SCREENING OF THE CRUDE ACETONE EXTRACTS OF *TOONA CILIATA, SERIPHIUM PLUMOSUM* AND *SCHKUHRIA PINNATA* FOR THEIR POTENTIAL ANTICANCER ACTIVITIES AGAINST HeLa CERVICAL CANCER CELLS

BY

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Biochemistry has not been previously submitted by me for a degree at this or any other University, that it is my work in design and execution, and that all material contained herein has been duly acknowledged.

Surname and Initials _____

Signature: _____ Date: _____

DEDICATION

This work is dedicated to my family, which taught me that education is the key that opens all possibilities; and the Muyimane family, whose support and encouragement I will forever cherish.

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Firstly, I have to thank God for being my pillar of strength through the years of my struggle. I am grateful that he was my eyes and ears throughout, and even when things got hard and unbearable he was always by me.

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LIST OF ABBREVATIONS

A

AIDS	Acquired Immunodeficiency syndrome
ATCC	American Type Culture Collection

B

Bax	Bcl-2-associated protein X
Bcl-2	B-cell leukemia-2

C

CEF	Chloroform: ethyl acetate: formic acid
CO_2	Carbon dioxide
°C	Degrees Celsius

D

DCM	Dichloromethane
DCF	Dichlorofluorescein
DCFH	Dichlorofluorescin
DES	Diethylstilboestrol
dH_2O	Distilled water
DMSO	Dimethylsulfoxide
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid

E

EMW	Ethyl acetate: methanol: water

F

FAD	Flavin adenine dinucleotide (oxidized)
FADD	Fas-associated death domain
FBS	Fetal bovine albumin
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
$FeCl_3$	Ferric chloride
FRAP	Ferric reduction antioxidant potential

G

GAE	Garlic equivalence

H

H_2	Hydrogen
H_2O	Water
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
HIV	Human Immunodeficiency Virus
HPV	Human papillomavirus

I

INF-γ	Interferon gamma

K

$K_3Fe\ (CN)_6$	Potassium ferricyanide

M

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

N

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
$NaNO_2$	Sodium nitrite
NF-$_k$B	Nuclear factor kappa-light chain –enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase

O

O_2	Molecular oxygen
O_2^-	Superoxide anion
OCs	Oral contraceptives
OH	Hydroxyl radical

P

PBS	Phosphate-buffered saline
PSN	Penicillin, streptomycin and neomycin

R

RNA	Ribonucleic acid
RNOS	Reactive nitrogen and oxygen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RTCA	Real time cell analysis

T

TCA	Trichloroacetic acid

TEA	Toluene: ethanol: ammonium hydroxide
TLC	Thin layer chromatography
TNF1	Tumour necrosis factor 1
Tris	Tris (hydroxymethyl) aminomethane

U

UV	Ultraviolet

V

VT	Venous thrombus

W

WHO	World Health Organization

Z

Zn^{2+}	Zinc ion

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ABSTRACT

Cervical cancer is the fourth most common cancer in females, and the seventh of all cancer types in both genders, with an estimated 500,000 new cases each year. As with liver cancer, a large majority (around 85%) of the global burden occurs in the less developed regions, where it accounts for almost 12% of all female cancers. About 90% of cervical cases are associated with human papillomavirus (HPV) as a causative agent and this virus is frequently transmitted through sexual contact involving exchange of fluids (Walboomers et al., 1997). Due to the ineffectiveness, undesirable side effects and costly treatment for the disease the current study was aimed at determining the anti-proliferative effects of extracts of selected medicinal plants for their anticancer activity on HeLa cell line *invitro*. In order to accomplish the outcome of this research study, medicinal plants (*Toona cilliata*, *Seriphium plumosum* and *Schkuhria pinnata*) from Limpopo Province (South Africa) with history of traditional use on cervical cancer-associated patients were selected.

The *Toona cilliata* plant leaves were collected from Tzaneen, area while *Seriphium plumosum* and *Schkuhria pinnata* leaves were collected from Mankweng area. The dried leaves were grounded into powder and extracted using acetone. Thereafter, extracted leaf materials of selected plants were subjected to fingerprint profiling using TLC silicon coated plates immersed in tanks with different mobile phases (TEA, CEF and EMW) of various increasing polarities since. The plates were sprayed with vanillin/H_2SO_4, dried and visualised under UV light. Scavenging ability of the plant extracts was determined through investigating the presence of antioxidant activities using 0.2% of the 2,2-diphenyl-1- picrylhydrazyl (DPPH) indicator. The quantitative presence of total phenolic and flavonoids contents was also determined using garlic and quercetin as standards, respectively. Quantitative antioxidant scavenging activities were also determined and ascorbic acid was used as a positive control. This was followed by quantitative determination of ferric reducing power and thereafter the EC_{50} values of the extracts were determined by linear regression. Cell proliferation or viability was determined using the 3-[4, 5 dimethylthiazol-2-yl]-2-5 diphenyltetrazolium (MTT) assay with actinomycin as a

positive control and untreated cells as the negative control. Apoptotic effects of the extracts were determined using the Annexin V Fluos staining kit. This was followed by determining whether apoptosis was calcium dependent or independent using a calorimetric assay.

In comparing the acetone extract yield per 10 g leaves of plants, *Toona cilliata* leaves exhibited the highest yield followed by *Seriphium plumosum* and with the least yield from *Schkuhria pinnata.* The finger print profile showed the prominent separation and was achieved from all the plants when using the non-polar TEA solvent. All plants were shown to contain extracts with varying levels of antioxidant activity especially when using CEF and EMW mobile phases. When evaluating the total phenolic and flavonoids contents all plant extracts exhibited presence of phenolic compounds with high presence observed in *Seriphium plumosum* and *Toona cilliata*. Extracts from *Seriphium plumosum* and *Toona cilliata* showed to have higher concentrations of phytochemicals that may be of a benefit in antioxidant activities as compared to *Schkuhria pinnata* in relation to the positive control and a similar trend were observed in the ferric reducing power assay. Extracts from *Seriphium plumosum* were shown to have the best IC_{50} scavenging values followed by *Toona cilliata* and *Schkuhria pinnata* respectively*.* All the plants exhibited free radical scavenging abilities with *Seriphium plumosum* shown to possess higher activities in comparison with the positive control. All the plants exhibited a dose-dependent cytotoxicity activity against the HeLa cervical cell line. Evidence of induced apoptotic activity was observed in HeLa cells when using extracts from *Seriphium plumosum* and *Toona cilliata.* Induction of apoptosis by plant extracts was shown to be *c*alcium dependent as there was a decrease in calcium concentration with a decrease in the number of viable cells. In conclusion, the leaf extracts from *Toona cilliata*, *Seriphium plumosum* and *Schkuhria pinnata* contain compounds of various polarities with free-radical, antioxidant and anti-cancerous activities that may be beneficial if further studies are conducted to identify chemical compounds that may inhibit anticervical cancer activities.

CHAPTER ONE: INTRODUCTION

1.1 Cervical cancer and their causative agents

Cervical cancer is in the rise, it is the fourth most common cancer in women, and the seventh overall, with an estimated 500, 000 new cases each year. As with liver cancer, a large majority (around 85%) of the global burden occurs in the less developed regions, where it accounts for almost 12% of all female cancers. About 90% is caused by the human papillomavirus (HPV) which is normally transmitted through sexual intercourse including oral and anal sex (Walboomers *et al.,* 1997). It is believed that 94% of the people who are sexually active have contacted the virus that is responsible for the disease (Chesson *et al.,* 2014). It has also been suggested that cervical cancer affects one in 41 South African women, and available reports from Statistics South Africa (2014) estimates that 16.84-million women over the age of 15 are at risk of the disease in South Africa. At present more than eight South African women die from the disease every day, and the World Health Organisation (WHO) predicts that this figure could rise to 12 by 2025. This has prompted the observance in the month of September of each year as the cervical cancer awareness month in South Africa, to highlight the impact of the disease. Test procedures such as Pap smears can be conducted at any local public health facility in South Africa. The procedure is quick and simple, and the results will reveal whether or not an individual is at risk of having cervical cancer (Finocchario -Kessler *et al*., 2016)

Cervical cancer is a slow and progressive cancer where normally symptoms can go unnoticed and can be detected through early diagnosis. Vaccines are available for the prevention of the disease and are mostly administrated to girls at the age of 9 until 12 years to prevent the pathogenesis of the disease although they are expensive and not affordable to the general populace. In males the virus has a tendency of disappearing without causing any harm (Botha and Dochez, 2012).

1.2 Localisation of the disease

In females, the tumour has the potential of moving across the transformation zone, which is the area that separates the exo-cervix which is made out of glandular tissue and ecto-cervix, which is made of the squamous epithelial tissue (Autierl *et al.,* 1996). A representative diagram of the female reproductive organ is shown in figure 1.

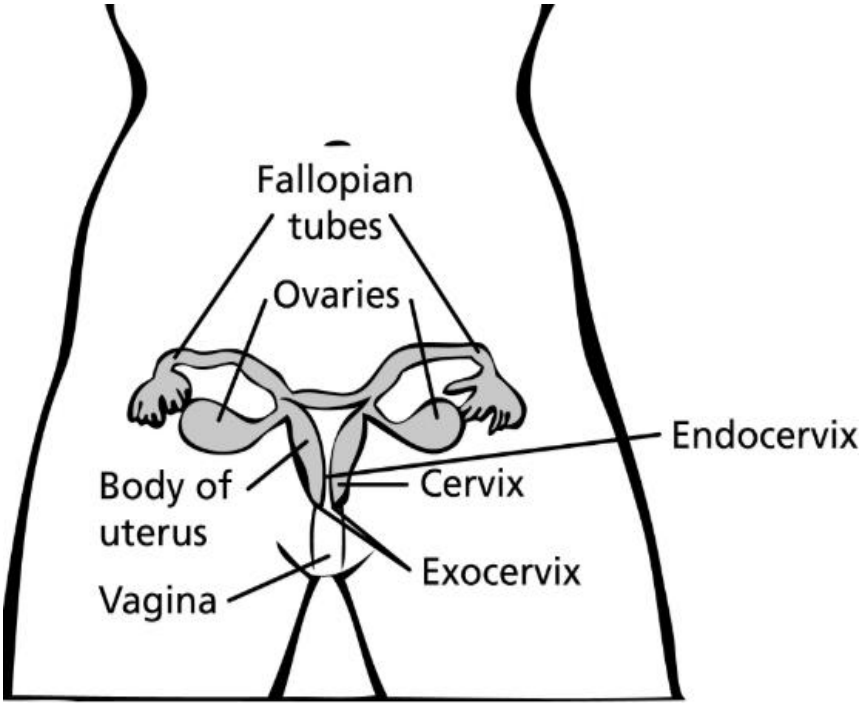

Figure 1: Diagrammatic representation of the different parts of the cervix. The diagram depicts the location of the cervix and the surrounding areas that would be immediately affected upon onset of cancer disease. The point of entry of viruses by sexual means is conspicuous it the architecture of the cervix anatomy (American Cancer Society 2014).

Tumours can be benign as they have the ability to be localised at the same position of a tissue or may be malignant, in this case they can migrate from one position to another while damaging health tissues after growing through the top layer of the cervical tissue. Malignant tumours invade underlying tissues while the process of relocating from the point of origin is termed metastasis, following which cells develop their own new stem cells leading to angiogenesis (Nishida *et al.,* 2006).

The cervix plays a major role in facilitating the movement of sperms from the vagina after deposition up to the uterus for fertilisation. The cervical mucus can also be used as a

measure of fertility in women during unprotected sex to prevent pregnancy. The thicker the mucus, the higher the indication of oestrogen produced during ovulation, which suggests higher chances of the possibility of conception (Nishida *et al.,* 2006).

1.3 Treatment for cervical cancer

A current treatment mostly used for benign tumours is surgery which can be directly used to remove the tumour with less chances of recurrence (Foley and Kathleen, 2011). Radiotherapy on the other hand has negative impacts on surrounding healthy cells following exposure of cancerous cells (Dueñas *et al.,* 2010). In cases of malignancy, chemotherapy, gene therapy and immunotherapy are mostly used. However, the tumours do have the ability to recur if it is not correctly done and is associated with side effects, expensive and inaccessible. Types of drugs available for the treatment of conditions are Cisplatin, Carboplatin, Paclitaxel (Taxol®) (Kitagawa *et al.,* 2015), Topotecan and Gemcitabine (Gemzar®). Side effects are associated with dark urine, body aches/pain, slow heart rate, white spots on the lips or tongue or inside the mouth and low blood counts respectively (Miller *et al.,* 1994). Cervical cancer treatment may be successful depending on various factors, including the stage of the cancer, the shape and size of the tumour, the general health of the woman, her age and her desire to have children in future (Miller *et al.,* 1994).

Cancer treatment depends on the type of cancer, the stage of the cancer (how much it has spread), age, health status, and additional personal characteristics. There is no single treatment for cancer and patients often receive a combination of therapies and palliative care. Treatments usually fall into one of the following categories: surgery, radiation, chemotherapy, immunotherapy, hormone therapy, or gene therapy. However, cervical cancer can be prevented through the use of vaccinating girls mostly between the age of eleven and twelve are catered for. The vaccines can be bivalent were human papillomavirus 16 and 18 are target and quadrivalent vaccines target HPV 6, 11, 16 and 18 (Botha and Dochez, 2012).

1.3.1 Surgery

Surgery is the oldest known treatment for cancer. If a cancer is benign, it is possible to completely cure a patient by surgically removing the cancer from the body. This is often

seen in the removal of a body part. After the disease has spread, however, it is nearly impossible to remove all of the cancer cells. Surgery may also be instrumental in helping to control symptoms such as bowel obstruction or spinal cord compression. Currently, when a tumour is removed surgeons also take out a “margin” of healthy tissue to make sure no malignant cells are left behind. It does need additional time to keep the patient sleeping so that the cells can be taken for testing in the laboratory. If there are no clear margins, the surgeon has to go back and remove more tissue (if possible) (Foley and Kathleen, 2011).

1.3.2 Radiation

Radiation treatment, also known as radiotherapy, destroys cancer by focusing high-energy rays on the cancer cells. This causes damage to the molecules that make up the cancer cells and leads them to commit suicide. Radiotherapy utilizes high-energy gamma-rays that are emitted from metals such as radium or high-energy x-rays that are created in a special machine. Early radiation treatments cause severe side-effects because the energy beams would damage normal, healthy tissues; however, technologies have improved so that beams can be more accurately targeted (Dueñas *et al.,* 2010). Radiotherapy is used as a standalone treatment to shrink a tumour or destroy cancer cells (including those associated with leukemia and lymphoma), and it is also used in combination with other cancer treatments (Gambhir *et al*., 2015).

1.3.3 Chemotherapy

Chemotherapy utilizes chemicals that interfere with the cell division process - damaging proteins or DNA - so that cancer cells will commit suicide. These treatments target any rapidly dividing cells (not necessarily just cancer cells), but healthy cells usually can recover from any chemical-induced damage while cancer cells cannot. Chemotherapy is generally used to treat cancer that has spread or metastasized because the medicines travel throughout the entire body. It is a necessary treatment for some forms of leukemia and lymphoma. Chemotherapy treatment occurs in cycles so the body has time to heal between doses. However, there are still common side effects such as hair loss, nausea, fatigue, and vomiting. Mixed therapies often include multiple types of chemotherapy or chemotherapy combined with other treatment options (Gambhir *et al*., 2015).

1.3.4 Immunotherapy

Immunotherapy aims to stimulate the body's immune system to fight the tumour. Local immunotherapy injects a treatment into an affected area, for example, to cause inflammation that causes a tumour to shrink. Systemic immunotherapy treats the whole body by administering an agent such as the protein interferon alpha that can shrink tumours. Immunotherapy can also be considered non-specific if it improves cancer-fighting abilities by stimulating the entire immune system, and it can be considered targeted if the treatment specifically tells the immune system to destroy cancer cells. These therapies are relatively young, but researchers have had success with treatments that introduce antibodies to the body that inhibit the growth of cancer cells. Bone marrow transplantation (hematopoietic stem cell transplantation) can also be considered immunotherapy because the donor's immune cells will often attack the tumour or cancer cells that are present in the host (Disis 2014).

1.3.5 Hormone therapy

Several cancers have been linked to some types of hormones, most notably breast and prostate cancer. Hormone therapy is designed to alter hormone production in the body so that cancer cells stop growing or are killed completely. Breast cancer hormone therapies often focus on reducing oestrogen levels (a common drug for this is tamoxifen) and prostate cancer hormone therapies often focus on reducing testosterone levels. In addition, some leukemia and lymphoma cases can be treated with the hormone cortisone (Cerhan *et al*., 2002)

1.3.6 Gene therapy

The goal of gene therapy is to replace damaged genes with ones that work to address a root cause of cancer or damage to DNA. For example, researchers are trying to replace the damaged gene that signals cells to stop dividing (the p53 gene) with a copy of a working gene. Other gene-based therapies focus on further damaging cancer cell DNA to the point where the cell commits suicide. Gene therapy is a very young field and has not yet resulted in any successful treatment (Saraswathi *et al., 2007;* Sonia *et al.,* 2008; Abuzeid *et al.,* 2011).

1.4 Predisposing factors of cervical cancer

Immunosuppression from chemotherapy, immune suppressive cytokine interleukin 10 and human immunodeficiency virus (HIV) damages the immune system and puts women at higher risk of HPV infection. This might, in part, explain the increased risk of cervical cancer in women with AIDS. A strong immune system may also be important in destroying cancer cells and slowing their growth and spread. In women with an impaired immune system from HIV, a cervical pre-cancer might develop into an invasive cancer faster than it normally would. Another group of women at risk of cervical cancer are those who have had an organ transplant (Chapman *et al.,* 2013).

Chlamydia is a relatively common kind of bacteria that can infect the reproductive system and can be spread by sexual contact. Chlamydia infection can cause pelvic inflammation, leading to infertility. Some studies have seen a higher risk of cervical cancer in women whose blood test positive for chlamydia infection compared to women with who do not. Women who are infected with chlamydia often have no symptoms and most often are aware that they are infected (Chumduri *et al.,* 2013).

There is evidence that suggests that taking oral contraceptives (OCs) for a long time increases the risk of cancer of the cervix (van Hylckama *et al.,* 2009). In one study, the risk of cervical cancer was doubled in women who took birth control pills longer than 5 years, but the risk returned to normal 10 years after they were stopped (Reid *et al.,* 2011). The American Cancer Society believes that a woman and her doctor should discuss whether the benefits of using OCs outweigh this very slight potential risk. A woman with multiple sexual partners should use condoms to lower her risk of sexually transmitted infections no matter what other form of contraception she uses.

Women who have had 3 or more full-term pregnancies have an increased risk of developing cervical cancer. One theory is that these women had to have had unprotected intercourse to get pregnant, so they may have had more exposure to HPV (Reid *et al.,* 2011). Also, studies have pointed to hormonal changes during pregnancy as possibly making women more susceptible to HPV infection or cancer growth (Brake and Lambert 2005). Another thought is that pregnant women might have weaker immune systems, allowing for HPV infection and cancer growth (Holtan *et al.,* 2009).

Women who are younger than 17 years and have had their first full-term pregnancy are almost 2 times more likely to get cervical cancer later in life than women who get pregnant at 25 years or older (Thompson cancer survival centre). Poverty is also a risk factor for cervical cancer. Many women with low incomes do not have ready access to adequate health care services, including Pap smear tests. This means they might not get screened or treated for cervical cancers and pre-cancers.

Diethylstilboestrol (DES) is a hormonal drug that was administered to some women to prevent miscarriage between 1940 and 1971. Women whose mothers took DES when pregnant are often called DES daughters. They develop clear cell adenocarcinoma of the vagina or cervix more often than would normally be expected (van Dirjk *et al.,* 2009). This type of cancer is extremely rare in women who are not DES daughters. There is about one case of this type of cancer in every 1,000 women whose mother took DES during their pregnancy. This means that about 99.9% of DES daughters do not develop these cancers. Related-DES clear cell adenocarcinoma is more common in the vagina than the cervix. The risk appears to be greatest in women whose mothers took the drug during their first 16 weeks of pregnancy.

The average age of women when diagnosed with DES-related clear-cell adenocarcinoma is 19 years. Since the use of DES during pregnancy was stopped by the Food and Drug Administration (FDA) in 1971, the youngest DES daughters are older than 35= (past the age of highest risk). However, there's no age cut-off even when women are safe from DES-related cancer. DES daughters may also be at increased risk of developing squamous cell cancers and pre-cancers of the cervix linked to HPV.

1.5 Free radicals in cancer formation

Oxygen free radicals are very reactive molecules which can react with every cellular component. They are normally produced in organisms involved in various biologic reactions. High levels of these partially-reduced O_2 species can give rise to functional and morphologic disturbances in cells. There is evidence to implicate oxygen free radicals as important pathologic mediators in many human disease processes (Martinen 1995).

More free radicals lead to oxidative stress that activates a variety of transcription factors like NF-κB and p53 to mention a few. Activation of these transcription factors can lead to

the expression of inflammatory cytokines, chemokines, cell cycle regulatory molecules, and anti-inflammatory molecules. The activation of inflammatory pathways leads to transformation of a normal cell to tumour cell, tumour cell survival, proliferation, chemo resistance, radio resistance, invasion, angiogenesis and stem cell survival (Reuter *et al*., 2010).

1.6 Symptoms of cervical cancer

Symptoms of cervical cancer do include irregular vaginal bleeding, vaginal discharge with an unpleasant odour, watery vaginal discharge, vaginal discharge tinged with blood, pelvic or back pain, and pain during sex, problems when urinating, problems when defecating and swelling of the legs (Bien 2016).

Irregular bleeding is the most common symptom of invasion cervical cancer. This bleeding may occur after sexual intercourse. It may also occur between menstrual periods. It can even occur in a postmenopausal woman whose menstrual periods have stopped. Vaginal bleeding in postmenopausal women indicates a serious medical problem. It requires a visit to a doctor (Bien 2016).

In younger women, minor bleeding irregularities can be easy to ignore. Spotting between periods may mean nothing at all. However, it can also be a sign of cervical cancer. Vaginal bleeding between periods or after sex is an alarming point of a cervical cancer symptom ((Bien 2016).

1.7 Draw backs of current cancer treatment and management methods

The current methods used for cancer treatment and management are their unaffordability, high price cost, inaccessibility and do elicit vast side effects. In case of surgery, if the disease is widely spread it is nearly impossible to remove all cancer cells. Traumatic experiences increase as some parts of surgery are difficult to deal with, whereas there would be no validity for sub-clinical metastases, while there are limitations in some treatments. Radiation therapy equipment is expensive, and therapy requires long period, which normally takes up to 1 to 2 months. The therapy leads to more complications, and even promotes loss of function caused by sector, while treatment is not good because it cannot completely eradicate cancer cells.

As for chemotherapy, drugs don't kill cancer cells only but also kill normal cells, this will therefore shorten the survival of patients over time. The department of tumour is not sensitive to the use of chemotherapy drugs because there is no clinical value. Chemotherapy cannot completely kill all the cancer cells *in vivo*, in a certain time, cancer will relapse or metastases.

1.8 The economic impact of cervical cancer globally and in South Africa

Lancet predicts that South Africa could see an increase of 78% in the number of cancer cases by 2030 and 75% increase globally is expected, leading to a total incidence of all new cancer-cases from 12.7 million in 2008 to 22.2 million by 2030. Cancer is one of the world's leading causes of death and has the greatest economic impact in the form of premature death and disability. The total global economic impact of premature death and disability resulting from cancer was $895 billion in 2008. The figure represents 1.5% of the world's gross domestic product (Snyman 2014).

1.9 The need for alternative treatment methods

The use of medicinal plants in treating different diseases has increased dramatically worldwide. Medicinal plants have a long historic therapeutic use and are rich in the antioxidants in the form of polyphenols which are useful in neutralizing reactive oxygen intermediates (Wang *et al.,* 2011). These plants can be used as directly prescribed or as processed to be ready for use in treating chronic diseases (Tang and Halliwell, 2010).

CHAPTER TWO: LITERATURE REVIEW

2.1 Phytochemicals and their role in human health

Phytochemicals are chemical compounds contained within plants and forms part of their metabolic needs. The compounds contained in plants are divided into primary metabolites such as sugars and fats, which are found in all plants and secondary metabolites, which differ from plant to plant and possess a more specific function (Meskin and Bidlack, 2002). Some plant secondary metabolites are used to fight against predations, while others are used as pheromones to attract insects for plant reproduction. Secondary metabolites and pigments possess therapeutic value most of which has been targeted in drugs research (Demain and Fang 2001).

As such, the bioactive non-nutritional chemical constituents in fruit, vegetables, grains, and other plant foods are associated with minimizing the risk of many life treating chronic diseases. Approximately 5000 phytochemicals are known for their health benefit. However, a majority of these phytochemical are yet to be discovered (Teplitski *et al*., 2000). A majority of the most abundant secondary metabolites contained in plants are considered to have antioxidant properties. Human cells are frequently interacting with various oxidizing agents, be it from inhaled air, consumed food, and water, or generated within the body via metabolic activities. The important factor is to keep in equilibrium and maintaining a balance between oxidants and antioxidants in order to sustain optimal physiologic conditions in the body. Overproduction of oxidants is associated with oxidative stress. To balance oxidative stress induced by free radicals, sufficient amounts of antioxidants are required as a supplement. Fruits and vegetables contain a wide variety of antioxidant compounds (phytochemicals) that may help protect cellular systems from oxidative damage and lower the risk of chronic diseases (Liu *et al.,* 2003).

Up to date studies reveals that natural compounds isolated from medicinal plants possess antioxidant activity; regulate gene expression in cell proliferation, cell differentiation, and tumour suppressor genes. The possible mechanism is associated with initiation of cell-cycle synchronization and programmed cell death, control of enzyme functions in detoxification, oxidation and reductions, recruitment of immune defence mechanism,

regulation of pathways associated with hormone metabolism, antibacterial and antiviral activity, inhibition of signal transduction pathways, anti-angiogenesis, inhibition of cell to cell interaction and invasion (Liu *et al.,* 2004).

2.2 Important classes of phytochemicals in cancer management

Consuming of phytochemical-rich foods such as colourful fruit, vegetables and herbs is associated with a lower risk of cancer and relapse after treatments, their anti-oxidant properties help to protect our DNA from environmental carcinogens.

Polyphenols (phenolics) are compounds that contain phenol rings. The phenolic compounds found in plants have been reported to be good reactive oxygen species scavengers and have shown promising effects as therapeutic drug for diseases caused by free radicals (shekhawat *et al*., 2010). Flavonoids on the other hand are 15-carbon compound that are present throughout the plant kingdom. Of importance, flavonoids are mainly known for their antioxidant, anti-carcinogenic, antimicrobial and anti-tumour properties (Borokini and Amotayo, 2012).

Polyphenols have direct anti-cancer mechanism of action via inflammation, modulation of cellular and signalling events involved in growth, invasion and metastasis (Thomas *et al.,* 2015). Antioxidants are compounds in food that play an important role by protecting our bodies against cancer. A variety of antioxidant compounds are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Antioxidants have the ability of trapping free radicals preventing them from leading to degenerative diseases (Miller *et al.,* 2000).

2.3 Mechanisms of anti-cancer activity phytochemicals

The biochemical mechanisms through which phytochemicals exert their anti-cancer effects are still being explored. The most published cancer prevention mechanism is through their antioxidant activity, elicited either through direct free radical absorption or through induction of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione via a variety of molecular mechanism. One of these mechanisms is activation of Nrf2, which switches on genes that code for antioxidant, as well as detoxification enzymes (Reuland *et al*., 2013).

Since medicinal plants have shown to be effective in producing phytochemical that inhibits progression in various molecular pathways *invitro*, specifically for the genes associated with cell proliferation, angiogenesis, metastasis and suppression of apoptosis which appears to work hand in hand with NF-κB as they promote oncogenesis and cancer therapy resistance. Recent experiments, through *in vitro* studies and the use of xenograft models of cancer, shows NF-κB inhibition as an important new approach for the treatment of certain hematological malignancies and as an adjuvant approach for a variety of cancers (Orlowski and Baldwin 2002).

On the other hand, some medicinal plants have shown to play a major role in enhancing apoptosis, since they detach anti-apoptotic family proteins Bcl2 from the cell membrane allowing the increase in attachment of the pro-apoptotic proteins the Bax. The attachment of the Bax family proteins leads to the activation of the gene p53 which is a tumour suppressor. The tumour suppressor protein plays a role in the system that pathologically eliminates damaged cells from an organism. Multiple signal pathways monitor the state of a cell and when damage or a fault is found that could cause heritable changes. In such instances p53 protein is activated to either coordinate the repair process or induce cell suicide. This also leads to the activation of caspases which are end proteases that hydrolyse peptide bonds. Although caspase-mediated processing can result in substrate inactivation, it may also generate active signalling molecules that participate in ordered processes such as apoptosis and inflammation. Accordingly, caspases have been broadly classified by their known roles in apoptosis like caspase 3, 6, 7, 8, and 9 (Wang *et al.,* 2015).

These studies have unrevealed multifaceted apoptotic mechanisms that are interleaved with other significant pathways, such as cell cycle, cellular metabolic and receptor transduction pathways. Apoptotic pathways that have been targeted by researchers for potential anticancer drug development (Baig *et al.,* 2016). The figure below represents the pathways.

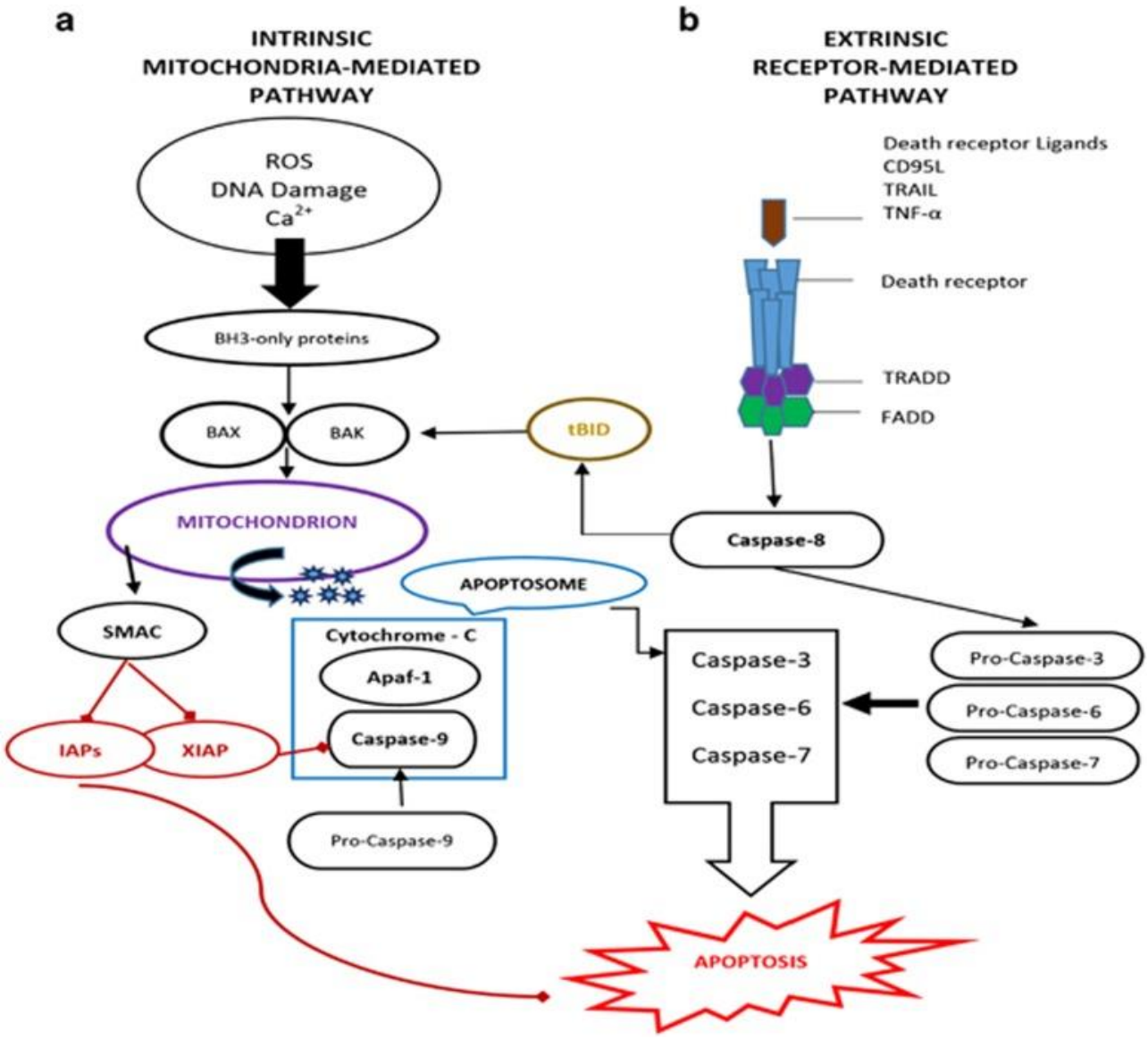

Figure 2: Representation of the pathways and caspases involved in apoptosis. The mitochondria-mediated intrinsic (**a**) and death receptor-mediated extrinsic (**b**) pathway. Apaf-1, apoptotic protease activating factor 1; FADD, Fas-associated death domain; TRADD, TNFR-associated death domain protein (Baig *et al.,* 2016).

2.4 Medicinal plants in South Africa with indication for cancer treatment

Reports on medicinal plants used in South Africa for cervical cancer related treatments are at a low level. Four plants used by traditional healers in treating cancer related abnormalities have been highlighted. Plants indicated in table 1 are currently known for their use as remedies in the treatment of cancer (Aboyade *et al.*, 2014), (Kotina *et al.,* 2014), (Bungu *et al.,*2006) and (Seloi *et al.,* 2008).

Table 1: Plants currently known for their use for anticancer activities in South Africa.

Scientific name	Traditional use	Parts used	Production areas
Sutherlandla frutescens	Treat fever, poor appetite, peptic ulcer, dysentery, cancer, diabetes, colds and flu, cough, asthma, kidney, liver failure, urinary tract infection and stress	Leaves and stems	Northern Cape, Eastern Cape, Kwazulu-Natal, Western Cape and Mpumalanga province of South Africa
Warburgia salutaris	Treat colds, fever, malaria, influenza, coughs, abdominal pain, constipation, cancer, rheumatism and stomach ulcers	Entire plant	North-eastern parts of Limpopo, KwaZulu-Natal and Northern Gauteng province of South Africa
Tulbaghia violacea	Treat fever, rheumatism, asthma, constipation, coughs, colds, destroy intestinal worms and cancer	Rhizomes, leaves, bulb and flowers	Eastern Cape, Kwazulu- Natal and Limpopo provinces South Africa
	Treat benign prostate, urinary tract infections,	Tuber (corm)	Eastern Cape, Kwazulu-Natal,

Hypoxis hemmerocallidea	testicular tumours, dizziness, heart weakness, nervous and bladder disorders	leaves and bulbs	Mpumalanga, Limpopo, Gauteng, North-west and Free State province of South Africa

2.5 Plants used in the study

The three selected plants *Toona cilliata, Seriphium plumosum* and *Schkuhria pinnata* were investigated in this study. Selection of these plant species was based on the information given by traditional healers during onsite interviews in relation to their historic use in the treatment of patients with cancer related symptoms.

Toona cilliata

Toona cilliata, belongs to the Meliaceae family and is commonly known as Toonee, Tuni in Hindi; Red ceder in English and Nandi in Sanskrit. It is mainly distributed in tropical Himalayas from the Indus Eastward and throughout the hills of Central and Southern India (Ayurvedic) (Negi 1993). *Toona cilliata* originates from tropical Asia and tropical Australia but is now much cultivated throughout the tropics for its timber and as an ornamental or as a wayside tree. It is extensively planted in tropical Africa, particularly in East and Southern Africa, but also locally in West Africa, Madagascar and Mauritius. It was recorded from Zambia and Zimbabwe as early as the beginning of 20 century. It has locally become naturalized in Southern Africa (Loupee D 2008). *Toona Cilliata* is traditionally used in chronic dysentery, ulcer, leprosy, fever, headache, blood complaints, as a cardiotonic, aphrodisiac, anthelmintic good for scabis and as an expectorant (Sunil Kumar). In Southern Africa, herbalists use *Toona cilliata* leaf infusion to treat venereal diseases. The bark is used as an astringent and tonic, to treat dysentery and to heal wounds. It is commonly planted as an ornamental tree, and in particular as a roadside tree, and reforestation. The flowers yield a reddish or yellowish dye, which has been used in tropical Asia to colour silk. The bark may be used for tanning leather and has been traditionally used to make twine and string bags. The plant has also been reported to reduce gastric volume, free acidity, total acidity and ulcer index. The plant extract also

has gastro protective activity and protect water immersion in stress induced ulcers (Malairajan *et al*., 2007). Carbohydrates, proteins, phytosterols, flavonoids, glycosides, tannins and phenolics are the compounds presence. The plant extract also has gastro protective activity and protect water immersion in stress-induced ulcers (Katende *et al.,* 1995).

Schkuhria pinnata

Schkuhria pinnata, belongs to the Asteraceae family and is commonly known as Canchalagua in Spanish. It also does have synonyms that are used differently from country to country. It is found abundantly in the inter-Andean valleys of Peru and has been introduced and cultivated in other countries. It can be found in Latin American, Mexico, Africa and even parts of southern Arizona and Texas where it has escaped cultivation and flourished as an annual weed.

The worldwide ethnomedical uses of *Sckhuhria pinnata* are as follows blood cleanser, many skin problems, as diuretic and for antimicrobial urinary tract problems, for malaria, pimples, blackheads and acne. Usually the entire plant is uprooted and chopped up and brewed into an infusion (fresh plant) or a decoction (dried plant).

Seriphium plumosum

This is a fynbos plant that is quite common and conspicuous for its beautiful grey-green to light grey – silvery foliage. On mismanaged or overgrazed fields or disturbed places, the plant can flourish and be hard to eradicate. It is a member of the Daisy Family (Asteraceae). Very little has been published on the physiological, phenological and ecological aspects of this plant. It is generally accepted to be mainly found on sandy and rocky soils with a low pH (Smit 1955, Krupko and Davidson 1961).

Soils with a clay content of up to 24% could still be encroached if the drainage is good, which could favour the establishment of this woody species (Wepener 2007). *Seriphium plumosum*, generally known as slangbos, bankrupt bush or vaalbos (formerly known as *Stoebe vulgaris*) is indigenous to South Africa and already widely distributed in various parts of the country (Eastern Cape, Free State, Mpumalanga, North West Province and Gauteng) (Schmidt *et al*., 2002, Snyman 2009). It is not wholly true that encroachment is

due to only mismanagement like overgrazing (Hatting 1953), as *Seriphium plumosum* rapidly spreads on a farm after first occurring there (Richter 1989).

The medicinal plant uses are heart problems, epilepsy, postural realignment, scoliosis, facades, keloids and cicatrisation.

2.5 Purpose of the study

2.6.1 Aim of the study

To determine the proliferative and carcinogenic effects of acetone extracts of selected medicinal plants on HeLa cells *invitro.*

2.6.2 Objectives

The objectives of the study were to:

i. Extract leaf material of selected plants (*Toona cilliata, Seriphium plumosum* and *Schkuhria pinnata*) using acetone.
ii. Determination of the fingerprint profile of acetone leaf extracts of selected plants using TLC.
iii. Qualitative determination of the presence of the antioxidants in the acetone leaf extracts.
iv. Determine the presence of phenolics, flavonoids, tannins, saponins, steroids, phlobatannins, glycosides, coumarins, proteins, anthraquinones and anthocyanins in the acetone extract of the selected plants.
v. Determine the total phenolic and flavonoids content of the acetone leaf extracts of the selected plants.
vi. Quantitative determination of antioxidant constituents from acetone extracts of the selected plants.
vii. Quantitative determination of ferric reducing power from acetone extracts of the selected medicinal plants.
viii. Evaluate the cytotoxic effect of acetone leaf extract of the selected plants on HeLa cells.
ix. Determine the apoptotic effect of acetone leaf extracts of selected plants on HeLa cells using fluorescence.

x. Determine the effect of the acetone extract of selected plants on calcium influx in HeLa cells after treatment with different concentration of the extracts.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Collection of plants

Plants used in the study were collected based on their traditional indication in the treatment of patients exhibiting symptoms of cervical cancer in Tzaneen and Mangweng (Limpopo Province) with the help of a traditional healer, Mr Mboweni. With *Seriphium plumosum* and *Schkuhria pinnata* being collected around Mangweng, while *Toona cilliata* was collected from Tzaneen. The leaves of the selected plants following collection were separated from their stems, dried at room temperature and ground using a commercial blender. The powdered material was stored at room temperature in glass bottles in the dark. The voucher specimens of the medicinal plants used are as follows *Seriphium plumosum* UNIN 121065, *Toona cilliata* UNIN 12331 and *Schkuhria pinnata* UNIN 121066. No human or animal models will be utilised.

3.2 Extraction of plant materials

The fine ground powder (10 g) of each plant material was subjected to extraction in a 1: 10 ratios with 100 ml acetone. The mixture was enclosed in glass bottles and placed in a shaker (Rochelle, South Africa) to allow a total extraction of as much compounds as possible. The extracts were filtered with Whatman filter paper no: 3 and were poured into empty pre-weighed containers. The extracts were then dried under a stream of air and the containers with dried solutes were weighed to obtain the mass of the extracts.

3.3 Phytochemical profile of plant material

Thin layer chromatography is considered the quickest and cheapest method used to separate compound according to their polarity (Stahl, 1969). As such extracts of the selected plants were reconstituted in acetone (1 mg/ml) and spotted on aluminium backed thin layer chromatography plates (TLC) (Macherey-NEGEL, GMBH & CO.KG) for analysis. An amount of 10 µl of each extract was loaded onto a TLC plate using a micropipette. The plates were further developed in three different mobile phase viz: chloroform: ethyl acetate: formic acid [CEF] 10:8:2 v/v/v) (intermediate polarity/acidic), ethyl acetate: methanol: water [EMW] 10:1, 35:1 v/v/v) (polar/neutral) and toluene: ethanol: ammonium hydroxide [TEA] 18:2:0.2 v/v/v) (non-polar/basic). The separated

compounds were visualized under ultraviolet light at wavelengths of 254 and 360 nm. Thereafter, TLC plates were sprayed with vanillin-sulphuric acid. Plates sprayed with vanillin-sulphuric (0.1 g vanillin (sigma): 28 ml methanol) acid were heated at 110˚C to visualize coloured bands on the plates.

3.4 Determination of antioxidant activity (DPPH assay)

The plant extracts were tested for scavenging activity using both qualitative and quantitative methods as described by (Katsube *et al.*, 2004). Thin layer chromatography (TLC) plates were used to separate compounds of the extracts with antioxidant activity. The 2, 2- diphenyl-1-picrylhydrazyl (DPPH) indicator, was sprayed on the plates to assist in viewing the antioxidant compounds. The quantitative method was performed using micro-titer plates, where 100 μl of each plant extract was serially diluted with water and was tested for antioxidant activity at various concentrations. DPPH (100 μl) was added into the wells after dilution. Ascorbic acid (vitamin C) was used as positive control and absorbance was read at 540 nm using M8500 UV-Visible spectrophotometer made in China.

3.5 Qualitative screening of various phytochemical constituencies from the selected medicinal plants

The phytochemicals tested for in the study using various methods are outlined below. The qualitative screening results were based on color change, persistence froth observed, ring and precipitates.

3.5.1 Phenols

The plant leaf extract of 0.1 g was added to 10 ml of distilled water. The solution was heated in a boiling water bath for 3 min and filtered. A 2 ml aliquot of the filtrate was placed in each of 3 test tubes. The filtrate in one of the test tubes was diluted with distilled water in the ratio 1:4. A blue or greenish colour of the sample was observed to draw an inference, indicating the presence of phenols (Harbone 1998).

3.5.2 Tannins

Briefly, 0.5 g powdered leaf samples were dissolved in 5 ml of distilled water, boiled gently followed by cooling. A volume of 1 ml of the solution was aliquoted into a test tube and 3

drops of ferric chloride solution was added. The greenish-black colour of the sample was observed to draw an inference, indicating the presence of tannins (Borokini and Omotayo, 2012).

3.5.3 Flavonoids

A total of 5 ml of diluted ammonia solution was added to a portion of the filtrate of each plant extract, followed by the addition of concentrated [H_2SO_4]. Yellow colour change was observed to draw an inference, indicating the presence of flavonoids (Borokini and Omotayo, 2012).

3.5.4 Saponins

Persistent froth forming test for saponins was used. Water (30 ml) was added to powdered 1 g of the leaf extract after which it was followed by a vigorous shaking and heating. The observation of persistence froth was observed to draw an inference, indicating the presence of saponins (Borokini and Omatayo, 2012).

3.5.5 Steroids

The Salkowski's test was used. About 2 ml of $CHCl_3$ was added to 2 ml of the leaf extract after which 2 ml of concentrated H_2SO_4 was added to the solution. A reddish-brown ring at the interface was observed to draw an inference, indicating the presence of steroids (Borokini and Omotayo, 2012).

3.5.6 Phlobatannins

Precipitate test method was performed were 2 ml Hcl (1%) was added into another 2 ml of the plant extract followed by heating. A red precipitate was observed to draw an inference, indicating the presence of phlobatannins (Yadav *et al.*, 2014*)*

3.5.7 Glycosides

Liebermann's test was performed to determine the presence of glycosides where 2 ml of the extract was mixed with 2 ml $CHCl_3$ and finally [CH_3COOH] added into the mixture. A violet to blue to green coloration was observed to daw an inference, indicating the presence of glycosides (Yadav *et al.*, 2014).

3.5.8 Proteins

Xanthroproteic test was performed by mixing 1 ml of the extract with 1 ml H_2SO_4 (conc.) after which a white precipitate was observed to draw an inference, indicating the presence of proteins (Yadav *et al.*, 2014).

3.5.9 Coumarins

Volume of 2 ml extract was mixed with 3 ml of (10%) NaOH, a yellow coloration was observed to draw an inference, indicating the presence of coumarins (Yadav *et al.*, 2014*).*

3.5.10 Anthraquinones

Borntrager's test method was used where 3 ml of the extract was mixed with 3 ml benzene followed by the addition of 5 ml NH_3 (10%). A pink colour was later observed to draw an inference, indicating the presence of anthraquinones (Yadav *et al.*, 2014*).*

3.5.11 Anthocyanins

Volume of 2 ml extract was mixed with 2 ml HCl (2N) and NH_3. A pinkish red to bluish violet coloration was observed to draw an inference, indicating the presence of anthocyanins (Yadav *et al.*, 2014*).*

3.6 Quantitative determination of secondary metabolites

3.6.1 Determination of total phenolic content

The total phenolics in extracts was determined spectrophotometrically using Folin-Ciocalteu phenol reagent method according to (Humadi and Istudor, 2009). Garlic acid was used as a standard and the total phenolics were expressed as mg/g gallic equivalence (GAE). Concentraction of 0.01, 0.02, 0.03 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. Concentration of 0.1 and 1 mg/ml of plant extract were also prepared in methanol and 0.5 ml of each sample were introduced into the test tubes and mixed with 2.5 ml of a 10-fold dilute Folin- Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 min at room temperature before the absorbance was read at 760 nm using (Beckman Coulter DU® 730 and Life Science UV-Visible spectrophotometer). All determinations were performed in triplicate. Folin-Ciocalteu reagent being sensitive to reducing compounds including polyphenols produce a blue color upon reaction which was measured spectrophotometric

(Savitree *et al.,* 2004). (Beckman Coulter DU® 730 and Life Science UV-Visible spectrophotometers was used) South Africa.

3.6.2 Determination of total flavonoids

The presence of flavonoids in the plant extracts was determined using aluminium chloride colimetric assay as described by (Zhishen *et al.,* 1999). Volume of 1 ml of plant extract was mixed with 4 ml of distilled water and subsequently with 0.30 ml of a $NaNO_2$ solution (10%). After 5 minutes, 0.30 ml solution $AICI_3$ (10%) was added followed by 2 ml of 1% NaOH solution to the mixture. Immediately, the mixture was thoroughly mixed and absorbance was determined at 510 nm versus the blank using T60 UV- Visible spectrophotometer (Roche, S.A). Standard curve of quercetin was prepared (0-12 mg/ml) and the results were expressed as quercetin equivalents (mg quercetin/gm dried extract).

3.6.3 Quantitative determination of antioxidant activity

The antioxidant activity of the selected plants and the antioxidant standard were assessed on the basis of radical scavenging effect of the stable 2, 2- diphenyl-1-picrylhydrazyl (DPPH) free radical. One hundred microliter of a 0.2% solution of DPPH radical in methanol was mixed with 100 µl of plant extracts. The concentrations of extracts ranged between 12.5 – 200 µg/ml. After mixing, they were left for 30 min at room temperature. The DPPH radical inhibition was measured at 540 nm (Moein and Moein, 2010) using Beckman Coulter Du®730 Life Science UV-Visible spectrophotometer. Tests were carried out in n=3 and Ascorbic acid (vitamin C) was used as positive control.

3.6.4 Ferric ion reducing power

The ferric ion reducing power of the different extracts were determined. Different concentrations (0 µg/ml-1000 µg/ml) of the extracts in deionised water (100 µl) were prepared. A blank was prepared without adding extract, while ascorbic acid was used as the reference standard. These were then mixed with phosphate buffer (250 µl) (pH 7.4) and concentration 0.2 M) together with potassium ferri-cyanide (250 µl) and incubated at 50°C for 20 min. After incubation, aliquots of trichloroacetic acid (250 µl) were added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant (250 µl) was mixed with distilled water (250 µl) and freshly prepared ferric chloride solution (50 µl). The absorbance of the samples was measured at 700 nm. Substances, which have reduction

potential, react with potassium ferri-cyanide (Fe^{3+}) to form potassium ferro-cyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric-ferrous complex that has an absorption maximum at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power which was measured at 700 nm using a microtiter-plate multimode detector (Promega-GlomaxMulti detection system). Percentage reducing power was calculated according to the following formula: Percentage reducing power = ((A_{700nm} of sample – 1) x 100)/ (A_{700nm} of blank).

3.7 Cell culture mantainance

HeLa cells obtained from the American Type Culture Collection (ATCC, Rockville, USA) were cultured and maintained in DMEM supplemented with 10% foetal bovine serum (Highveld Biologicals (Pty) Ltd, Lyndhurst, RSA) at 37˚C in an atmosphere of 5% CO_2. For experiments, cells were seeded at a density of 2×10^4 cells/well and then treated with various concentrations (50-1000 μg/ml) of the extract of the selected plants. Actinomycin D was used as a positive control at a concentration of 25 μg/ml.

3.8 Evaluation of the effects of the selected plant extracts on HeLa cell proliferation using the MTT assay

The MTT principle is a standard colorimetric assay for measuring cellular proliferation or cell viability. HeLa cells were seeded in 96 well tissue culture plates at a density of 2 x 10^4 cells/ml and treated with various concentrations (50 – 1000 μg/ml) of extract for 24 hours. In brief, after 24 hours of incubation, MTT solution (2.5 mg/ml) was added to each well (20 μl). The cells were incubated for a further 4 hours at 37˚C and the supernatant was removed. The pellet was then dissolved by the addition of 100 μl of DMSO. The anti-proliferative activity of extracts was determined following the conversion of tetrazolium by the enzyme dehydrogenase to purple formazan (Mossman, 1983) which directly represent the number of viable cells. The absorbance was measured at 560 nm using a Glomax microtiter plate (Promega, U.S.A). Actinomycin D was used as positive control while untreated cells where used as negative control.

Percentage viability = (A_{490nm} of sample) x 100) / (A_{490nm} of control)

3.9 Determination of induction of apoptotic activity effect of the selected plant extracts on HeLa cells

The cells were seeded over a cover slip and put inside a six well plate with culture media containing 10% FBS irrespectively for 24 hours. The cells were then treated with different concentrations of the extracts followed by incubation for 24 hours. The slips were washed by 1xPBS followed by addition of paraformaldehyde and incubated for 30 min. After incubation it was followed by a washing step with PBS and Annexin FITC solutions was added onto the cover slips followed by incubation for 30 min. The cover slips were mounted on the slides with glycerol applied on them to prevent friction. This was followed by viewing them under fluorescent of X60 magnitude using different activation of the blue, green and red lights. Viable cells will be observed as green. On the other hand, dead cells will be observed as red following overlaying with green colour surrounding the outer membrane (Rieger *et al.,* 2011); this represents a cell which would have under gone apoptosis.

3.10 Determination of effects of selected plant extracts on calcium influx in HeLa cells

Calcium colorimetric assay was used to determine if apoptosis induced by the acetone leaf extracts of *Seriphium plumosum, Toona cilliata* and *Schkuhria pinnata* was calcium dependent or independent. Cells at a density of 1x 10^6 were incubated for 24 hours and treated with different concentration ranges of 50 μg/ml - 1000 μg/ml. Actinomycin D was used as the positive control at a concentration of 25 μg/ml. Chromogenic reagent of 90 μl and calcium assay buffer of 60 μl were added in each well followed by mixing gently. The reaction was incubated for 10 min in the dark, followed by measuring of absorbance at 575 nm using a Glomax microtiter plate (Promega, U.S.A). Where apoptosis is calcium dependent there will be a decrease in the number of cells, as well as in calcium concentration (Giorgi *et al.,* 2008).

CHAPTER FOUR: RESULTS

4.1 Mass extracted from plant materials

Three plants with history of traditional medicine use were selected and leaves of these plants were collected for study since leaves were traditional used for treating abdominal pain of diseases. The three medicinal plants *Seriphium plumosum, Toona cilliata and Schkuhria pinnata* were collected and an amount of 10 g of leaves from each of the plants was harvested for extraction of medicinal components.

The quantity of leaf extracts obtained after extracting the plants where plant material of 10 g from the leaf extracts of *Seriphium plumosum, Toona cilliata and Schkuhria pinnata* was thoroughly extracted with 100 ml of absolute acetone. The extracted materials obtained there were as follows *Seriphium plumosum* (0.0432 g), *Toona cillliata* (0.1786 g) and *Schkuhria pinnata* (0.0295 g).

After extracting all the plant extracts using acetone *Toona cilliata* showed to have the highest materials extracted followed by *seriphium plumosum* and lastly *Schkuhria pinnata*.

4.2 TLC profiles of acetone extracts of selected plants

The finger print profile of the three acetone extracts was determined using TLC plates developed in TEA, CEF and EMW mobile phases (figure 3). There was a decrease in separation of compounds as the polarity of the mobile phases was increasing. The compounds circled were those visible under UV light at wavelengths 255 nm and 360 nm. The reactivity of compounds was enhanced on the chromatograms using aluminium silicon coated plates. A compound of the same plant with the same molecular weight and same polarity migrates the same distance on the aluminium silicon coated plate eluted in the same mobile phase. Polar compounds would dissolve and separate better in the EMW mobile phase, while the intermediate polar compounds would dissolve and separate better in the CEF mobile phase, whereas non-polar compounds would dissolve and migrate better in the TEA.

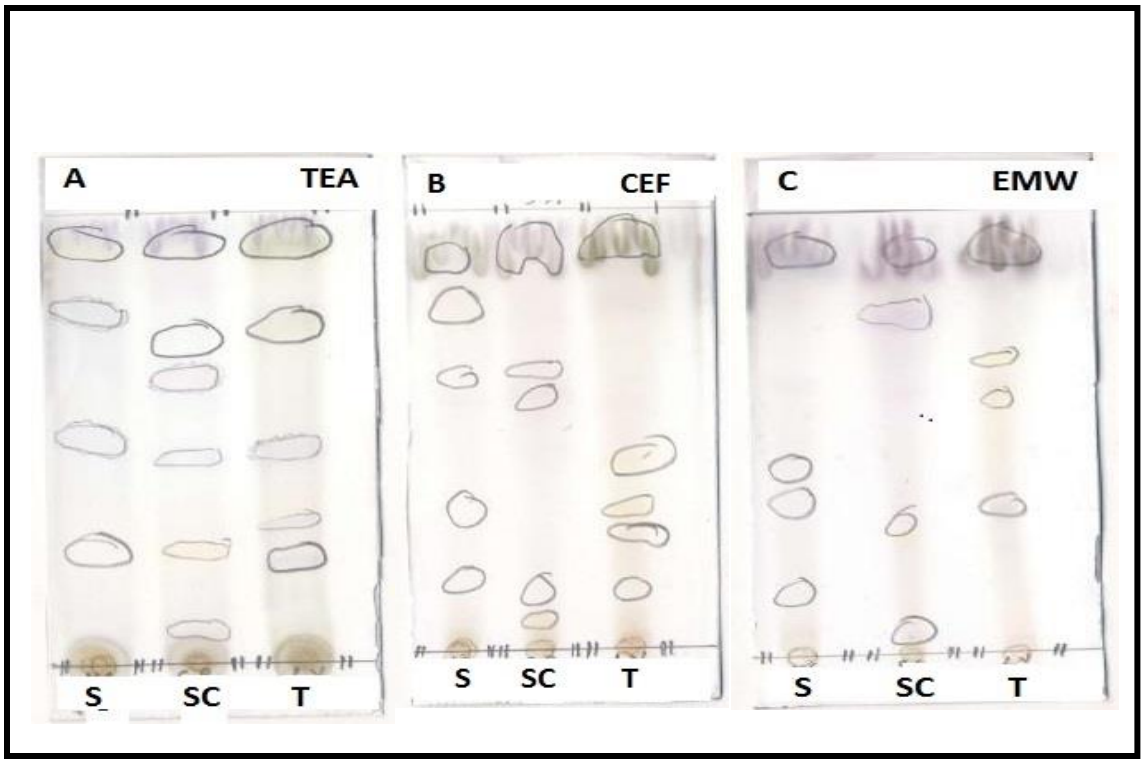

Figure 3: Thin layer chromatograms of acetone leaf extracts obtained from *Seriphium plumosum, Schkuhria pinnata* and *Toona cilliata* developed with TEA (A), CEF (B) and EMW (C) as mobile phases. The chromatograms were sprayed with vanillin/H_2SO_4 and heated in an oven at 110^{o}C for colour development.

Table 2: Relative frequency and quantified values of acetone leaf extracts of *Seriphium plumosum, Schkuhria pinnata* and *Toona cilliata.*

	Seriphium plumosum	***Schkuhria pinnata***	***Toona cilliata***
TEA	# 0,3cm × 0,441%	# 0,11cm × 0,216%	# 0,3cm × 0,389%
CEF	# 0,2cm × 1%	# 0.09cm × 0,504%	# 0.2cm × 0,326%
EMW	# 0,2cm × 1%	# 0.09cm × 0,41%	# 0,5cm × 0,32%

Keynote: Relative frequency (#) and quantified percentage values (×) from the three plant extracts.

After finger printing was performed in three different mobile phases, in TEA *Schkuhria pinnata* showed to have more number of bands followed by *Toona cilliata* and *Seriphium plumosum* when using the non-polar TEA mobile phase. In the intermediate polar solvent all the plant extracts showed to have the same number of bands even though they migrated different distances as it was also noticed from the polar solvent were they also had the same number of bands but migrated different distances. In the TEA mobile phase all the plants did have non polar secondary metabolites given that there was migration that is represented by the bands. With the R_f values of 0.3 cm where found from *Seriphium plumosum* and *Toona cilliata* and 0.11 cm for *Schkuhria pinnata* which implies that the two plants had almost have the same types of the non-polar metabolites. Plate B in the CEF mobile phase there was a decrease in the migration of the solvents in all the plants where the R_f values were *Seriphium plumosum* 0.2 cm, *Schkuhria pinnata* 0.09 cm and *Toona cilliata* 0.2 cm. In the polar EMW mobile phase there was no change in R_f values of *Seriphium plumosum* 0.2 cm, *Schkuhria pinnata* 0.09 cm while there was an extreme change for *Toona cilliata* 0.5 cm this indicates that there was an increase in the presence of polar metabolites in the plant.

4.3 Determination of antioxidant activity of acetone extracts of selected plants

The antioxidant activity of the leaf extracts can be evaluated using qualitative and quantitative determination methods. The qualitative method is used to determine the antioxidant compound separated on TLC plates and the representative chromatogram of extracts eluted in different solvents is presented on (figure 4). The yellow spots/bands against the purple background represent compounds with antioxidant activity. The TEA mobile phase was unable to move the antioxidant compounds from the base of the plates in the extracts. The antioxidants compounds which were not able to migrate in TEA were able to migrate in both intermediate polar and polar mobile phases (CEF and EMW) respectively. In CEF *Seriphium plumosum* show a compound with antioxidant activity of R_f 0.80 cm, while *Schkuhria pinnata* and *Toona cilliata* show a compound activity with R_f 0.40 cm respectively. In EMW, *Seriphium plumosum* show compound antioxidant activity with R_f 0.40 cm, *Schkuhria pinnata* had compound antioxidant activity with R_f 0.32 cm and *Toona cilliata* had compound antioxidant activity with R_f 0.60 cm.

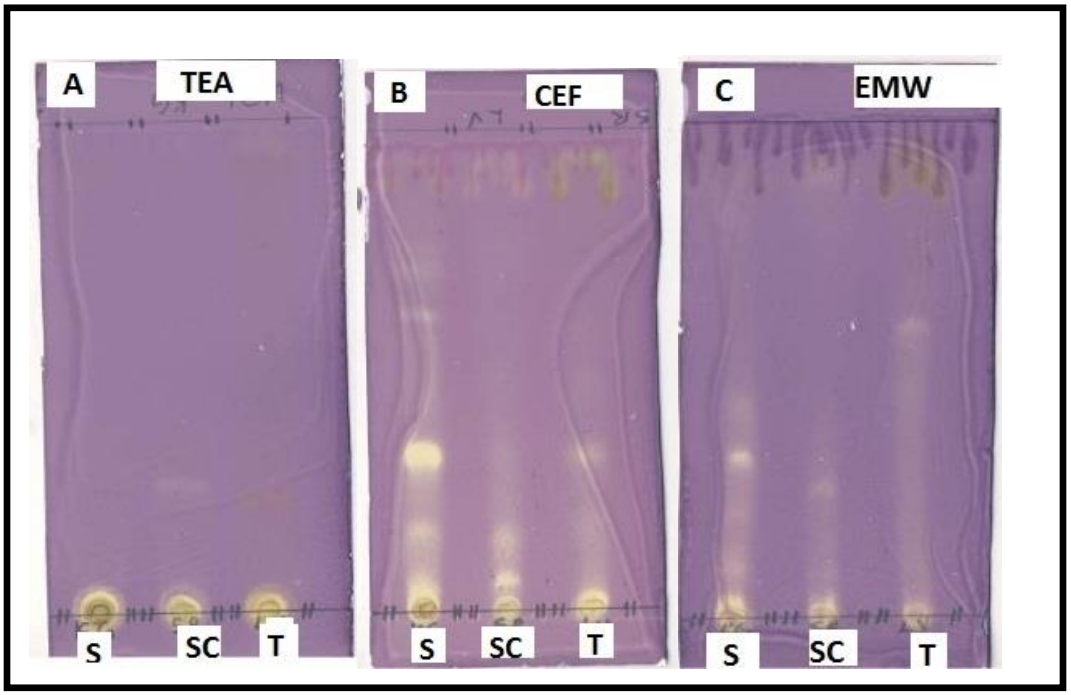

Figure 4: Compounds with antioxidant activity on TLC plates in *Seriphium plumosum, Schkuhria pinnata* and *Toona cilliata* developed with TEA (A), CEF (B) and EMW (C) as mobile phases. The chromatograms were sprayed with 0.2% DPPH.

Table 3: Quantified values of acetone leaf extracts of *Seriphium plumosum, Schkuhria pinnata* and *Toona cilliata.*

	Seriphium plumosum	***Schkuhria pinnata***	***Toona cilliata***
TEA	1%	30,5%	1%
CEF	2,3%	0,6%	0,4%
EMW	1%	0,5%	0,3%

Keynote: Quantified percentage values from the three plant extracts.

The more discolouration of the goldish colour indicates the higher the presence and scavenging activity of the sample. *Seriphium plumosum* show to have the highest presence of the antioxidants in CEF and EMW solvent systems followed by *Toona cilliata*

and lastly *Schkuhria pinnata* whereas all the plants show shown to have the same characteristics in the TEA solvent.

4.4 Evaluating of the presence of secondary metabolites in the acetone extracts of the selected medicinal plants

Acetone leaf extracts were tested for the presence of secondary metabolites using chemical methods and the results are presented in (table 4). All the leaf extracts did show the presence of phenolics, flavonoids, tannins and steroids as compared to saponins anthraquinones, phlobatannins and anthocyanins which were not detected in all the extracts. The presence of phenols was observed by the formation of blue colour, flavonoids by yellow coloration, tannins by green and steroids by a reddish-brown ring. However, saponins were not detected due to the failure of the formation of persistent froth and emulsion. Only *Toona cilliata* was shown to be positive for the presence of glycosides, while coumarins were observed to be present in all the extracts. On the other hand only *Schkuhria pinnata* tested positive for the presence of proteins that was indicated by the formation of a white precipitate.

Table 4: Types of secondary metabolites detected in the acetone leaf extracts of *Seriphium plumosum, Schkuhria pinnata* and *Toona cilliata* using the chemical methods.

Type of secondary metabolites	Type of extract		
	SPLA	SCPLA	TCLA
Phenolics	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Saponins	–	–	–
Steroids	+	+	+
Phlobatannins	–	–	–
Glycosides	–	+	+
Coumarins	+	+	+
Proteins	–	+	–
Anthraquinones	–	–	–
Anthocyanins	–	–	–

Keynote: (+) presence; (–) absence; *Seriphium plumosum* (SP) leaf (L) acetone (A) extract, *Schkuhria pinnata* (SCP) leaf (L) acetone (A) extract, *Toona cilliata* (TC) leaf (L) acetone (A) extract.

4.5 Determination of phenolic contents of the acetone extracts of the selected plants

The total phenolics in extracts was determined spectrophotometrically using Folin-Ciocalteu`s phenol reagent (figure 5). Concentration of 0.1 and 1 mg/ml of leaf extracts was used. The graph below represents the concentrations of phenolic contents determined in acetone extracts. *Seriphium plumosum* and *Toona cilliata* are shown to have the same concentration of phenolic contents, while *Schkuhria pinnata* had the least.

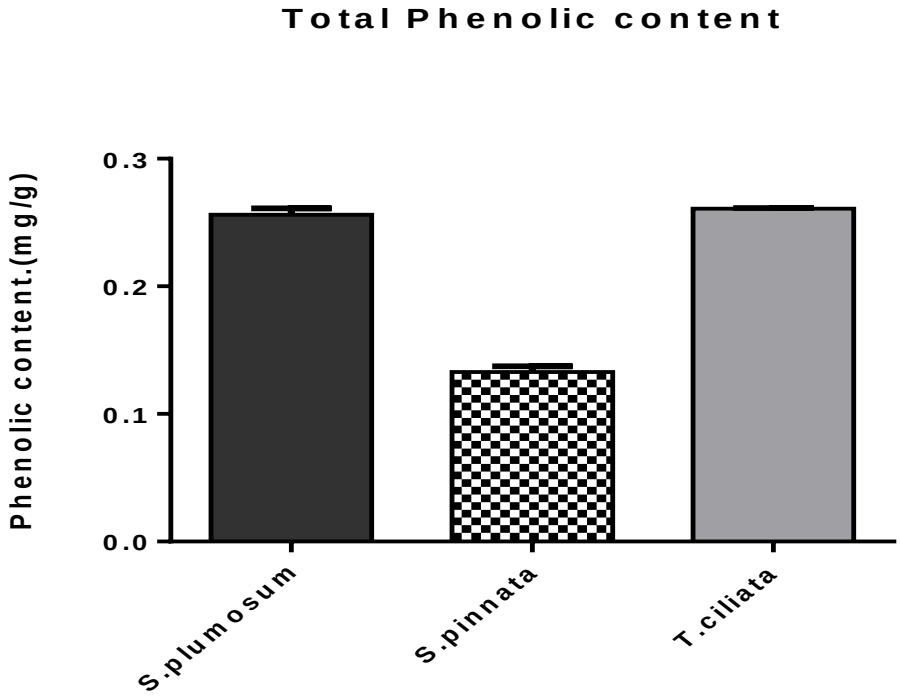

Figure 5: Determination of the total phenolic contents of *Seriphium plumosum, Schkuhria pinnata* and *Toona ciliata* acetone extracts. Garlic acid was used as a standard and the total phenolics were expressed as mg/g gallic equivalence (GAE). Concentration of 0.01, 0.02, 0.03 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol.

4.6 Determination of total flavonoids contents of the acetone extracts of the selected medicinal plants

The flavonoid content of extracts was measured by the aluminium chloride colorimetric assay. *Seriphium plumosum* was shown to have more flavonoids contents followed by *Toona cilliata* extracts and lastly *Schkuhria pinnata.*

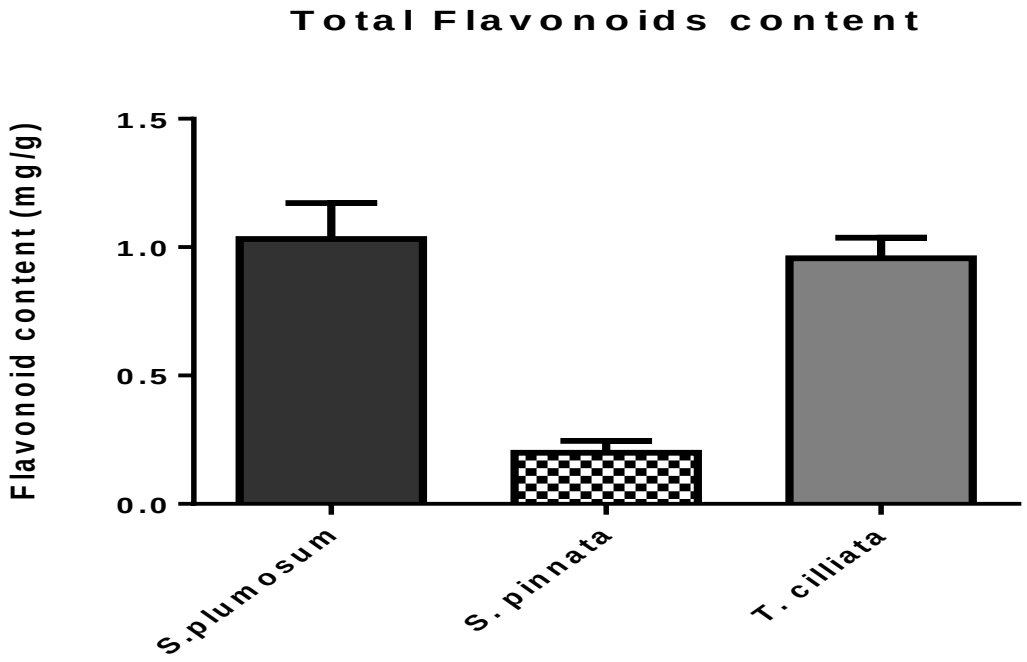

Figure 6: Determination of the total flavonoids contents of *Seriphium plumosum, Schkuhria pinnata* and *Toona cilliata* acetone extracts. Quercetin was used as a standard and the total flavonoids were expressed as mg quercetin equivalence (QE). Concentration range used was 20 – 100 µg/ml.

4.7 DPPH scavenging activities of the acetone extracts of the selected medicinal plants

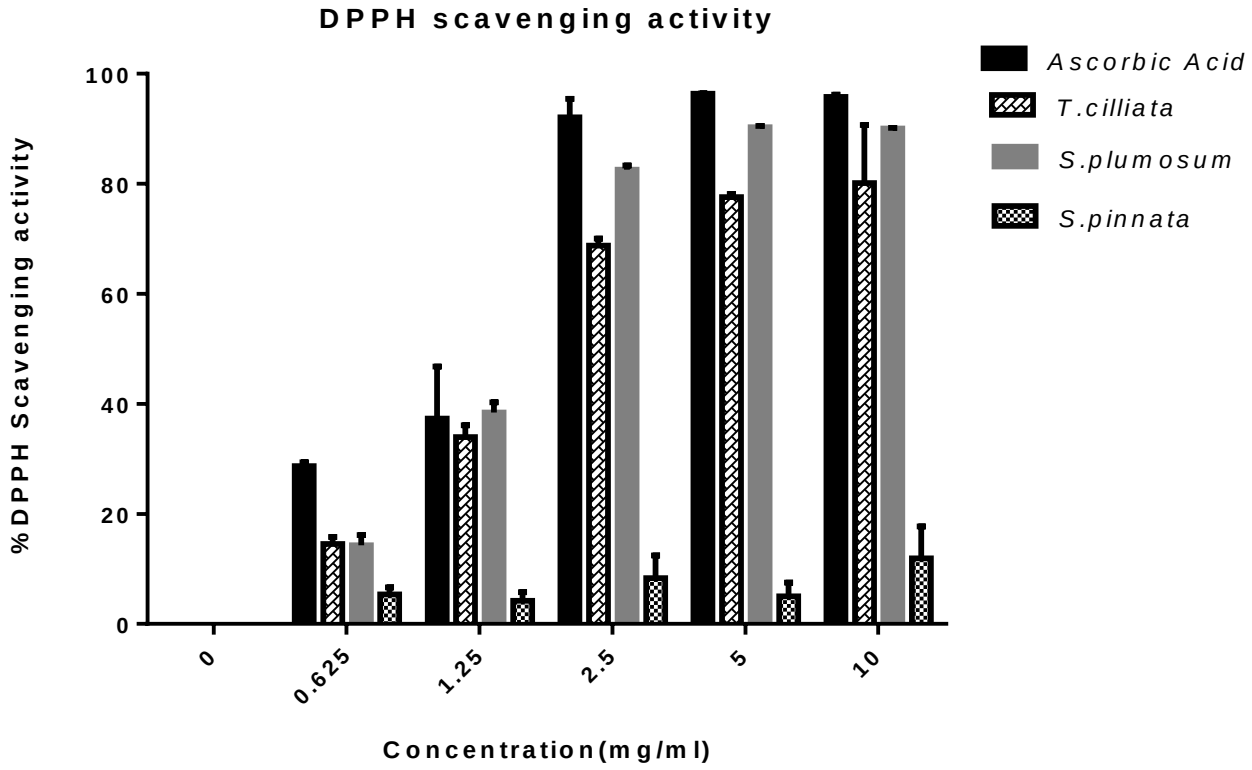

Figure 7: Quantitative antioxidant scavenging activities of the acetone leaf extracts obtained from *Seriphium plumosum*, *Toona cilliata* and *Schkuhria pinnata*. These acetone leaf extracts from these three plants were assayed at a concentration ranging from 0.625 - 10 mg/ml. Ascorbic acid was used as a positive control.

DPPH scavenging activities shown to increase with increase in concentration in all the extracts with *Seriphium plumosum* and *Toona cilliata* extracts showing the highest scavenging activities relative to the control.

4.8 Ferric reducing power of the acetone extracts of the selected medicinal plan

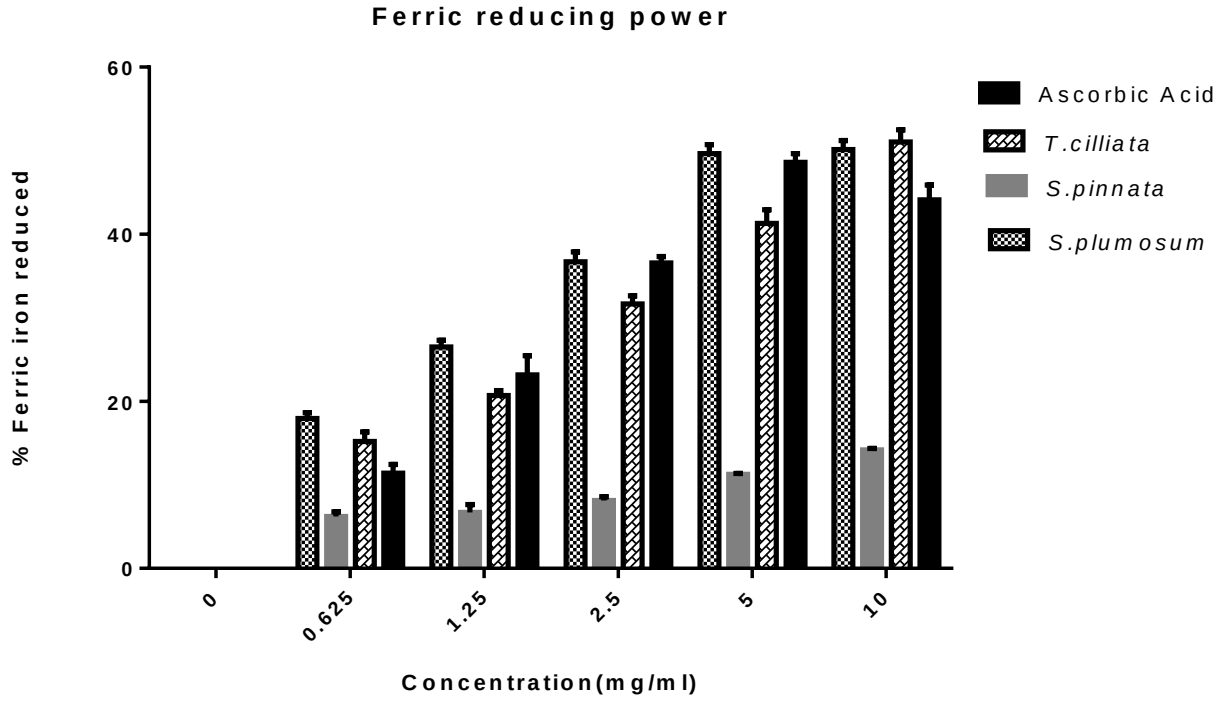

Figure 8: Reducing power assay showing ferric iron reduction of the acetone leaf extracts obtained from *Seriphium plumosum*, *Toona cilliata* and *Schkuhria pinnata*. These acetone extracts obtained from the three plants and assayed at a concentration ranging from 0.625 - 10 mg/ml. Ascorbic acid was used as the positive control.

The quantitative method determines the reducing power through an electron donation from one substance to an oxidizing agent of the extracts. *Seriphium plumosum* followed by *Toona cilliata* extracts showed more reducing power as compared to *Schkuhria pinnata* (figure 8). As concentration of the extracts increases the reducing power increases as well.

4.9 IC_{50} Values of the acetone extracts of the selected medicinal plants

Table 5: Represents the IC_{50} values of *Seriphium plumosum, Toona cilliata and Schkuhria pinnata* extracts for their DPPH scavenging assay and their ferric reducing power. *Seriphium plumosum* showed the greatest antioxidant scavenging activity followed by *Toona cilliata* and lastly *Schkuhria pinnata*.

Plant Extract	IC_{50} (mg/ml)	
	DPPH scavenging	Ferric reducing power
***S. plumosum* extract**	**1,573**	**3,374**
***T. cilliata* extract**	**1,837**	**4,678**
***S. pinnata* extract**	**12,361**	**38,037**
Ascorbic acid	**1,387**	

The plant extract with the lowest IC_{50} DPPH scavenging value has the most ability to neutralise the oxygen intermediates through donating an electron since it has a value closer to that of our positive control which is Ascorbic acid.

4.10 Cell proliferation determination of the acetone extracts of the selected plants

The MTT assay was used to determine the effect of the acetone leaf extracts of *Seriphium plumosum, Toona cilliata and Schkuhria pinnata* on the HeLa cell viability. Cells at a density of 1x 10^6 were incubated for 24 hours and treated with different concentrations ranges of 50 μg/ml -1000 μg/ml. Actinomycin D was used as the positive control at a concentration of 25 μg/ml (figures 9,10 and 11). Cell viability is shown to decrease with increase in concentration with all the different plants, with the effects of the extracts shown to be cytotoxic at the lowest concentration tested against HeLa cells.

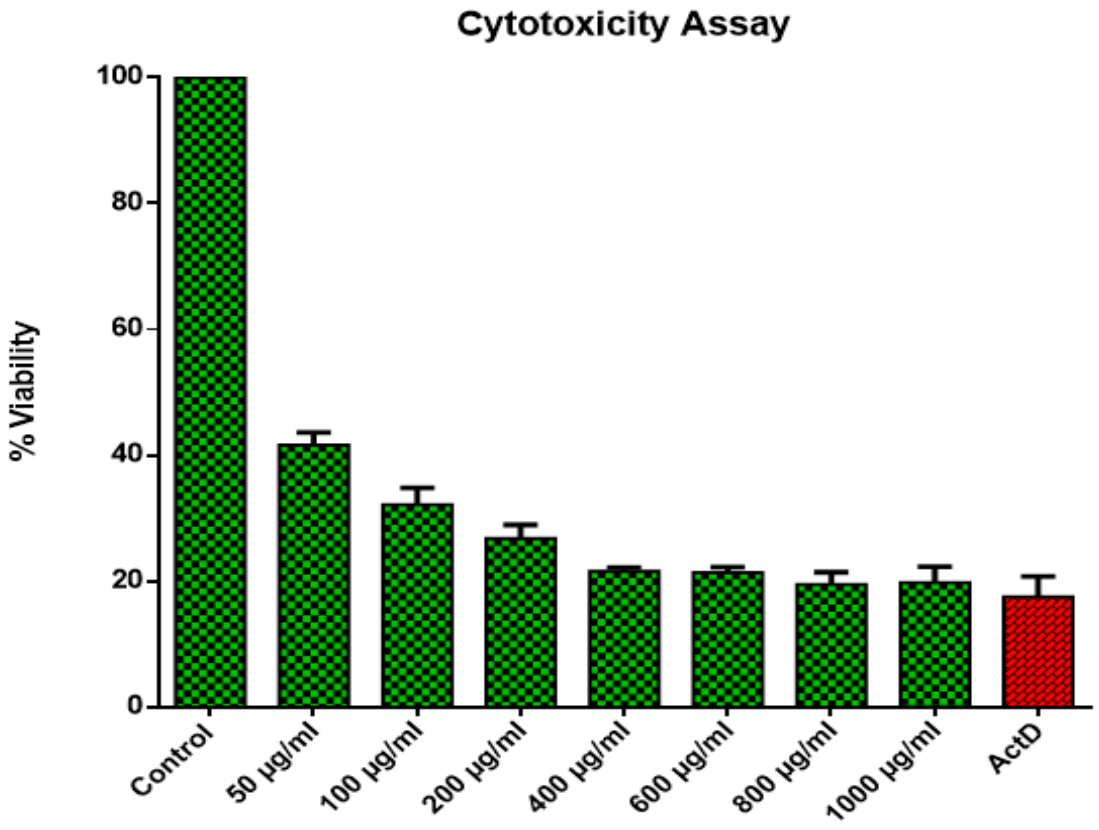

Figure 9: Cytotoxicity effects of *Seriphium plumosum* acetone leaf extracts on HeLa cells. Cells at a density of 1x 10^6 were incubated for 24 hours and treated with different concentrations ranges of 50 μg/ml -1000 μg/ml.

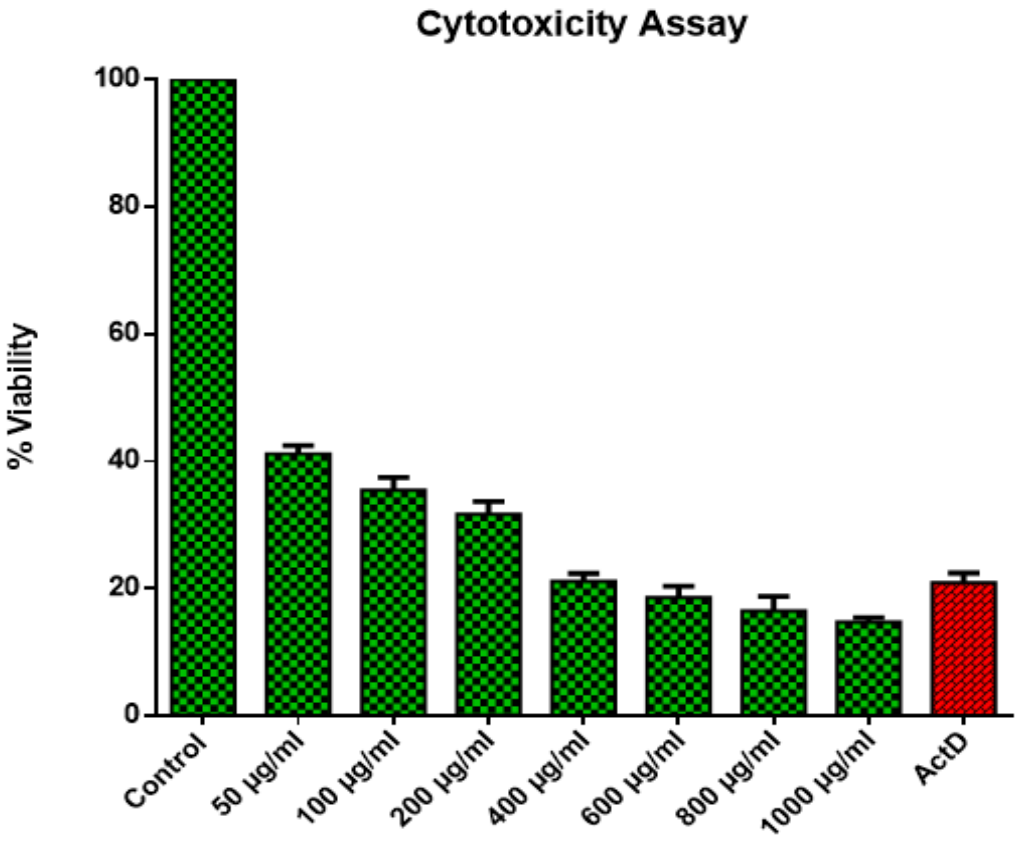

Figure 10: Cytotoxicity effects of *Toona cilliata* acetone leaf extract on HeLa cells. Cells at a density of 1x 10^6 were incubated for 24 hours and treated with different concentrations ranges of 50 µg/ml -1000 µg/ml.

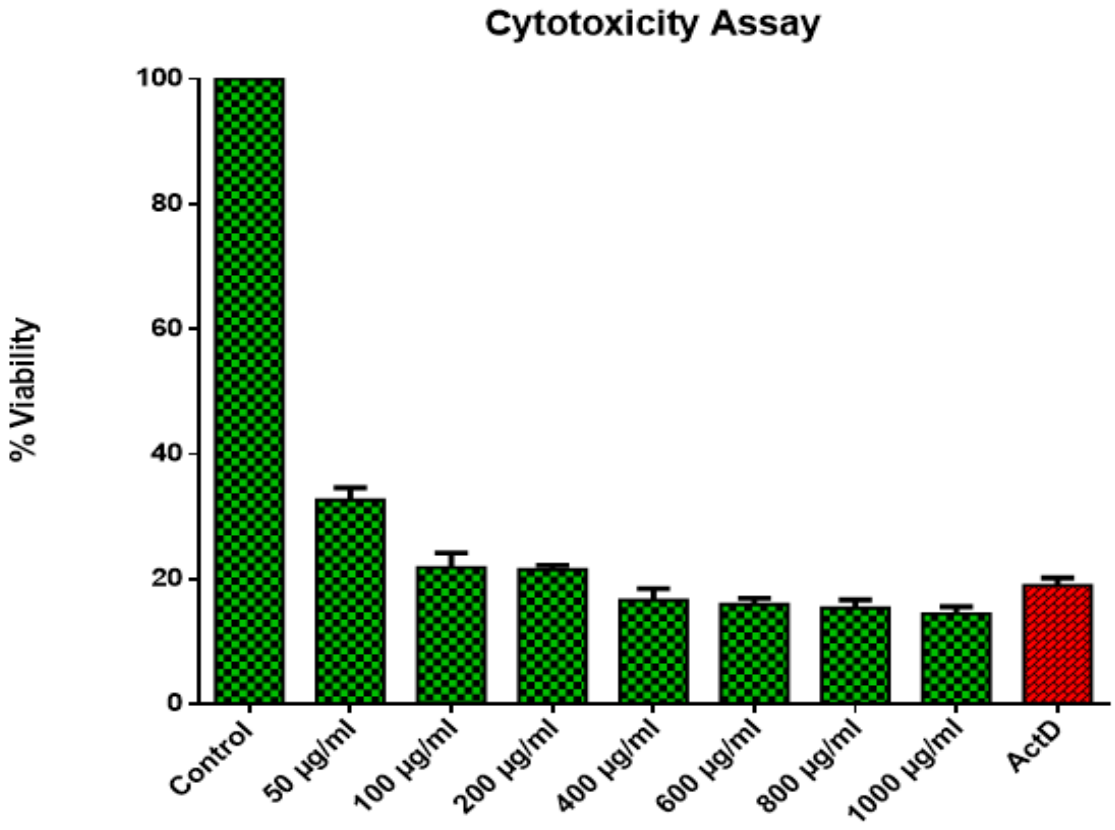

Figure 11: Cytotoxicity effects of *Sckhukhria pinnata* acetone leaf extract on HeLa cells. Cells at a density of 1x 10^6 were incubated for 24 hours and treated with different concentrations ranges of 50 µg/ml -1000 µg/ml.

4.11 Determination of apoptotic effects of the selected plant extracts

Annexin-V and PI apoptosis detection assay was used to test for the ability of the acetone leaf extracts of *Seriphium plumosum, Toona cilliata and Schkuhria pinnata* to induce apoptosis. All the cells were treated with 400 μg/ml using different plant extracts since it was close to be the average of my concentrations range. Cells stained positive for both Annexin-V and PI as shown in (figure 12). All the pictures represented in (A) were stained with DAPI to show apoptotic nuclei identified through condensation of chromatin at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. The high the concentration of the blue colour represents nuclei considered with abnormal phenotype. As deduced from the figures 12, 13 and 14 below *Seriphium plumosum* was shown to have the highest concentration of the stain followed by *Toona ciliata* and lastly *Schkuhria pinnata*.

Propidium iodide can be used to evaluate if a cell has undergone apoptosis through its ability to enter the cell when the cell membrane has flipped as represented in pictures (C). The red colour shows the degree of DNA degradation and chromatin condensation which is a characteristic of a cell which would have gone apoptosis. In figures 12, 13 and 14 cells are shown to take up the green stain in them from which represents filliping of phosphatidylserine in the cell membrane as in pictures (B).

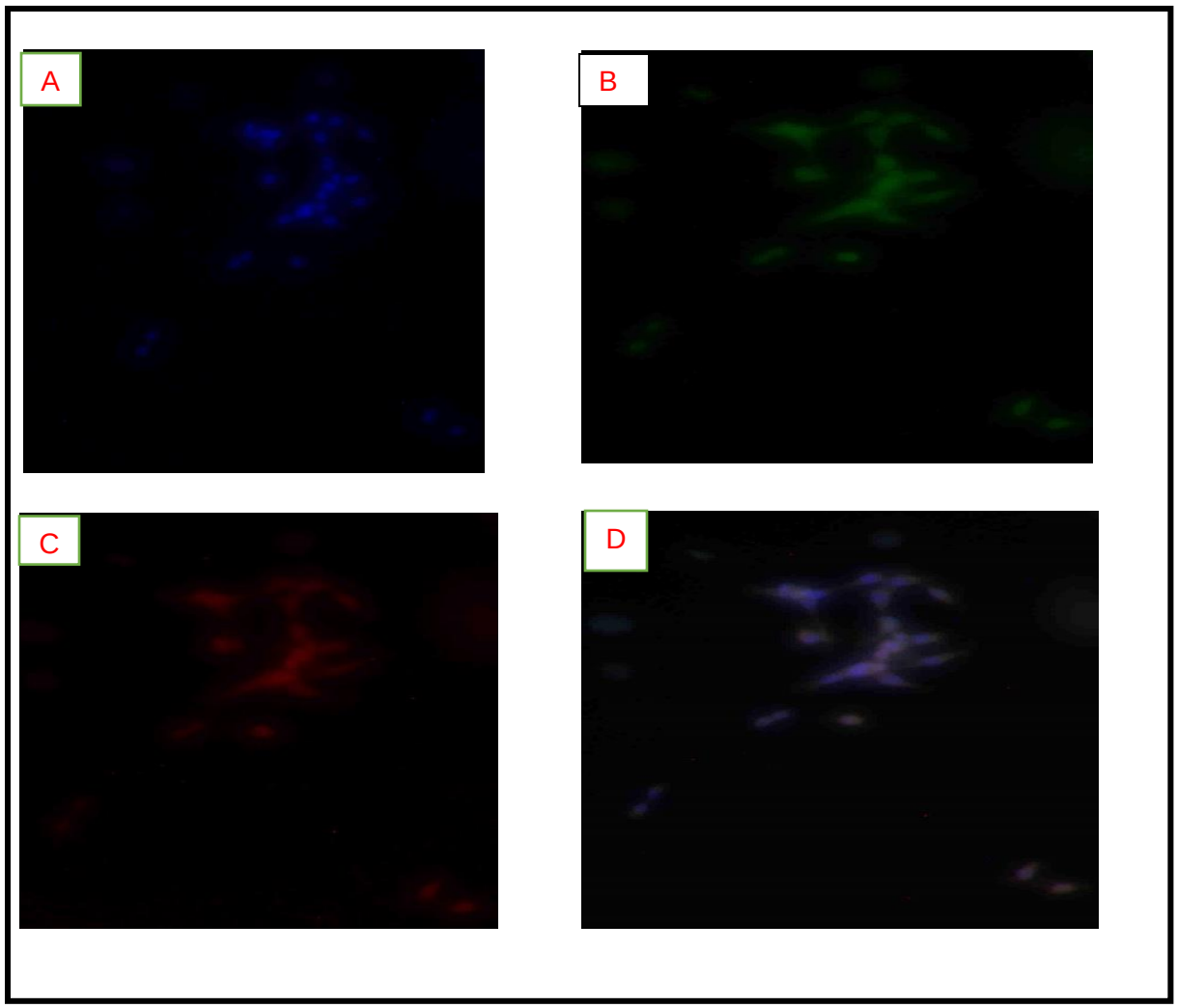

Figure 12: Apoptotic effects of *Seriphium plumosum* acetone leaf extract. Cells were cultured at a density of $6x10^6$ cells/ml, on coverslip in micropetri-dishes. Cells were treated with 400 μg/ml extract for 24 hrs. After 24 hrs, cells were stained with Annexin- V / PI for 30 min. Pictures was then captured at 60x magnification with Nixon Ti- E inverted microscope (Nikon, Japan). A= image of the nucleus stained with DAPI which fluoresce blue. B= image of phosphatidylserine flipping stained with Annexin – V which fluoresce green. C= image of DNA degradation and chromatin condensation stained with PI which fluoresce. D= image of overlying colours in A, B and C.

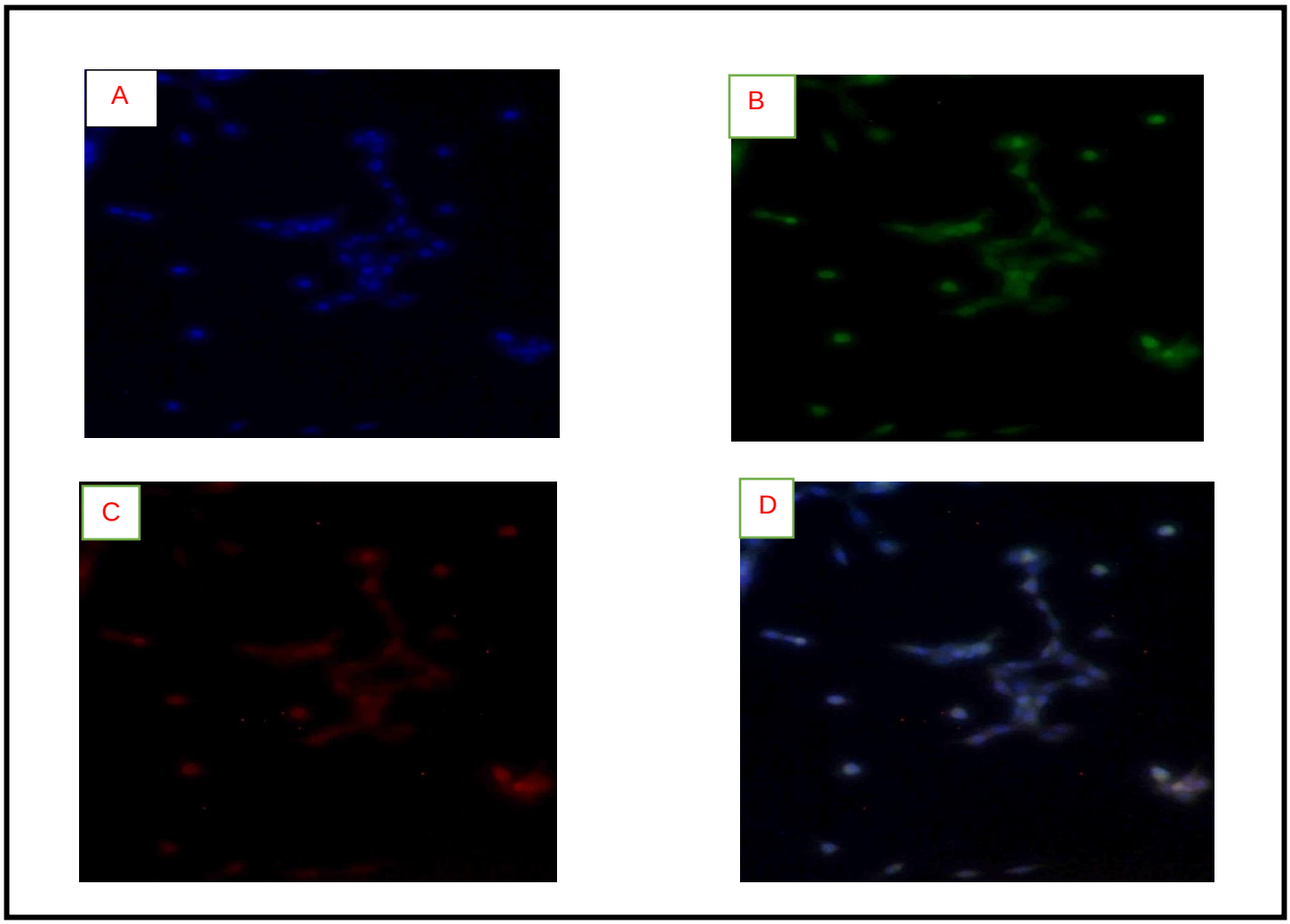

Figure 13: Apoptotic effects of *Toona cilliata* acetone leaf extract. Cells were cultured at a density of $6x10^6$ cells/ml, on coverslip in micropetri-dish. Cells were treated with 400 μg/ml extract for 24 hrs. After 24 hrs, cells were stained with Annexin- V / PI for 30 min. Pictures was then captured at 60x magnification with Nixon Ti- E inverted microscope (Nikon, Japan). A= image of the nucleus stained with DAPI which fluoresce blue. B= image of phosphatidylserine flipping stained with Annexin – V which fluoresce green. C= image of DNA degradation and chromatin condensation stained with PI which fluoresce. D= image of overlying colours in A, B and C.

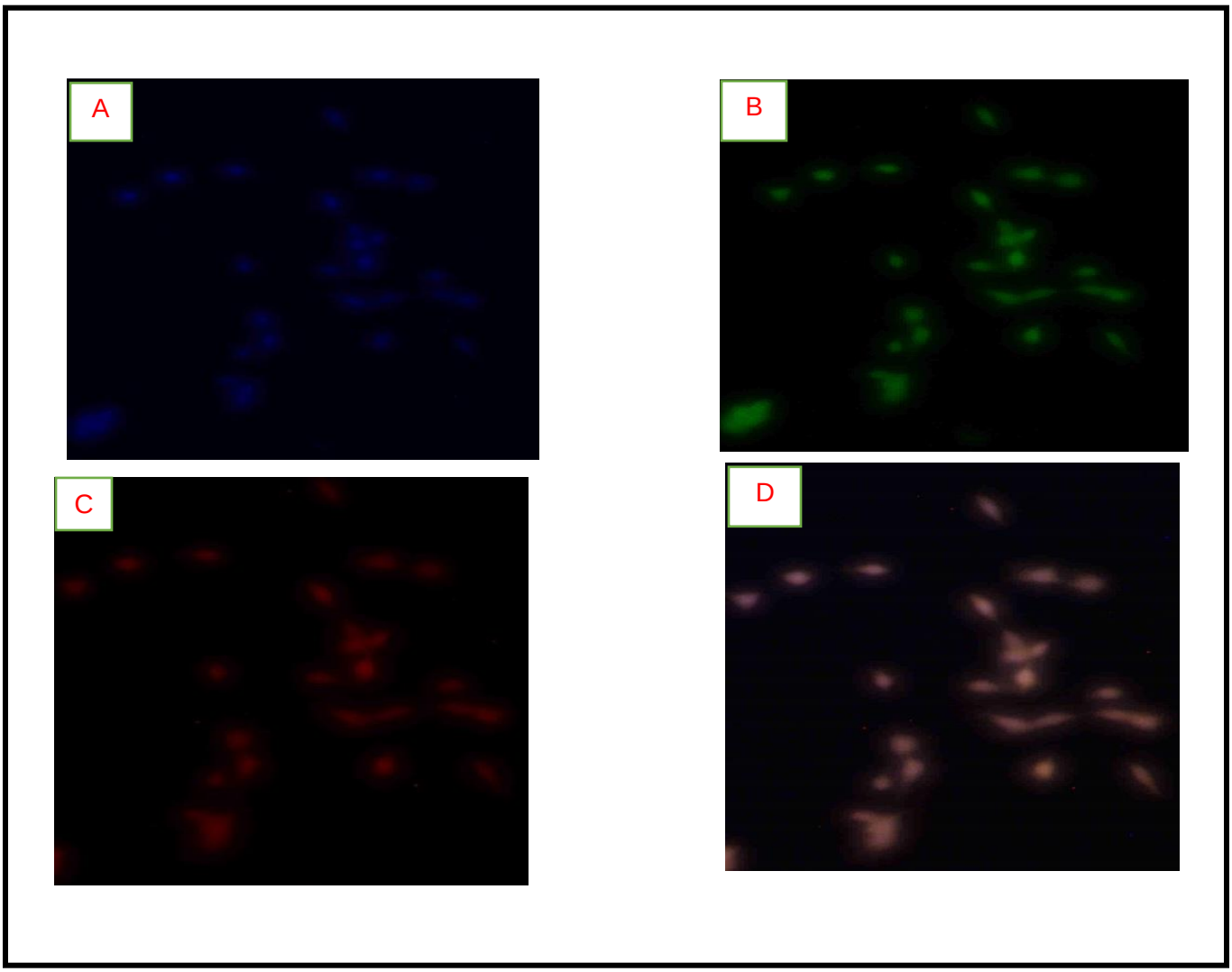

Figure 14: Apoptotic effects of *Schkuhria pinnata* cetone leaf extract. Cells were cultured at a density of $6x10^6$ cells/ml, on coverslip in micropetri-dish. Cells were treated with 400 μg/ml extract for 24 hrs. After 24 hrs, cells were stained with Annexin- V / PI for 30 min. Pictures was then captured at 60x magnification with Nixon Ti- E inverted microscope (Nikon, Japan). A= image of the nucleus stained with DAPI which fluoresce blue. B= image of phosphatidylserine flipping stained with Annexin – V which fluoresce green. C= image of DNA degradation and chromatin condensation stained with PI which fluoresce. D= image of overlying colours in A, B and C.

4.12 Determination of Calcium influx from acetone extracts of the medicinal plants

Calcium colorimetric assay was used to determine if apoptosis induced by the acetone leaf extracts of *Seriphium plumosum, Toona cilliata and Schkuhria pinnata* was calcium dependent or independent. Actinomycin D was used as the positive control at a concentration of 25 μg/ml (figures 15, 16 and 17). The numbers of cells are shown to decrease with a decrease in calcium concentration in cells exposed to all plant extracts. *Seriphium plumosum* show a higher effect on the concentration of calcium release followed by *Toona cilliata* and lastly by *Schkuhria pinnata*.

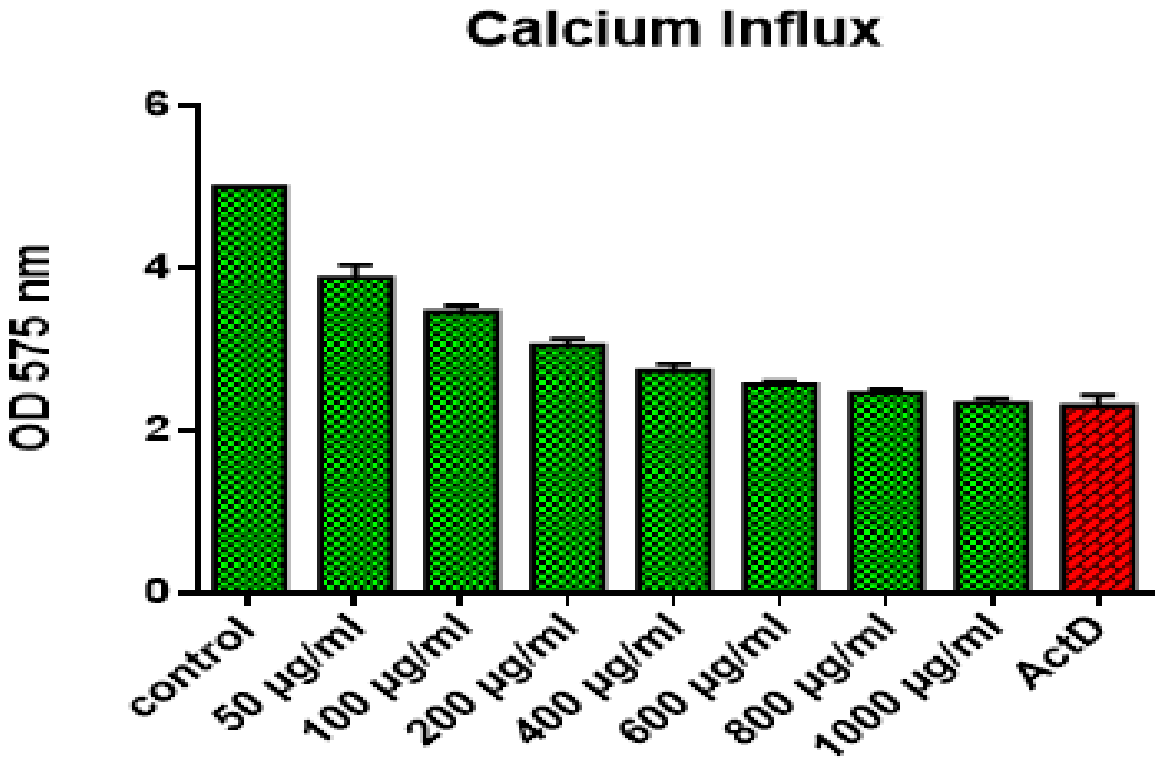

Figure 15: Evaluation of the effects of *Seriphium plumosum* acetone leaf extract on calcium concentration in the HeLA cells. Cells were seeded at a density of $1x10^6$ cells/ ml and treated 50 μg/ml – 1000 μg/ml for 24hrs. Followed by addition of chromogenic reagent of 90 μl and calcium assay buffer of 60 μl were added in each well followed by mixing gently.

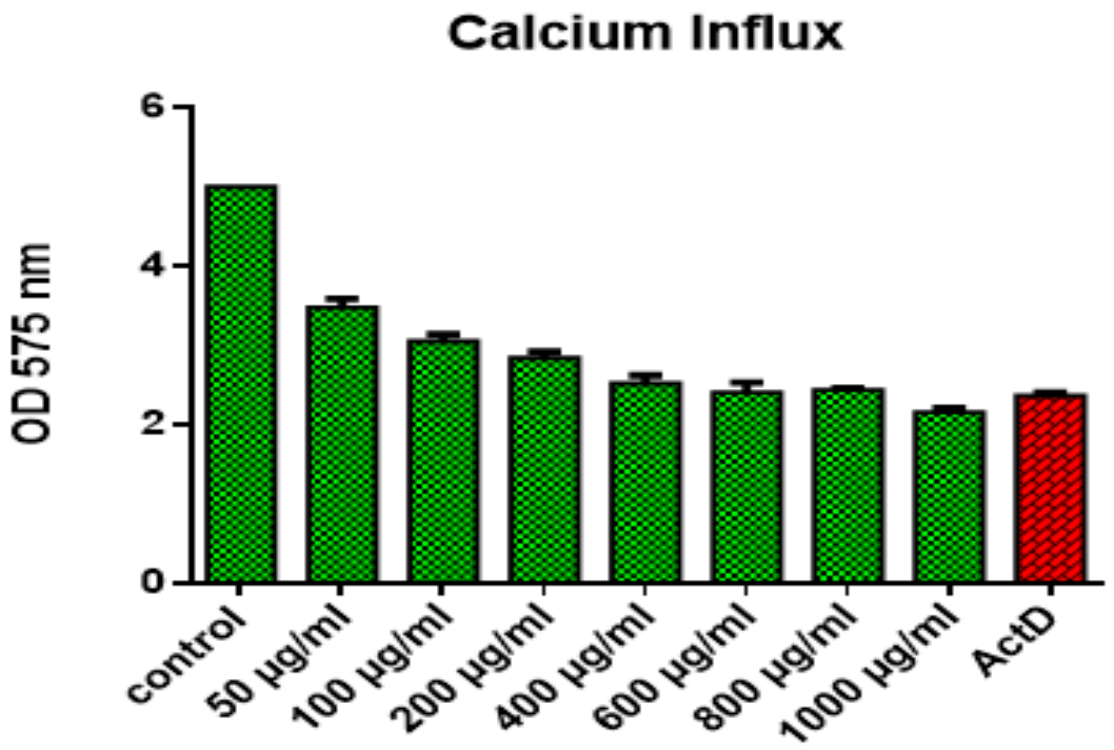

Figure 16: Evaluation of the effects of *Toona cilliata* acetone leaf extract on calcium concentration in the HeLa cells. Cells were seeded at a density of $1x10^6$ cells/ ml and treated 50 µg/ml – 1000 µg/ml for 24hrs. Followed by addition of chromogenic reagent of 90 µl and calcium assay buffer of 60 µl were added in each well followed by mixing gently.

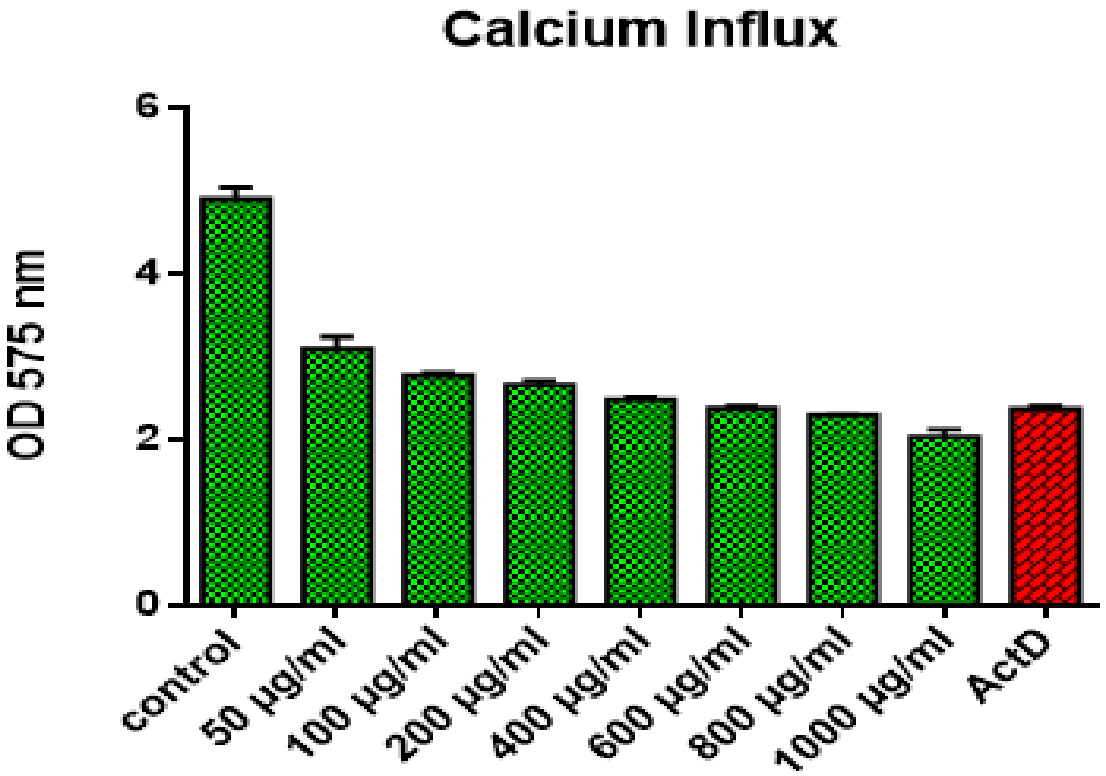

Figure 17: Evaluation of the effects of *Schkuhria pinnata* acetone leaf extract on concentration in the cells. Cells were seeded at a density of $1x10^6$ cells/ ml and treated 50 µg/ml – 1000 µg/ml for 24hrs. Followed by addition of chromogenic reagent of 90 µl and calcium assay buffer of 60 µl were added in each well followed by mixing gently.

CHAPTER FIVE: DISCUSSION AND CONCLUSION

Cervical cancers have become one of the most common cancers in women. About 8% of women in South Africa are considered to be harbouring the disease and the incidence rate is on the rise (Botha and Dochez 2012). The causative agent of the disease is the human papillomavirus which is mainly transmitted sexually or through skin contacts, to compound the problem current treatments methods are unaffordable, limited and is associated with vast side effects in patients. Hence, the search for medicinal plants to be used as possible alternative since they are affordable, accessible and effectively potent with fewer or no side effects have increased dramatically and can be key in unveiling the present societal dilemma surrounding cervical cancer Boadu and Asase, (2017). The current study was aimed at investigating and documenting various bioactivities of three plants that are traditionally used for the management of cervical cancer. The investigated bioactivities included phytochemical analysis, antioxidant activities, cell proliferation analysis, and apoptosis analysis. Crude extracts were prepared from the dried plant material of the three selected plants using different solvents, depending on their varying polarity. Traditional healers use water as a solvent, mainly because of its availability and none toxic effect. In this study, leaves of selected plants were extracted using acetone since we were targeting to extract intermediate polar compounds at a ratio of 1: 5 (g/ml).

The TLC finger printer profiles were developed in different mobile phases for each specific metabolite. The R_f values suggest the presence of secondary metabolites that are contained within the acetone leaf extracts of the selected plants.

The qualitative secondary metabolites analysis of the plant extracts showed the presence of phytochemicals such as phenolics, flavonoids, tannins, coumarins and steroids as compared to saponins, phlobatannins, anthraquinones and anthocyanins which were shown to be absent in all the extracts. Only *Toona cilliata* and *Schkuhria pinnata* were shown to test positive for the presence of glycosides and *Schkuhria pinnata* for the presence of proteins.

Toona cilliata have been previously screened for its anticancer proliferative effects against MCF-7 breast cancer cell line with phytochemicals like tannins and coumarins detected (Nasi *et al*., 2013).

The different compounds that were qualitatively identified to be present in the different extracts were further separated on thin layer chromatography plates. The compounds had various R_f values that suggest the presence of secondary metabolites that are contained within the acetone leaf extracts. These were responsive to different wavelengths of UV-light and resulted in different coloured bands in the presence of acidic vanillin. All the observations concurred with the fact that there were many different compound classes which were qualitatively identified in the plant extracts.

Compounds within plant extracts were shown to have antioxidant activity and were detected through qualitative DPPH assay. The compounds were further quantified spectrophotometrically. *Seriphium plumosum* showed the lowest IC_{50} value in the DPPH scavenging assay which explains its better ability to neutralise the oxygen intermediates, followed by *Toona cilliata* and lastly *Schkuhria pinnata* since obtained value was shown to be closer to Ascorbic acid (positive control). The free radical scavenging activities of the leaf extracts were evaluated qualitatively by spraying the developed chromatograms with DPPH. The appearance of yellow colour against a purple background on TLC plates was shown to be indicative of the presence of antioxidants. The degree of decolouration is suggestive of hydrogen donation ability of antioxidant constituents in the extract (Kagan and Flythe, (2014). Compounds with antioxidant capabilities helps enzymes to ameliorate toxic free radical intermediates. Antioxidants are understood to reduce the risk of many conditions such as rheumatoid arthritis, cardiovascular diseases, Alzheimer's diseases, cancer and Parkinson's diseases.

Toxicity is essential in discovering and producing safe products in form of new drugs. To achieve this in vitro study are required as a first step in toxicological studies. MTT was performed to determine the cytotoxic effects of acetone leaf extracts on HeLa cervical cancer cells. The method is based on the ability of the mitochondrial succinate-dehydrogenase to transform the MTT tetrazolium salt to a formazan product, which is proportional to the number of viable cells present (Saravanan *et al*., 2013).

It was observed that all the plants were cytotoxic to the cervical cancer HeLa cells with *Schkuhria pinnata* showing to be more cytotoxic followed by *Toona cilliata* and lastly by *Seriphium plumosum* in all different concentrations.

After treating the cells with different concentrations calcium influx was determined through the use of a calorimetric assay. All the plant leaf extracts showed to have a decrease in calcium concentration as the concentration in the extracts was increasing.

Apoptosis is important since it does not promote continuous growth of cells that finally leads to cancer. Determination of apoptotic effects of the acetone leaf extracts was performed against cervical cancer HeLa cells. The method is based on the degradation of the nucleus, condensation of the chromatin and flipping of the phosphatidylserine in the cell membrane (Galluzzi *et al*., 2007). In all the three plants it was observed that apoptosis was induced due to the ability of the cells in having a greenish surrounding which represent the flipping of the membrane the central part of the cell to have a reddish colour showing cell death. *Seriphium plumosum* and *Toona cilliata* did show to have higher activities of inducing apoptosis, since it can also be supported by the darker colour inside the cells as compared to the other plant. Calcium-transporting proteins, which are involved in the cellular process of apoptosis, still constitute a novel area of research in oncology. Not all aspects of oncogenic calcium homeostasis have been investigated, and its roles in different types of cancer are only just beginning to be understood. New studies of calcium-transporting proteins are necessary to allow the development of new chemotherapies, as are fresh models and technical approaches (Marchi and Pinton 2016).

Catechins plays a major role in dissociating anti apoptotic proteins from the cell membrane and allowing the attachment of proapoptotic proteins which leads to the movement of excess calcium from the endoplasmic reticulum to the mitochondrial in resulting in cell death through calcium influx (Yang *et al*.*,* (2014).

In conclusion, all leaf extracts were shown to contain various phytochemical constituents. TLC profile revealed the presence of more compounds in all the three leaf extracts in the intermediate polar solvent followed by the polar solvent system. *Seriphium plumosum*

showed more antioxidant activity as compared to the other extracts. All leaf extracts were shown to contain secondary metabolites such as phenolics, flavonoids, tannins, coumarins and steroids with the absence of saponins, phlobatannins, anthraquinones and anthocyanins.

Glycisides were found to be only present in *Schkuhria pinnata* and *Toona cilliata* and *proteins* in *Schkuhria pinnata.* The extracts were shown to be cytotoxicity to the HeLa cells with *Schkuhria pinnata* being more cytotoxic as compared to *Seriphium plumosum* and *Toona cilliata. Seriphium plumosum* and *Toona cilliata* had high scavenging activities and ferric reducing power due to their low IC_{50} value as compared with *Schkuhria pinnata*. The apoptotic induced from the activities of *Seriphium plumosum* and *Toona cilliata* were comparable as against that observed for *Schkuhria pinnata.*

In general, even though all the plants did prove to be effective, however further studies are required to determine the chemical structures that may have led to the results obtained against HeLa cell line *invitro*. *Seriphium plumosum* and *Toona cilliata* reflected to have almost the same characteristics of determining the anti-proliferative effects of anticancer activities against the cervical cancer HeLa cells as compared with *Schkuhria pinnata.* The unique findings are that no acetone leaf extracts from all three plants have been tested against the cervical cancer cell line even though it has been found out that *Toona cilliata* and *Schkuhria pinnata* have the potential of inhibiting cervical cancer cells after being extracted using other forms of solutions while less information have been said concerning *Seriphium plumosum* the use of the plant as a medicinal plant.

CHAPTER SIX: REFERENCES

Aboyade, M. O., Styger, G., Gibson, D. and Hughes, G. (2014). *Sutherlandia frutescens*: The Meeting of Science and Traditional Knowledge. *Journa*l of *Alternative* and *Complementary Medicine*. 20: 71-76.

Abuzeid, M. W., LI, D. and O' Malley Jr. B., W. (2011). Gene therapy for head and neck cancer. *Otorhinolaryngol*. 70: 141-151.

Autierl, P., Coibion, M., Huet, F. and Grivegneel, A. R. (1996). Transformation zone location and intraepithelial neoplasia of the cervix uteri. *British Journal of Cancer*. 74: 488-490.

Avenant, P. (2015). Production Scientist Report on the National bank bush (*Seriphium plumosum*) Survey.*Agriculture. Forestry and Fisheries.* 4-38.

Baig, S., Seevasant, I., Mohamad, J., Mukheem, A., Huri, Z. H. and Kamarul, T. (2016). Cell death and disease. Potential of apoptotic pathway-targeted cancer therapeutic research: Where do we stand? *Advanced Journal List*. 7: e2058.

Bien, M. (2016). Human Papillomavirus (HPV) and Cervical Cancer. Healthline. 1-5.

Boadu, A. A. and Asase, A. (2017). Documentation of Herbal Medicines Used for the Treatment and Management of Human Diseases by Some Communities in Southern Ghana. *Evidence-Based Complementary and Alternative Medicine.*1-12.

Borokini, T. I. and Omotayo, F. O. (2012). Phytochemical and ethanobotanical study of some selected medicinal plants from Nigeria. *Journal of Medicinal Plants Research.* 6: 1106-1118.

Botha, M. H. and Dochez, C. (2012). Introducing human papillomavirus vaccines into the health system in South Africa. *Vaccine*. 30: 28-34.

Brake, T. and Lambert, P. F. (2005). Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse

model. *Proceedings of the National Academy of Science of the United States of America*. 102: 2490-2495.

Bungu, L., Frost, L. C., Brauns, C. S. and Van de Vente. M. (2006). Tulbaghia violacea inhibits growth and induces apoptosis in cancer cells in vitro. *African Journal of Biotechnology*. 5: 1936-1943.

Cerhan J. R., Habermann T. M., Vachon C. M., Putnam S. D., Zheng W., Potter J. D. and Folsom A. R. (2002). Menstrual and reproductive factors and risk of non-Hodgkin lymphoma: the Iowa Women's Health Study. *Cancer Causes Control*. 13*:* 131-136.

Chakraborty, D., Ravi, V. and Chakraborty, P. (2010). Phytochemical evaluation and TLC protocol of various extracts of *Bombaxceiba* Linn. *International Journal of Pharmaceutical Science and Research.* 1: 8-10.

Chapman, J. R., Webster, A. C. and Wong, G. (2013). Cancer in the Transplant Recipient. *Cold Spring Harbor Perspectives Medicine*. 3: 15677.

Chesson, H. W., Dunne, E. F., Hariri, S. and Markowitz, L. E. (2014). The estimated lifetime probability of acquiring human papillomavirus in the United States. *Sexually Transmitted Diseases*. 41: 660-664.

Chumduri, C., Gurumurthy, R, K., Zadora, P, K., Mi, Y. and Meyer, T, F. (2013). Chlamydia Infection Promotes Host DNA Damage and Proliferation but Impairs the DNA Damage Response. Untreated Chlamydia Infections Linked to Cervical and Other Gynecological Cancers. *Cell host and microbe.* 13: 746-750.

Demain, A. L. and Fang, A. (2001). The Natural Functions of Secondary Metabolites. *Advances in biochemical in Biochemical Engineering Biotechenology*. 69: 1-39.

Disis, M. L. (2014). Mechanism of Action of Immunotherapy. *Tumor Vaccine Group*.5: 3-13.

Dueñas, G. A., Cetina, L., Coronel, J. and Martínez, B. D. (2010). Pharmacotherapy options for locally advanced and advanced cervical cancer. *Drugs*. 70: 403-432.

Foley, K. M. (2011). The treatment of pain in the patient with cancer. *Cancer Journal for clinicians.* 36: 294-215.

Fulda, S. (2013). How to target apoptosis signaling pathways for the treatment of pediatric cancers. *Frontiers in Oncology*. 3: 22.

Galluzzi, L. Zamzami, N. de La Motte Rouge, T. Lemaire, C. Brenner, C. and Kroemer, G. (2007). Methods for the assessment of mitochondrial membrane permeabilization in apoptosis. *Apoptosis*. 12: 803-13.

Gambhir, S., Vyas, D., Hollis, M., Aekka A. and Vyas, A. (2015). Nuclear factor kappa B role in inflammation associated gastrointestinal malignancies. *World Journal of Gastroenterology.* 21: 3174-3183.

Giorgi. C., Romagnoli, A., Pinton. A. and Rizzuto. R. (2008). Ca2+ Signaling, Mitochondria and Cell Death. *Current Molecular Medicine*. 8: 119-130.

Han, X., Shen, T. and Lou, H. (2007). Dietry polyphenols and their biological significance. *International Journal of Molecular Sciences*. 950-988.

Hatting, E. R. (1953). Observation on the ecology of Seriphium plumosum Levyns. *Empire Journal of Experimental Agriculture*. 21: 84.

Havsteen, H. B. (2002). The biochemistry and medical significance of the flavonoids. Pharmacology and Therapeutics. 96: 62-202.

Holtan, S. G., Creedon, D. J., Haluska, P. and Markovic, S. N. (2009). Cancer and Pregnancy: Parallels in Growth, Invasion, and Immune Modulation and Implications for Cancer Therapeutic Agents. Mayo Clinic Proceedings. 84: 985-1000.

Hunadi, S. S. and Istudor, V. (2009). Lythrum salicaria (purple loosestrife). Medicinal use, extraction and identification of its total phenolic compounds. *Farmacia*. 57: 192-200.

Kagan, A. I and Flythe, D. M. (2014). Thin-layer Chromatographic (TLC) Separations and Bioassays of Plant Extracts to Identify Antimicrobial Compounds. *Journal of Visualized experiments.* 85: 51411.

Katende B., Birnie A. and Tengnas B. (1995). Useful trees and shrubs for Uganda.Identification, Propagation and Management for Agricultural and Pastoral Communities. Regional Soil Conservation Unit (RSCU), Swedish International Development Authority (SIDA). 1-710.

Katsube, T., Tabata, H., Ohta, Y., Yamaski, Y., Anuurad, E., Shiwaku, K. and Yamane, Y. (2004). Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin- Ciocalteu assay. *Journal of Agriculture Food Chemistry*. 5: 2391-2393.

Kitagawa, R., Katsumata, N., Shibata, T., Kamura, T., Kasamatsu, T., Nakanishi, T., Nishimura, S., Ushijima, K., Takano, M., Satoh, T. and Yoshikawa H. (2015). Paclitaxel Plus Carboplatin versus Paclitaxel plus Cisplatin in Metastatic or Recurrent Cervical Cancer: The Open-Label Randomized Phase III Trial JCOG0505. *Journal clinical oncology.* 23: 2129-2135.

Kotina, L. E., Van Wyk, E. and Tilney, M. P. (2014). Anatomy of the leaf and bark of *Warburgia salutaris* (Canellaceae), an important medicinal plant from South Africa. *South African Journal of Botany*. 94: 177-181.

Krupko, I. and Davidson, R., S. (1961). An experimental study of *Seriphium plumosum* in relation to grazing and burning. *Empire Journal of Experimental Agriculture*. 29: 114.

Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *The Journal of nutrition.* 134: 3479-3485.

Luseba, D. and Van der Merwe, D. (2006). Ethnoveterinary medicine practices among Tsonga speaking people of South Africa. *Ondrstepoort Journal of Veterinary Research.* 73: 115-122.

Malairajan, P., Gopalakrishnan. G., Narasimhan, S., Veni, K., J., K. and Kavimani, S. (2007). Anti-ulcer activity of crude alcoholic extract of *Toona ciliata*Roemer (heart wood). *Journal of Ethnopharmacology*. 110: 348-351.

Marchi, S. and Pinton, P. (2016). Alterations of calcium homeostasis in cancer cells. *Current Opinion in Pharmacology*. 29: 1-6.

McGaw, L. J., Lall, N., Meyer, J. J. M. and Eloff, J. N. (2008). The potential of South African plants against Mycobacterium infections. *Journal of ethnopharmacology.*119: 482-500.

Meskin, M. S., Bidlack, W. R., Davies, A. J. and Omaye, S. T. (2002). *Phytochemicals in nutrition and health,* CRC Press.

Miller, A. A., Hargis, J. B., Lilenbaum, R. C., Fields, S. Z., Rosner, G. L. and Schilsky, R. L. (1994). "Phase I study of topotecan and cisplatin in patients with advanced solid tumors: a cancer and leukemia group B study." *Journal of Clinical Oncology*. 12: 2743-2750.

Miller, H. E., Rigelhof, F., Marquart, L., Prakash, A. and Kanter, M. (2000). Whole grain products and antioxidants. *Journal of Cereal Foods World*. 45: 59-63.

Moein, S. and Moein, R. (2010). Relationship between antioxidant properties and phenolics in Zhumeriamajdae. *Journal Medicinal Plants Research.* 4: 517-521.

Molassiotis, A., Fernadez-Ortega, P., Pud, D., Ozden, G., Scott, J.A., Panteli, V., Margulies, A., Browall, M., Magri, M. and Selvekerova, S. (2005). Use of complementary and alternative medicine in cancer patients: a European survey. *Annals of oncology.* 16: 655-663.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods.* 65: 55-63.

Muthaura, C. N., Rukunga, G. M., Chhabra, S. C., Omar, S. A., Guantai, A. N., Gathirwa, J. W., Tolo, F. M., Mwitari, P. G., Keter, L. K., Kirira, P. G. and Kimani, C. W. (2007). Antimalarial activity of some plants traditionally used in Meru district of Kenya. *Phytotherapy Research.* 21: 860-867.

Nathan, C. (2002). Points of control in inflammation. *Nature.* 420: 846-852.

Negi, S. S. (1993). Biodiversity and its conservation in India.

Nisa, S., Bibi, Y., Zia, M., Waheed, A. and Chaudhary, M. F. (2013). Anticancer investigations on Carissa opaca and Toona ciliata extracts against human breast carcinoma cell line. *Pakistan Journal of Pharmaceutical Sciences*. 26: 1009-1012.

Nishida, N., Yano, H., Nishida, T., Kamura, T. and Kojiro, M. (2006). Angiogenesis in cancer. *Vascular Health Risk Management*. 2: 213-219.

Orlowski, R. Z. and Baldwin, A. S. (2002). NF-κB as a therapeutic target in cancer. *Trends in Molecular Medicine*. 8: 385-389.

Reid, R., Leyland, N., Wolfman, W., Allaire, C., Awadalla, A., Best, C., Dunn, S., Lemyre, M., Marcoux, V., Menard, C., Potestio, F., Rittenberg, D., Singh, S., and Senikas, V. (2011). Oral contraceptives and the risk of venous thromboembolism. *International Journal Gynaecologists Obstetricians*. 112: 252-256.

Reuland, D. J., Khademi, S., Castle, C. J., Irwin, D. C., McCord, J. M., Miller, B.F. and Hamilton, K.L. (2013). Upregulation of phase II enzymes through phytochemical activation of Nrf2 protects cardiomyocytes against oxidant stress. *Free Radical Biology and Medicine*. 56: 102-111.

Reuter, S., Gupta, S. C., Chaturvedi, M. M. and Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology and Medicine*. 49: 1603-1616.

Richter, C. (1989). Belangrike indringerplante en die beheer daarvan in the sentrale grasveldgebiede van die Vrystaatstreek Agriculture. 18: 19-23.

Rieger, A. M., Nelson, L. K., Konowalchuk, J. D. and Barreda, D. R. (2011). Modified Annexin V/Propidium Iodide Apoptosis Assay for Accurate Assessment of Cell Death. *Journal of Visualized Experiments*. 50: 2597.

Saraswathi, T. R., Kavitha, B. and Priyadharsini, J. V. (2007). Gene therapy for oral squamous cell carcinoma: *Journal of Clinical and Diagnostic Research*. 18: 120-123.

Saravanan, B. C., Sreekuman, C., Bansal, G. C., Ray, D., Rao, J. R. and Mishra, A. K. (2013). A rapid MTT colomerimetric assay to assess the proliferative index of two Indian strains of *Theileria annulata*. *Veterinary Parasitology*. 113: 211-216.

Savill, J. and Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature.* 407: 784-788.

Savitree, M., Isara, P., Nittaya, S. L. and Worapan, S. (2004). Radical Scavenging Activity and Total Phenolic Content of Medicinal Plants Used in Primary Health Care. *Journal of Pharmaceutical Sciences*. 9: 32-35.

Schmidt E, Lotter M and McCleland W. (2002). Trees and Shrubs of Mpumalanga and Kruger National Park. *Jacana Media Publishers*. 668.

Shekhawat, N. and Vijayvergia, R. (2010). Investigation of anti-Inflammatory, analgesic and antipyretic Properties of *Madhuca indica* GMEL. *International Journal of Molecular Medicine Advanced Sciences*. 6: 26-30.

Smit, J. B. J. (1995). Die slangbosprobleem. Farming in South Africa. 85: 1-5.

Snyman, J. (2014). Cancer.South Africa: 78% increase in cancer by 2030.

Sonia, A. B., Julian, C. T., Jorge, C. S., Miguel, A. G., Naytra, E. C. and Antonia B. M. (2008). Gene therapy in the management of oral cancer: review of the literature. *Medicina Oral Patologia Oral y Cirugia Bucal*. 13: 15-21.

Sun, B., Richardo- da- Silvia, J. M. and Spranger, I. (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. *Journal of Agriculture and Food Chemistry*. 46: 4267-4274.

Tang, Y. S. and Halliwell, B. (2010). Medicinal plants antioxidants: What do we learn from cell culture and *Caenorhabditis elegans* studies? *Biochemical and Biophysical Research Communication*. 394: 1-5.

Teplitski, M., Robinson, J. B. and Bauer, W. D. (2000). Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-

dependent behaviors in associated bacteria. *Molecular Plant-Microbe Interactions*. 13: 637-648.

Thomas, R., Butler, E., Macchi, F. and Williams. M. (2015). Phytochemicals in cancer prevention and management? *British Journal of Medical Practitioners*. 8: 815.

Van der Merwe, D. (2000). *Use of enthnoveterinary medicinal plants in cattle by Setswana- speaking people in the Madikwe area of the North West Province* (Doctoral dissertation, University of Pretoria).

Van Hylckama Vlieg, A., Helmerhorst, F. M., Vandenbroucke, J. P., Doggen, C. J. and Rosendaal, F. R. (2009). The Venous Thrombotic Risk of Oral Contraceptives, Effects of Oestrogen Dose and Progestogen. 339: 2921.

Walboomers, J. M., Meijer, C. J., and Pathol, J. (1997). Do HPV-negative cervical carcinomas exist. 181: 253-254.

Wang, S., Meckling, K. A., Marcone, M. F., Kakuda, Y. and Tsao, R. (2011). Can phytochemical antioxidant rich foods act as anti-cancer agents? *Food Research International*. 44: 2545-2554.

Wang, Y., Yu, H., Zhang, J., Gao, J., Ge, X. and Lou, G. (2015). Hesperidin inhibits HeLa cell proliferation through apoptosis mediated by endoplasmic reticulum stress pathways and cell cycle arrest. *Biology of Medicine Centre*. 15: 682.

Wat, C., MacRae, W., Yamamoto, E., Towers, G. and Lam, J. (1980). Phototoxic effects of naturally occurring polyacetylenes and α-terthienyl on human erythrocytes. *Photochemistry and photobiology.* 32: 167-172.

Wen, C. C., Kuo, Y. H., Jan, J. T., Liang, P. H., Wang, S. Y., Liu, H. G., Lee, C. K., Chang, S. T., Kuo, C, J., Lee, S. S., Hou. C. C., Hsiao, P. W., Chien, S. C., Shyur, L. F. and Yang, N. S. (2007). Specific plant terpenoids and lignoids possess potent antiviral activities against severe acute respiratory syndrome coronavirus. *Journal of Medicine and Chemistry*. 50: 4085-4095.

Wepener, J. P. (2007). The control of *Stoebe vulgaris* encroachment in the Hartenbeesfontein area of the North West Province.Unpublished MSc thesis, North West University, Potchefstroom. 135.

World Health Organization. (2009). Human papillomavirus vaccines. WHO position paper. Weekly Epidemiological Record. 84: 118-131.

Yadav, M., Chatterji, S., Gupta, S. K. and Watal, G. (2014). Preliminary phytochemical screening of six medicinal plants used in traditional medicine. *International journal of pharmarcy and pharmaceutical sciences*. 6: 0975-1491.

Yang, S. C., Wang, H., Chen, X. J. and Z, J (2014). Effects of Tea Catechins on Cancer Signaling Pathways. *Enzymes*. 36: 195-221.

Zhishen, J., Mengcheng, T. and Jianming, W. (1999). The determination of flavonoins contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*. 64: 555-559.