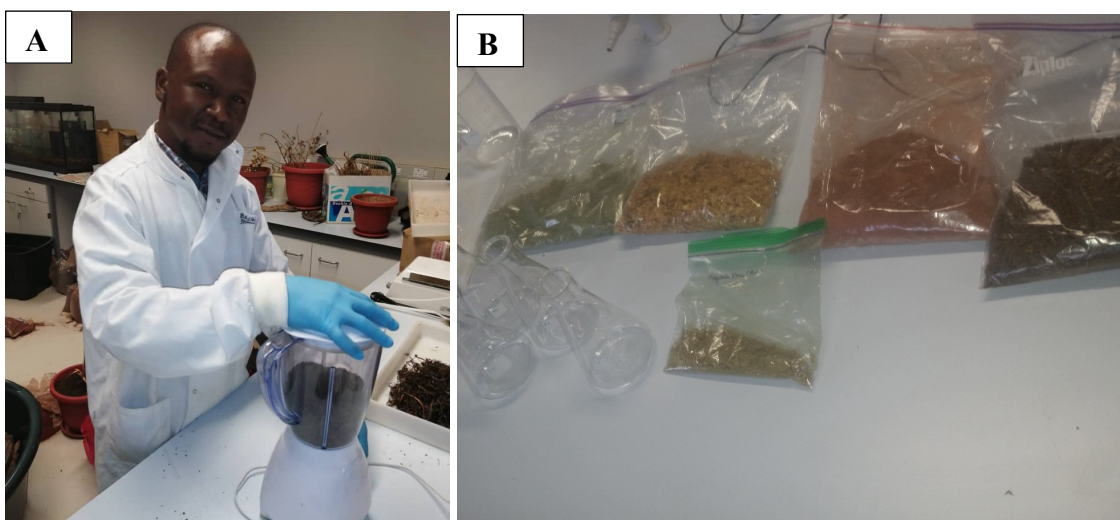


Figure B1: Plant samples being crushed in a blender (A) and samples being packaged in zip-lock bags for storage in a 4°C cold room prior to the extraction of phytochemicals from the samples (B).



Figure B2: Plant extracts being filtered immediately after sonication (A) and filtration done on the bench top (B).

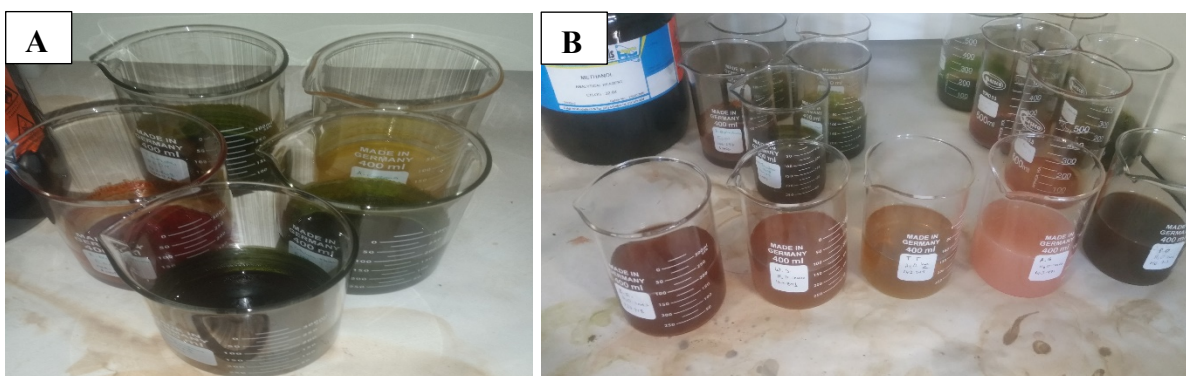


Figure B3: Plant extracts in beakers were allowed to dry in the fume-hood

Appendix C

Survey questionnaires that were administered on traditional healers

A survey on the use of medicinal plant to alleviate and/or cure signs and symptoms of cancer

Target respondent: Traditional healers (affiliated with Botswana **Dingake** Association), Street vendors and community around cities in Botswana

Intended purpose: the information gathered through this questionnaire will be useful for my research as a MSc biological science student

Section A: Demographic questions

1.
 - a. male ☐
 - b. female ☐
2. What is your age group?
 - a. 0-29 ☐
 - b. 30-39 ☐
 - c. 40-49 ☐
 - d. 50+ ☐

SECTION B: this is the section that shows the signs and symptoms of cancer, please fill accordingly

Vomiting or nausea

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Are there any other medicinal plant used

If yes please name them

Sexual transmitted infections

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

Are there any other medicinal plant used

If yes please name them

Change in bowel

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Are there any other medicinal plant used

If yes please name them

Unusual vaginal discharge

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Are there any other medicinal plant used

If yes please name them

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Are there any other medicinal plant used

If yes please name them

Persistent fatigue

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Are there any other medicinal plant used

If yes please name them

Nagging cough or hoarseness

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Abnormal bleeding between periods and after sex

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Are there any other medicinal plant used

If yes please name them

Recurring infections which will not clear with usual treatment

Which medicinal plant is mostly used to treat such illness?

Where is it found (suitable climatic conditions and soil)?

How is the portion prepared?

How long does it take for the medication to work?

Which compound is found in this plant that help cure and/or alleviate the disease

Are there any other medicinal plant used

If yes please name them

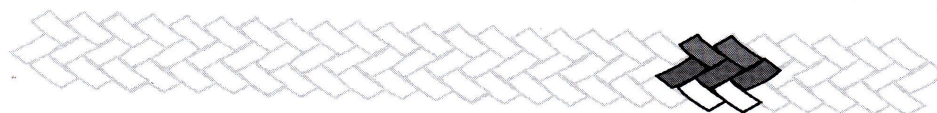
Appendix D

Table D1: Composition of DMEM (Dulbecco's Modified Eagle's medium) used during cell culture in this study (ThermoFisher Scientific)

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75.0	30.0	0.4
L-Arginine hydrochloride	211.0	84.0	0.39810428
L-Cystine 2HCl	313.0	63.0	0.20127796
L-Glutamine	146.0	584.0	4.0
L-Histidine hydrochloride-H ₂ O	210.0	42.0	0.2
L-Isoleucine	131.0	105.0	0.8015267
L-Leucine	131.0	105.0	0.8015267
L-Lysine hydrochloride	183.0	146.0	0.7978142
L-Methionine	149.0	30.0	0.20134228
L-Phenylalanine	165.0	66.0	0.4
L-Serine	105.0	42.0	0.4
L-Threonine	119.0	95.0	0.79831934
L-Tryptophan	204.0	16.0	0.078431375
L-Tyrosine disodium salt dihydrate	261.0	104.0	0.39846742
L-Valine	117.0	94.0	0.8034188
Vitamins			
Choline chloride	140.0	4.0	0.028571429
D-Calcium pantothenate	477.0	4.0	0.008385744
Folic Acid	441.0	4.0	0.009070295
Niacinamide	122.0	4.0	0.032786883
Pyridoxine hydrochloride	206.0	4.0	0.019417476
Riboflavin	376.0	0.4	0.0010638298
Thiamine hydrochloride	337.0	4.0	0.011869436
i-Inositol	180.0	7.2	0.04
Inorganic Salts			
Calcium Chloride (CaCl ₂) (anhyd.)	111.0	200.0	1.8018018
Ferric Nitrate (Fe(NO ₃) ₃ ·9H ₂ O)	404.0	0.1	2.4752476E-4
Magnesium Sulfate (MgSO ₄) (anhyd.)	120.0	97.67	0.8139166
Potassium Chloride (KCl)	75.0	400.0	5.3333335
Sodium Bicarbonate (NaHCO ₃)	84.0	3700.0	44.04762
Sodium Chloride (NaCl)	58.0	6400.0	110.344826
Sodium Phosphate monobasic (NaH ₂ PO ₄ ·H ₂ O)	138.0	125.0	0.9057971
Other Components			
D-Glucose (Dextrose)	180.0	4500.0	25.0
Phenol Red	376.4	15.0	0.039851222

Appendix E

Verification report of plant species by Department of National Museum and Monuments,
Herbarium Unit



**BOTSWANA
national
museum**

Department of National Museum
and Monuments
331 Independence Avenue
Gaborone, Botswana
Private Bag 00114
Tel: (267) 3610400
Fax: (267) 3902797

Our Reference: DNMM 8/2/1 (3)

3rd May 2023

Botswana International University of Science and Technology
Private Bag 16
Palapye
Tel: 4900117

Attention: Dr Gaobotse

Dear Sir,

VERIFICATION OF PLANT SPECIMENS

The Department of National Museum and Monuments, through the Herbarium Unit have received the plant specimens from your institution on the 28th April 2023 in order to provide assistance on the verification of the plant names.

The plants have been identified as follows:

Collector NO	FAMILY	SCIENTIFIC NAME	COMMON NAME	LOCAL NAME
001BRAG.F-BIUST	Malvaceae	Azanza garckeana	Snot apple	Morojwa
002BREE.P-BIUST	Fabaceae	Elephantorrhiza elephantina	Elands bean	Mositsane
003BRPO.P-BIUST	Portulacaceae	Portulaca oleracea	Common purslane	Serepe / Selele
004BRTT.P-BIUST	Zygophyllaceae	Tribulus terrestris	Devil's thorn	Mosetlho / Tshetlho
005BRWS.P-BIUST	Solanaceae	Withania somnifera	Winter cherry	Moarasope / Modikaseope

Thank you

Yours Faithfully



Daniel Mafokate

For/DIRECTOR, DEPARTMENT OF NATIONAL MUSEUM AND MONUMENTS

Our Vision: World leader in environmental sustainability



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