PHYTOCHEMICAL PROFILE, IN SILICO MOLECULAR DOCKING ANALYSIS AND ANTI-CERVICAL CANCER EFFECTS OF *Rhamnus prinoides* and *Grewia villosa* EXTRACTS

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JULY, 2025

DECLARATION

I, Kamau Sally Wambui, duly declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award.

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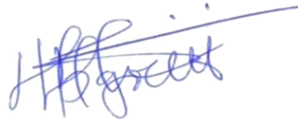

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DEDICATION

This work is first and foremost dedicated to the Almighty GOD to WHOM I owe everything, to my parents, Geoffrey Kamau and Mary King'ara for their unfettered support during my studies and to my brother, Solomon Kababii for his love, support, encouragement and always standing by me.

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ABBREVIATIONS AND ACCRONYMS

APAF-1	Apoptotic protease activating factor-1
ATCC	American Type Cell Collection
BCL	B-cell lymphoma family
CT values	Cycle Thresh-hold values
CTMDR	Center for Traditional Medicine and Drug Research
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ERBB2	Avian Erythroblastic leukemia viral oncogene homolog 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC/MS	Gas Chromatography/Mass Spectrometry
GLOBOCAN	Global Cancer Observatory
HeLa	Human cervical cancer cell line
IARC	International Agency for Research on Cancer
KEMRI	Kenya Medical Research Institute
LMICs	Low-to middle-income countries
MEM	Minimum Essential Media
mRNA	Messenger Ribonucleic acid
MTT	3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium
NCD	Non communicable disease
NCI	National Cancer Institute
NFκB	Nuclear factor kappa B
PCR	Polymerase Chain Reaction
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
VEGF	Vascular Endothelial Growth Factor
VERO-CCl 81	Monkey Kidney cells
WHO	World Health Organization

ABSTRACT

Globally, cancer is the biggest cause of illness and mortality. Cancer comes second in prevalence in non-communicable disease in Kenya, after cardiovascular diseases. Cervical cancer is the leading cause of deaths in Kenya, resulting in as much as 11% of all cancer-related deaths. Presently there are several ways to treat cervical cancer: hysterectomy, radiation therapy, and chemotherapy. Chemotherapy is the most often utilized treatment because in Kenya, cervical cancer is typically diagnosed in its advanced stages. Although effective, chemotherapy is plagued by a myriad of challenges including severe side effects that greatly diminish the quality of living for the affected patients, the prohibitive cost of medical treatment and development of chemo-resistance to the chemotherapeutic drugs. Plant derived products are a feasible alternative in alleviating some of the challenges facing chemotherapy, especially in cervical cancer cases. Historically, *Rhamnus prinoides* and *Grewia villosa* plants have been utilized to cure and manage cancer and inflammatory illnesses. This study was undertaken to evaluate the phytochemical profile, the selective anti-proliferative activity of the extracts from *R. prinoides* and *G. villosa* root barks and their effects in limiting cell migration *in vitro*. In accordance with standard procedures, the 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) method was utilized to determine the anti-proliferative effects of the extracts *in vitro,* the *in vitro* scratch assay was used to determine the effects of the extracts in limiting cervical cancer cell migration while gas chromatography/mass spectrometry analysis was employed to identify extract specific compounds. The probable targets for the compounds found in the active extracts were identified using a variety of online databases and applications, and Pyrx software was utilized for molecular docking. *R. prinoides* ethyl acetate extract exhibited the most anti-proliferative effect having an IC_{50} value of 77.87 µg/ml while *G. villosa* ethyl acetate extract having an IC_{50} value of 100.70 µg/ml, similarly, exhibited the highest anti-proliferative effect among the extracts from *G. villosa*. The ethyl acetate extracts of both plants also had the highest selectivity indices with the *R. prinoides* extract having 4.40 and the *G. villosa* extract having a selectivity index of 2.48. The ethyl acetate extracts were also shown to inhibit cell migration *in vitro* with the IC_{50} concentration having the highest inhibitory effect after 48 hours. Phenols, triterpenoids, hydrocarbons, alkaloids, fatty acid esters were identified in the crude, hexane and ethyl acetate extracts of *R. prinoides* and *G. villosa*. 2,6,10-trimethyltetradecane and Benzene_1-methylundecyl compounds in the ethyl acetate extract of *G. villosa* and squalene, 3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene and Olean-12-en-3.beta.-ol,acetate compounds in the ethyl acetate extract of *R. prinoides* interacted with various oncogenic proteins with high binding affinity energies (<-4 kcal/mol). Network pharmacological analysis revealed that the compounds interacted with various proteins from key oncogenic pathways including the PI3K/Akt signaling pathway, carbon pathways in cancer and the EGFR tyrosine kinase resistance pathway. The ethyl acetate extracts of both plants upregulated TP53 mRNA levels while concurrently downregulating EGFR, ERBB2, and AKT1 mRNA levels. Additionally, the ethyl acetate extract of *R. prinoides* upregulated Bax mRNA levels while downregulating Bcl-2 and NF-kB mRNA levels, while the ethyl acetate extract of *G. villosa* upregulated Caspase 3 levels. In conclusion, the results from this study demonstrate the potential of *Grewia villosa* and *Rhamnus prinoides* extracts against cervical cancer *in vitro* and lay a solid foundation into further studies using the plant extracts for the development of drugs in cervical cancer therapy.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Cervical cancer originates in the cervix which comprises the ecto-cervix (the most distal part of the cervix) and an endo-cervix (the most proximal and inner part of the cervix). Atypical vagina bleeding, atypical discharge, pain in the pelvic region and dyspareunia are some of the symptoms associated with cervical cancer (Feudo *et al*., 2022).

After lung, colorectal, and breast cancers, cervical cancer is the fourth most diagnosed cancer in high-income nations in women. In 2018, 80% of all new cases world-wide occurred in low-to middle-income countries (LMICs) (Arbyn *et al*., 2020; Ferlay *et al*., 2021).

Breast and cervical cancer are the most diagnosed cancers in women living in LMICs, respectively. The burden is borne chiefly in low-resource regions or among people from lower socioeconomic regions. Cervical cancer ranks fourth with incidence rates of 7.4 in 100,000 women and death rates of 2.2 in 100,000 women. These rates vary according to the geographic location; in comparison to high-income countries, the rates notably higher in LMICs, with Eastern Africa recording incidence rates of 40.1 in 100,000 women and Kenya having crude rates of 19.4 in 100,000 women rising to 31.3 in 100,000 women when age is added as a factor (Arbyn *et al*., 2020; WHO 2021; Monk *et al*., 2022).

Although cervical cancer is ranked second in incidence, it predominates in mortality for Kenyan women, partly owing to diagnoses in the advanced stages, with a dismal prognosis, and as well

as diagnoses in the more advanced years, typically between 45 and 70 years old (Arbyn *et al*., 2020).

Despite government efforts, such as the free HPV vaccine and screening, cervical cancer remains a leading cause of mortality, partly due to late diagnosis and limited treatment options (Ng'ang'a *et al*., 2018). The United Nations has set a broad goal to decrease early deaths by NCDs by one third by the year 2030 as a response to the severe challenge that NCDs pose to human development and to slow the upsurge of NCDs worldwide (Ferlay *et al*., 2021).

The current treatments for cancer in Kenya today are radiotherapy, chemotherapy and surgery. They carry a heavy burden on the patients when it comes to the cost of treatment, the severity of the off-target effects of the treatment and the risk of complications, especially in surgery, which includes a risk of death. Therefore, there is an immediate and necessary need to find cost-effective, more active, and less toxic alternative treatments (Degu *et al*., 2017; Shaffi *et al*., 2024).

Traditional African medicine has been in use on the continent since time immemorial. A substantial portion of the population in Sub-Saharan Africa still depends on Traditional African Medicine for their healthcare needs (James et al., 2018) About half of novel conventional medicines that are released into the market today are plant-derived (Belete, 2019). Several commercial drugs sold today have been developed from medicinal plants. These include paclitaxel which is an anti-tumor drug developed from the bark of *Taxus brevifolia*, artemisinin which is an anti-malarial drug developed from *Artemisia annua*, digoxin used to treat heart

conditions such as heart failure developed from *Digitalis lanata*, atropine from *Atropa belladonn*a, aspirin from *Filipendula ulmaria*, among others. Furthermore, over 60% of all cancer drugs being used today originate from plants (Bhanot *et al*., 2011.).

Rhamnus prinoides and *Grewia villosa* are among the plants that are used in treatment today. In Kenya, the roots, leaves and stem extracts of *R. prinoides* have been used in treating sexually transmitted diseases, arthritis, as an analgesic for backaches and stomachaches, pneumonia and as nutritional supplements. Furthermore, it has been used to treat infections of the throat, nose, and ears. Treatments for sexually transmitted diseases and malaria have made use of the root extracts (Nigussie, Alemu, *et al*., 2021). *G. villosa* has been used traditionally in the Mbeere and Embu communities to treat and manage prostate and breast cancers (Kareru, 2007). The two plants, therefore, serve as excellent candidates for research on their anti-proliferation activity. This study, therefore, was conceived to bio-screen extracts from *R. prinoides* and *G. villosa* for their potential against cervical cancer.

1.2 Statement of the problem

In 134 out of 183 countries, cancer is the leading cause of early mortality, and in another 45, it ranks third or fourth, as per the WHO World Cancer Statistics 2020. Worldwide, cancer-related premature deaths in those aged 30 to 69 make up around 29.8% of all non-communicable disease-related deaths (WHO, 2020). The burden for cancer has increased by a factor of 4 in LMICs when compared to high income nations. According to the GLOBOCAN estimates, in females, the cancers that are mostly identified in prevalence and mortalities are Cervix uteri, breast and esophagus (Ferlay *et al*., 2021). In Kenya, according to a survey by the ministry of health between 2017 and 2021, cancer takes the third place as a leading cause of mortality after

infectious diseases and cardiovascular diseases, respectively. Currently, cancer accounts for approximately 37,000 new cases with a mortality of close to 28,000 cases. This is estimated to rise by 70% in the next two decades (Njue and Barsigo, 2019).

In women, cervical cancer comes fourth in terms of prevalence globally. Infection by the human papilloma virus (HPV), with HPV serotypes 16, 18, 31 and 45 being the most implicated, is the biggest risk factor for the development of cervical cancer (WHO, 2020). Globally, cervical cancer ranks third in mortality after esophageal and breast cancers (Ferlay *et al*., 2021). In Kenya, cervical cancer accounts for about 11% of all new cases in both genders and about 10% of all deaths linked to cancer in both genders (Njue and Barsigo, 2019).

In the high-income countries, early diagnosis has significantly decreased the mortality rates although the incidences remain high. Despite the countrywide disbursement of the HPV vaccine to young school going girls and young women, the incidence rates in the country for cervical cancer is still high, owing partially to the high mean ages of diagnoses, between 45 and 70 and the cancer diagnosis is made in the late stages. Recurrence of cervical cancer after treatment with chemotherapy in the late stage usually displays high drug resistance, therefore chemotherapy is not as effective. These are among the reasons for the heavy disease burden in the country (Arbyn *et al*., 2020; Jedy-Agba *et al*., 2020; Feudo *et al*., 2022).

The main limitations facing the current chemotherapeutic agents were serious side effects ranging from hair loss, nausea, weight loss and an increased risk of drug resistance within the

first two years post-treatment for cases of locally advanced cervical cancer (Hyeok *et al*., 2021; Feudo *et al*., 2022).

The medicinal value of these plants had not been scientifically validated. Their phytochemical profile, selective activity against cervical cancer cells and safety in normal cells remained unexplored prior to this study to the best of my knowledge.

1.3 Justification of the study

Phytochemicals-plant-derived secondary metabolites-have led to the development of many modern drugs, including paclitaxel for cancer treatment. There are many clinical trials ongoing in the world evaluating the effectiveness of the addition of phyto-therapy to the drug regimen of patients suffering from cancer (Greenwell & Rahman, 2015). The use of plants as lead agents as well as in combination therapy for the treatment and management of cervical cancer is a feasible option that should be further explored.

R. prinoides extracts had previously been shown to contain a high quantity of glycosides and flavonoids which had been proven to have anti-cancer properties in cells both *in vivo* and *in vitro* (Chen *et al*., 2020). *G. villosa* extracts had previously been used in the Embu community in Kenya for the management of cancer (Kareru, 2007). Scientific evidence for the anti-proliferation activity of these plants was scarce, which necessitated this study to fill that gap in literature.

1.4 Research Questions

1. What is the phytochemical composition of *R. prinoides* and *G. villosa* extracts?
2. Do *R. prinoides* and *G. villosa* extracts inhibit cervical cancer cell proliferation and are the effects selective?
3. What are the compounds putative targets that can be identified in the extracts?
4. Do *R. prinoides* and *G. villosa* extracts have effect on gene expression of key genes involved cancer?

1.5 Objectives

1.5.1 General objective

To determine anti-proliferative activities, phytochemical profiles, putative targets, and gene expression effects of *R. prinoides* and *G. villosa* plant extracts on cervical cancer cells.

1.5.2 Specific objectives

1. To determine the anti-proliferative effects of *R. prinoides* and *G. villosa* extracts against cervical cancer cell lines (HeLa) and their safety in normal cells (Vero CCL-81) using MTT assay
2. To determine phytochemical composition of *R. prinoides* and *G. villosa* extracts
3. To elucidate potential putative targets of the compounds in cervical cancer cells through network pharmacology and molecular docking approaches
4. To determine the effects of *R. prinoides* and *G. villosa* extracts on the gene expression of hub genes

CHAPTER TWO

LITERATURE REVIEW

2.1 Cancer

Cancer refers to the continued multiplication of cells that can be either linked to a mutation in which the cells are able to avoid regulatory signals or bypass terminal signals. Growth and multiplication of cells under normal circumstances is a tightly regulated process in the body and it only occurs to replace older cells to maintain organs and tissues such as the epithelial layer of cells and in the bone marrow or because of aging or injury. In cancer development, however, the process becomes aberrant as cells transform from normal to neoplastic. The new cells continue to divide and multiply, losing their morphology and leading to the formation of solid tumors. Many factors can lead to carcinogenesis, such as exposure to known carcinogens such as tobacco, radiation, asbestos, infection by viruses such as HPV that leads to the development of cervical cancer, and genetics which predisposes a person to developing a certain type of cancer (Phan & Croucher, 2020).

The progression of cancer occurs in stages known as hallmarks which highlight the transitioning of cells from normalcy to neo-plasticity and finally to cancerous states. This transformation is primarily driven by the accumulation of genetic mutations in proto-oncogenes and tumor suppressor genes, resulting in loss of growth control and evasion of apoptosis. There are currently eight cancer hallmarks, and they are acquiring the ability to sustain continued cell division, evading growth signals and suppressors, resisting programmed cell death, enabling endless proliferation, acquiring vasculature, inducing invasiveness and migration, reprogramming cellular metabolism and evading the immune system. All these factors culminate

in metastasis; the development of multiple tumors in several body regions (Yuan *et al*., 2019; Hanahan, 2022).

2.2 Molecular Basis of cancer

Genes have been implicated with the onset and progression of cancer, particularly those that are directly engaged in cell division and death. Cancer can originate and spread because of gene alterations. Ordinarily, the genes exist in an intricate balance that governs how cells divide and multiply. These are genes that promote tumor growth (oncogenes) and those that inhibit tumor growth (tumor suppressor genes) (Hanahan & Weinberg, 2011).

In the cancer life cycle, two primary mutations are key; mutations that promote increased production of proteins that are key to growth and cell division and mutations that lead to the inactivation of transcription of key genes that regulate growth or that lead to cell death. Mutations can cause over-stimulation of proto-oncogenes or the down-regulation or silencing of tumor suppressor genes (Cadoná *et al*., 2022).

Proto-oncogenes and their receptors are actively involved in cancer progression and development through the various signaling pathways and cascades that they are involved in. The pathways activated lead to the activation of growth stimulatory signals and cascades and influence and activate the growth stimulatory signals of neighboring cells through cell-to-cell signaling within the tissue. Receptor activation triggers cascades that activate transcription factors that bind to the genes responsible for the production of proteins that enhance proliferation (Hanahan & Weinberg, 2011; Hanahan, 2022b).

2.3 Mechanistic view of carcinogenesis

Carcinogenesis refers to when a normal cell starts the process of conversion to a pre-cancerous state and finally to cancerous and metastatic state. Many factors have been linked to carcinogenesis from infections to chemical carcinogens to exposure to ionizing radiation.

2.3.1 DNA lesions

When bases or chromosomes break, they are referred to as lesions. They can happen naturally or endogenously, or they can be induced by external forces. When they occur naturally, they are referred to as background. DNA lesions have the propensity to produce mutations as the cells divide. The number of mutations produced by exogenous mutagens is far higher than those produced by endogenous mutagens. DNA repair mechanisms can manage endogenous mutations while they are overwhelmed by the number of exogenous mutations. The lesions that escape repair tend to lead to mutations as the cell divides. Accumulations of these mutations leads to the transition to cancerous states (Barnes *et al*., 2018).

2.3.2 Cell Division

Among the most implicated mutations in the development of cancer are deletions, translocations and point mutations. Cell division can occur naturally in a cell due to aging or injury usually to replace old cells. When cells are mutated, however, the rate of cell division increases exponentially when compared to background cell division. Increasing cell division indiscriminately, increases the rate of errors and mutations in the new daughter cells which can lead to the initiation of a cell to a cancerous state (Marte, 2004)

2.3.3 Cell Cycle checkpoints

The process by which cells must undergo in mitosis is called the cell cycle. The most important checkpoint is at the late G1 phase to S phase. The checkpoints are designed to detect lesions and stop the replication of cells with mutations. The cell cycle checkpoint is not perfect, when cells

with mutations escape the checkpoints, cancer can be a result (Kastan & Bartek, 2004; Williams & Stoeber, 2012).

2.3.4 Defense Mechanisms

The cell has innate mechanisms to combat DNA lesions and to limit the number of mutations in the cells before and during cell division. This is mainly through DNA repair enzymes that check the DNA for mutations and stops cell division before mutations occur. These enzymes are inducible; therefore, they are triggered by the presence of certain mutagens. A flaw in the detection of DNA damage by these enzymes can lead to the development of cancer (Tell & Wilson, 2010; Chen, 2013; Torgovnick & Schumacher, 2015).

2.4 Risk factors associated with carcinogenesis

2.4.1 Diet

Calorie restriction diets rich in protein have been implicated in having a protective effect against cancer (Brandhorst & Longo, 2016). Antioxidant rich diets, mostly found in vegetables or fruits, have previously been implicated in limiting cancer development and progression. They help protect the cells against DNA damage due to the oxidizing end products of various metabolic pathways and also exogenous mitogens (Fuchs-Tarlovsky, 2013; Hecht *et al*., 2024). Animal fats, raw fish and red meat on the other hand have been implicated as potential initiators of carcinogenesis in colon cancer. Studies have shown that consumption of more than 100g of red meat daily is associated with a 17% increase in colorectal cancer risk (English *et al*., 2004; Ferguson, 2010). The method of cooking food has also been shown to be involved in carcinogenesis as potentially carcinogenic compounds can be formed during the cooking process such as nitrosamines, polycyclic hydrocarbons and heterocyclic amines(Dai *et al*., 2002; Di Maso *et al*., 2019) . In developed countries, tobacco use has been implicated in the carcinogenesis of lung cancer (Vineis *et al*., 2004).

2.4.2 Chronic Inflammation and infections

During infection, the immune system employs various mechanisms to combat chronic inflammation which include leukocytes and phagocytes. During the destruction of these pathogens, immune cells use a host of mutagenic and oxidizing agents including nitrogen peroxide, hydrogen peroxide, superoxide and hypochlorite (Yu *et al*., 2022). These substances can cause DNA damage especially when they are released for a prolonged period during chronic infection which has been implicated in cancer initiation (Multhoff *et al*., 2012; N. Singh *et al*., 2019). Various infections have been inculpated in carcinogenesis including Hepatitis B and C in liver cancer (Ringehan *et al*., 2017) and the HPV which has been connected to carcinogenesis of cervical cancer although its action has not been linked to inflammation. HPV, the main etiological agent for cervical cancer, does not necessarily act via inflammation but exerts oncogenic effects by disrupting tumor suppressor pathways such as p53 and pRb (Schiffman *et al*., 2007; Crosbie *et al*., 2013; Petry, 2014;). Schistosomiasis infection and *Helicobacter pylori* infection has also been linked to colon cancer development (De Flora & Bonanni, 2011).

2.4.4 Hormones

Long term use of sex hormones such as estrogen has been heavily inculpated in carcinogenesis of various cancers including endometrial cancer. Estrogens are thought to increase the cell division rate while progesterone are thought to decrease the frequency in which the cells divide. In addition, an increase in the rate of proliferation of breast cancer cells has been exhibited in the presence of estrogens (S. B. Brown & Hankinson, 2015; Brinton *et al*., 2016; Rodriguez *et al*., 2019)

2.4.5 Occupational Hazards

Chronic exposure to chemicals classified as carcinogenic by the International Agency for

Research on Cancer has been linked to cancer development. Industries in aluminum production, coal gasification, coke production and mining have been associated with carcinogenesis of lung cancer while the rubber industry has been linked to the development of leukemia while welding has been connected to the formation of melanoma (Cogliano *et al*., 2011).

2.4.6 Sun exposure

The ultraviolet rays emitted by the sun have long been attributed to the emergence of skin cancer. Chronic exposure especially to children that leaves sun burns has been implicated mostly (Andreassi, 2011).

2.5 Hallmarks of cancer

Cancer cells develop in stages as they progress from normalcy to neoplastic states and finally to metastatic states. These stages are called hallmarks as a cancer cell moves across the different stages it becomes more and more cancerous (Hanahan, 2022). There are currently eight cancer hallmarks; continued proliferative signaling, resisting growth suppressors, resisting apoptosis, enabling continued replication, gaining the ability to form vasculature, enabling invasion and metastasis, reorganizing cell metabolism and evading death by the immune system (Cadoná *et al*., 2022).

2.5.1 Continued proliferation

Cells proliferate through growth signals that set off processes that induce the mitotic pathway that leads cells to produce daughter cells. This signaling is however limited in normal cells. In cancerous states, however, cells can bypass the mechanisms set up by the cells to limit proliferation and they are able to continuously divide. They are believed that they accomplish this by using growth factors that attach to tyrosine kinase receptors on cell surfaces, thereby activating pathways that promote sustained cell proliferation or circumvent cell cycle checkpoints, allowing for continuous division (Hanahan & Weinberg, 2011; Hanahan, 2022b).

2.5.2 Evading growth suppressors

Sustaining proliferation is not enough to sustain cancer cells, they must also evade innate mechanisms set up by cells to limit growth which is tightly linked to the activation of tumor suppressor genes. Two such proteins activated by tumor suppressor genes are the RB (retinoblastoma like protein) and TP53 (tumor protein 53). The RB protein is critical in the cell cycle as it determines whether a cell is fit to complete the cycle and make a daughter cell or whether to stop the cell cycle while TP53 is mainly involved in pathways that activate apoptosis in cells. In cancerous states, silencing of genes that code for growth suppressors has been shown (Hanahan & Weinberg, 2011; Hanahan, 2022).

2.5.3 Resisting cell death

As an adaptation of irreparable injury or aging, is the initiation of programmed cell death (apoptosis). Cancer cells evade this normal trajectory of cells and immortalize themselves despite the stress that they undergo such as DNA damage (P. Singh & Lim, 2022). They are able to do this by up-regulating the genes that favor survival such as the genes that encode for BcL-2 (B-cell Lymphoma like protein 2) family of regulatory proteins that are anti-apoptotic in favor of survival and the PI3-kinase-AKT-mTOR signaling pathway that similarly blocks apoptosis, while at the same time down-regulating apoptotic genes such as tumor protein 53 (Wong, 2011; Letai, 2017).

2.5.4 Enabling continued replication

Normal cells are limited in the number of times they can multiply. This is due to two phenomena, senescence which is a state in which cells are not able to proliferate but remain viable and crisis which is when cells die due to depletion of telomeres (Schmitt *et al*., 2022). Cancer cells evade death in crisis phase through generation of the telomerase enzyme and they escape. Cells that can evade senescence and emerge from crisis are able to replicate indefinitely

and attain immortality (Hanahan & Weinberg, 2011; Hanahan *et al*., 2022).

2.5.5 Accessing vasculature

Once the cancer cells acquire immortality, they grow until they form a solid tumor. Once the tumor is formed it undergoes a crisis due to limited access to nutrients and oxygen. In response, tumors form the tumor neo vasculature through a process called angiogenesis. Tumors are believed to achieve this hallmark through the induction of angiogenic genes such as the vegetative growth factors (VEGF) and thrombospondin-1 (TSP-1). These genes are switched off after embryogenesis (Tonini *et al*., 2003; Nishida *et al*., 2006; Rajabi & Mousa, 2017).

2.5.6 Invasion and metastasis

A tumor enlarges to a point where cells start migrating from the original site and colonizing distant sites. Normal cells are kept in place due to processes including cell-cell communication and attachments through molecules to the extracellular matrix. These adhesion molecules are often severely dysregulated in cancer cells. Cadherins are the ones most affected. Cells start by invading local tissues and organs, then they enter into the local bloodstream and lymphatic vessels through a process coined intravasation, then they escape the blood vessels and form micro-tumors in new sites, a process called extravasation and finally they form macro-tumors in new sites, a process called colonization (Leber & Efferth, 2009; Jiang *et al*., 2015).

2.5.7 Reprogramming cell metabolism

Due to the initial restriction of oxygen and nutrients, tumors use glycolysis primarily to metabolize their glucose, a phenomenon that is termed the Warburg effect after the man who discovered it. As the tumor gains vasculature and normal levels are attained, they develop a hybrid system incorporating both glycolysis and normal oxidative metabolism (Barba *et al*., 2024). This allows for enough generation of ATP to sustain their high levels of proliferation. As a result, there are many mitochondrial mutations observed in cancer cells. Key genes which are

master regulators of metabolism including *p53* and *c-myc* are found to be significantly deregulated in cancer cells (Hammoudi *et al*., 2011; Jang *et al*., 2013; Wu & Zhao, 2013).

2.5.8 Escaping the immune system

Immune surveillance is the mechanism via which the immune system detects, identifies, and eradicates nascent malignant cells. Immune cells such as Natural killer (NK) and cytotoxic T cells are the champions majorly tasked with immune surveillance. The secretion of large amounts of immune suppressors such as TGF-β and chronic inflammation are implicated as mechanisms of evasion of the immune system by quenching immune cells and a phenomenon known as immune-editing whereby cancer cells shed markers that may target them in the immune system from their surface receptors (Finn, 2012; M. Candeias & S. Gaipl, 2016;Gonzalez *et al*., 2018).

2.6 Cervical cancer

Cervical cancer is cancer which originates from the cervix. The cervix consists of an epithelium of stratified squamous cells (ectocervix) and a mucus secreting epithelium made up of columnar cells (endocervix). The junction between these two layers (squamo-columnar junction) is believed to be the area of highest risk of neoplastic transformation. Most cervical cancer tumors originate in the ectocervix and are mostly composed of squamous cells (up to 77% of all cervical cancer tumors) (Small Jr *et al*., 2017). The main clinical symptoms linked to cervical cancer include post-coital vaginal bleeding, irregular bleeding, pain in the pelvic region, dyspareunia and watery vaginal discharge. Cervical cancer is usually asymptomatic until the late stages. Diagnosis is made by a physician after physical investigation and confirmation through examination of tissue biopsies (Lea & Lin, 2012; Hyeok *et al*., 2021).

After diagnosis is made cervical cancer is then staged clinically which determines the method of treatment and is also a clinical indicator of survival. Surgery (hysterectomy and/or lymphadenectomy) is the operative procedure that is recommended for early stages of cervical cancer while late stages usually involve a combinatory treatment of radiation therapy and chemotherapy (Shepherd, 2012).

2.6.1 Risk factors associated with cervical cancer

2.6.1.1 Human Papilloma Virus (HPV) infection

HPV is a papilloma virus belonging to the family *Papovaviridae*. HPV is a small, naked virus (not enveloped), having a diameter of 55 nm. It is comprised of a capsid, icosahedral in shape, made up of at least two proteins, L1 and L2, and 72 capsomers. It has a single circular DNA molecule that is double-stranded. The oncogenicity of HPV variations varies; the most carcinogenic HPV serotypes are 16, 18, 31, and 45. Infection with multiple sero-types of HPV is also possible and it is usually associated with more severe cervical cancer cases (Schiffman *et al*., 2007; Petry, 2014).

HPV is identified in more than 90% of cervical cancer cases and serves as a important cause in malignant progression of cervical cancer to invasiveness. From infection to disease, it takes about fifteen years. Transmission of HPV primarily transpires by direct skin contact with sexual activity being the main mode of transmission although contact to contaminated clothing and surfaces can also lead to infection through fomites. High sexual activity with diverse partners increases the risk of contracting the infection and also sexual activity at an early age (de Sanjose *et al*., 2010; Petry, 2014).

2.6.1.2 Co-morbidities with HPV infection

A correlation between cigarette smoking and an increase in the risk of development of cervical cancer has been made in previous studies, both active and passive smoking. Chronic smoking is

associated with a higher chance of the HPV virus not being cleared completely due to the decreased immune-surveillance and overall immune suppression created by smoking. Cigarettes also contain some mutagenic compounds which can contribute to carcinogenesis of cervical cancer (Fonseca-Moutinho, 2011).

Identification and clearance of HPV is cell-mediated by the immune system therefore conditions that can impair or reduce the efficiency of the immune system to eradicate the HPV infection may increase the risk of cancer development. These include infection by the human immune-deficiency virus, co-infection of HPV with human herpes simplex virus 6 and 7 and cytomegalovirus infection (Raju, 2015).

The regulatory (upstream) region of HPV can be turned on by steroid hormones including progesterone which is the primary ingredient of oral contraceptives and dexamethasone as it contains similar sequences as those of the glucocorticoid responsive elements. This has led to an implication of the carcinogenesis of the prolonged use of oral contraceptives and cervical cancer (Hellberg, 2012).

2.6.2 Cervical cancer burden

Global cancer figures for 2020 indicate that, roughly 604,000 cases with 342,000 mortalities globally, cervical cancer ranks fourth among malignancies in women, after lung, breast, and colon-rectal cancers, respectively. It is, however, first in mortality rates in women with up to 19.9% of all cancer cases followed by breast cancer with 19.8%. In 2020, Eastern Africa had the most incidences of cervical cancer in the world, accounting for up to 40% of all cases with 28.6% of all deaths being from cervical cancer (Arbyn *et al*., 2020; Sung *et al*., 2021; Deo *et al*., 2022).

Most cases, up to 80%, of cervical cancer occur in LMICs (Jedy-Agba *et al*., 2020), which may partly be due to limited resources for screening which leads to detection of cervical cancer in the invasive stage. The prognosis for cervical cancer goes down significantly the later the diagnosis is made (Ng'ang'a *et al*., 2018).

The ministry of health in Kenya conducted a survey between 2017 and 2021, cancer is ranked third in terms of mortality behind infectious diseases and cardiovascular diseases. In Kenya, cervical cancer is the accounts for approximately 11% of all new cases in both genders and 10% of all deaths linked to cancer in both genders, which is the most fatalities due to cancer in the country. Currently, in terms of incidence, there are approximately 37,000 new cases with consequent mortality rates of up to 28,000 cases. This is estimated to rise by 70% in the next two decades (Njue and Barsigo, 2019).

2.6.3 Chemotherapy in cervical cancer

Cervical cancer is diagnosed in the late stages in low-to-middle countries where the only treatment options available are radical hysterectomies and/or chemotherapy. Chemotherapy is given either through single agents or multiple agents combined, depending on the clinical stage of the disease. Chemotherapy is given either orally or as intraperitoneal injections.

2.6.3.1 Single therapy agents

Many compounds and agents have been used to treat cervical cancer lesions and tumors at different stages of disease. These include paclitaxel (an substance that makes microtubules more stable via binding to the N-terminal of β-tubulin subunit leading to its phosphorylation which increases microtubule polymerization cascading to cell death) (Alqahtani *et al*., 2019), topotecan

(a camptothecin which blocks topoisomerase-I activity by making Topo-I DNA cleavable complex more stable which increases the number of single-stranded breaks in the DNA causing cell death) (Drummond *et al*., 2010), ifosfamide (a synthetic analogue of cyclophosphamides, it is an alkylating agent that acts through interference with DNA replication) (Ensergueix *et al*., 2020; Sprangers & Lapman, 2020), vinorelbine (an antimitotic agents that acts as a microtubule destabilizing agent that leads to microtubule depolymerization leading to high rates of spindle destruction) (Bisogno *et al*., 2019), pegylated liposomal doxorubicin (an anthracycline antibiotic that acts by intercalating DNA thereby inhibiting DNA polymerase and consequently inhibiting DNA synthesis) (Meredith & Dass, 2016) and cisplatin (a platinum based apoptotic agent that interacts with DNA forming adducts) (Brown *et al*., 2019). Cisplatin exhibits the highest activity with a dosage of 50mg/m^2 of tumor mass.

2.6.3.2 Combination therapy

Cisplatin has been used combines with many other substances such as methotrexate, vinblastine, bleomycin, mitomycin C, however, the combinations that had the highest activity overall were cisplatin and ifosfamide, cisplatin and ifosfamide and 5-Fluorouracil, paclitaxel and cisplatin, and cisplatin and topotecan (Elit & Hirte, 2014).

2.6.4 Downsides of chemotherapy

Although effective in reduction of tumor masses and inhibiting metastasis, chemotherapeutic drugs are laden with many downsides in their therapeutic roles in cancer, both long term and short term. The major limitations of chemotherapeutic agents are severe side effects both during and after chemotherapy and the carcinogenic nature of the agents themselves. Combination therapies, although more effective, have more severe toxicities compared to single agent therapies (Bida *et al*., 2024).

2.6.4.1 Side effects

Side effects can be categorized according to the part of the body most affected. Those affecting the Central Nervous System lead to fatigue, nausea, alopecia, anxiety, depression, loss of appetite leading to weight loss, insomnia, peripheral neuropathy, headaches and numbness, those affecting the Gastrointestinal tract lead to constipation, heartburn, indigestion, diarrhea, incontinence, bloody discharge and flatulence, those affecting the kidney lead to nephropathy, kidney damage and dysuria, those affecting the circulatory system lead to anemia and thrombocytopenia and those affecting the immune system lead to neutropenia (Assi *et al*., 2021).

Secondary conditions such as increased propensities to infections due to a decrease and efficiency of the immune system due to malnutrition and diminished immune cells can arise as a consequence of chemotherapy (Nesher & Rolston, 2014). In addition to the side effects especially in late-stage cervical cancer and recurrent tumors, there is increased risk of the emergence of multidrug resistance to the chemotherapeutic drugs (Hyeok *et al*., 2021).

2.6.4.2 Chemotherapeutic agents as carcinogens

Alkylating agents are carcinogenic in nature. Common anti-cancer drugs that are also alkylating agents include platinum agents such as carboplatin and cisplatin, cyclophosphamide and chlorambucil. Tamoxifen, which is effective against breast cancer, can cause endometrial cancer as it is an anti-estrogen. Phorbol ester, which is effective against leukemia, was not used for the longest time because of its carcinogenic nature (Blagosklonny, 2005).

2.6.5 Natural products in cervical cancer

Natural products have historically served as important sources of therapy for cancer including cervical cancer. Diverse natural products have been evaluated and shown to limit the *in vitro* proliferation of cervical cancer cells and compounds have been isolated for drug discovery. In

addition, natural products are known to have milder side effects compared to conventional chemotherapeutic agents in use today. Effective anti-cancer drugs in use today developed from natural products include vinca alkaloids (vincristine and vinblastine) (Dhyani *et al*., 2022). The plants that have previously been evaluated for anti-proliferative acidity of cervical cancer cells *in vitro* include *Azadirachta indica (*methanolic stem bark*)*, *Moringa oleifera (*aqueous and ethanolic leaf extracts*)*, *Curcuma longa (*essential oils*)* and *Rhamnus sphaerosperma (*crude and ethanolic extracts, emodin compound*)*(Santos *et al*., 2016; Moreira *et al*., 2018; Hyeok *et al*., 2021; Pandey & Khan, 2021).

Given the limitations of conventional chemotherapy and the growing interest in safer plant-derived alternatives, it is critical to explore indigenous plants such as *Rhamnus prinoides* and *Grewia villosa* which have ethnomedicinal relevance yet remain underexplored in cervical cancer models.

2.7 *Rhamnus prinoides*

2.7.1 Description of the plant

In Kenya it is known as "Mukarakinga" in the Kikuyu community (Mathiu, 2016) and "Olkonyil" in the Maasai community (Muthee *et al*., 2011). It is a small tree or shrub as shown in Figure 2.1, which is domiciled in the Rhamnaceae family. It is found in most African countries including Kenya(exotic), Ethiopia, Uganda, Tanzania, Eritrea, Angola, Malawi, Mozambique, Zambia, Zimbabwe, Cameroon, Democratic Republic of Congo and South Africa (Nigussie *et al*., 2021).

Figure 0.1 A picture of *Rhamnus prinoides* taken in Mount Kenya region (Source author)

2.7.2 Ethnobotanical uses of Rhamnus prinoides.

In Kenya, traditionally, the plant has been used in the treatment of acidity, rheumatoid inflammations, pneumonic symptoms as well as the flu, stomach ailments, back pain, to manage malnutrition as well as to manage sexually transmitted infections such as gonorrhea. Decoctions made from the leaves are used in treatment of flu-like symptoms, stomach ailments, to alleviate pain, malaria, diarrhea and ringworm infections (Muthee *et al*., 2011; Mathiu, 2016; Nigussie, Alemu, *et al*., 2021). In Tanzania, a root decoction in combination with other plants is used for treatment of colic while in Ethiopia, a leaf decoction combined with various other plants is taken before meals as a preventative measure for contracting sexually transmitted diseases (Nigussie, Alemu, *et al*., 2021), as animal de-wormers, (Bekele & Mussa, 2009), treatment of hepatitis and leech infestations (Nigussie, Alemu, *et al*., 2021). The leaves can also be made into an ointment and used to relieve joint pains (Chen *et al*., 2020).

2.7.3 Phytochemicals present.

A qualitative study conducted by Teklit Amabye on the phytochemicals present in *R. prinoides* roots noted the that triterpenes, saponins, tannins, phenols, glycosides, cardiac glycosides and resins were present (Amabye, 2016).

2.7.4 Biological activities of the extracts

R. prinoides root extracts have previously been tested for anti-inflammatory as well as antioxidant properties (Chen *et al*., 2020), the leaves for radical scavenging activity using DPPH and anti-oxidant activity (De Souza *et al*., 2019; Manoharan *et al*., 2019; Negash *et al*., 2021) and *in vitro* antibacterial activity (Molla *et al*., 2016; Campbell, 2020; Geta & Kibret, 2020). Thus far *R. prinoides* has not been tested for *in vitro* anti-proliferation activity.

2.8 *Grewia villosa*

2.8.1 Description and distribution

It is part of the Tiliaceae family. In Kenya, it is referred to locally as "Mubuu" by the Mbeere community in Embu County, in Central Kenya (Jared Misonge *et al*., 2019). It is a short tree that can reach four meters in height. It has wide serrated leaves. It grows in the dryer regions in East Africa, Sudan, India, Pakistan, Egypt, Cape Verde Isles, Tropical Africa, Arabia, and East Indies. Its stem and leaves are covered in silky hair when it is young. Flowers yellow-red-brown, in small clusters. Cymes umbel, 4-6 flowered, axillary or leaf-opposed; Sepals 5, lanceolate, 8 - 10 × 1.5 - 2 mm, acute at apex, Petals 5, oblong densely hairy Stamens 25 - 30, filaments c. 5 mm long, Ovary subglobose densely villous, style 4-lobed. Fruit is soft drupes and hairy when ripe, red brown, about 1 cm in diameter, 1-2 seeds per drupe. The ripe fruits are eaten as food and the leaves and the fruits are used as feed (Bashir *et al*., 1982; Kareru, 2007; Sureshkumar, 2017).

2.8.2 Ethno-medicinal uses

In India, the dried fruit is ground to a powder then dissolved using water and used to treat stomach ailments, the roots are dried and the root powder in water is taken orally to alleviate conditions such as diarrhea, the boiled crushed root is given to relieve body aches as well as coughs, a mixture of *G. villosa* roots in combination with the roots of *Grewia tenax* and *Grewia. flavascens* is given to treat syphillis, also in the treatment of tuberculosis as well as smallpox. Powders made from the barks and roots are used to relieve urinary tract infections. Fresh leaves as well as fruits are given to animals after they have given birth to help in expelling the placenta (Kumar *et al*., 2022; Sureshkumar, 2017). In Kenya in the Mbeere community in Embu County, in Central Kenya, the decoction from roots is given for treatment for prostate and breast cancers (Kareru, 2007). *G. villosa* has not been tested for *in vitro* anti-proliferation activity thus far.

2.9 Role of Network Pharmacology and Molecular Docking in Cancer Drug Discovery

Cancer is a disease that is complex in nature that affects multiple biological processes and that necessitates polypharmacological approaches for therapy. Network pharmacology is a systems approach that creates links between genes targeted by compounds in a plant and genes associated with a particular disease to create a "disease-target protein-drug" network. Molecular docking approaches helps us to discover and predict the interactions of ligands in binding sites of protein macromolecules (Zhao *et al*., 2024). One such example is imatinib used for the treatment of oral squamous carcinoma targets several tyrosine kinases in its anti-proliferative activity (Ma *et al*., 2024). Additionally, network pharmacology helps us to identify the molecular mechanism of action of plant compounds which can help in the optimization of its activity. Many small molecule inhibitors have been discovered and used in cancer therapy and network pharmacology and molecular docking approaches can help narrow down on the small molecules potentially interacting with the compounds. The flowchart below shows the processes involved in obtaining

the targets associated with different compounds (Chandran *et al*., 2017).

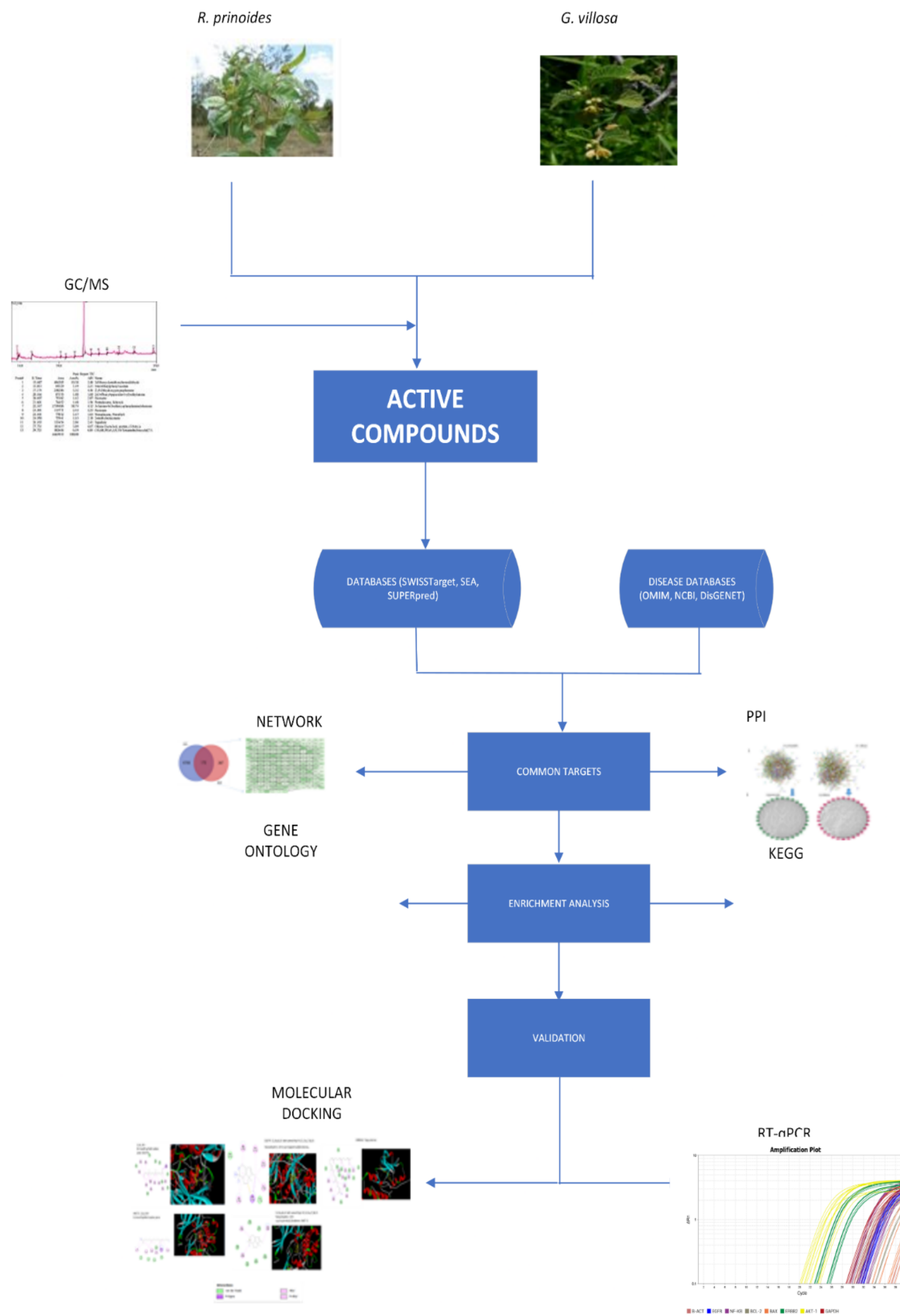

Figure 0.2 Diagram showing in silico workflow

2.9.1 Genes under study

2.9.1.1 Epidermal Growth Factor Receptor

One of the ways of regulating growth in normal cells is through receptor tyrosine kinase signaling. Cancer cells can increase signaling of these tyrosine receptors through continued activation of the receptors and desensitization to halting signals. This aberrant over activation of the receptors may be due to persistent binding of their ligands which include, epiregulin (EP), epidermal growth factor (EGF), and transforming growth factor (TGF), among others to EGFR. One of the receptors commonly activated in cervical cancer among many other cancers is the epidermal growth factor receptor. The human EGFR family comprises four closely related receptors; EGFR, ERBB2, ERBB3 and ERBB4. This receptor is involved in activation of cascades that favor the growth of cells. These pathways include the PI3K/Akt cascade, STAT cascade and Src kinase cascade (Lurje & Lenz, 2010).

The PI3K/Akt pathway regulates apoptosis, tumor invasion as well as metastasis. EGFR interacts with ERBB3 activating PI3K which then activates Akt1. Akt1 then recruits and activates the mTOR that leads to the translation of proteins favoring cell growth and proliferation (Bahrami et al., 2017a).

EGFR regulates the STAT pathway through JAK (Janus kinase) dependent and JAK independent mechanisms. STAT proteins are cytoplasmic transcriptional factors that are usually inactive unless activated. STAT proteins bind to the SH2 domain of phosphotyrosine kinase residues, after which the dimers relocate to the nucleus where they induce the production of gene targets.

The interaction between EGFR and STAT3 was shown to promote tumor development and progression (Jackson & Ceresa, 2017).

SRC kinase activation was shown to promote the initiation and advancement of multiple cancer types by mechanisms such as cell proliferation, migration, adhesion, and tumour angiogenesis. The SRC kinase protein can be activated through receptor tyrosine kinases such as EGFR or independently. When it is activated independently it can lead to induction of EGFR therapy resistance (Chen *et al*., 2018).

EGFR which is activated in both cancerous and non-cancerous tissues, has been found to be dysregulated in cervical cancer tumors and therefore serves not only as a key biomarker for cervical cancer progression and prognosis, but also as an important target for cervical cancer therapy. Agents against EGFR have also been developed and proposed for cervical cancer treatment (Soonthornthum *et al*., 2011). While EGFR is a known driver of cervical cancer, it is hypothesized in this study that specific phytochemicals such as tannins and saponins present in *R. prinoides* may interfere with EGFR signaling pathways through binging pocket, potentially inhibiting PI3K/Akt activation.

2.9.1.2 Erb-B Receptor Tyrosine Kinase 2

Also referred to as HER2, ERBB2 is a part of the EGFR family. Being a transmembrane protein, it consists of region outside the cell membrane, a middle transmembrane region and an inner tyrosine kinase domain (intracellular). A study by Perez-Regadera *et al* (2009) found that in the locally advanced stage of cervical cancer, ERBB2 was an important marker of a poorer prognosis.

ERBB2 signaling is heavily controlled in normal cells. ERBB2 plays a primary part in promoting growth and survival. Tumors have an aberrant expression of ERBB2 protein usually over expressed, there is also an over expression of the ligands for the ERBB2 protein leading to extended expressions and activation of downstream signaling which promotes cell proliferation, induction of angiogenesis, cell migration, cell differentiation and inhibition of apoptosis. Inhibition of ERBB2 has previously been proven to limit the cell cycle progression, activate apoptosis and inhibit the production of pro-angiogenic factors (Lurje & Lenz, 2010).

2.9.1.3 AKT1

It is also identified as protein kinase B alpha (PKBα). AKT1 is expressed in different types and has been linked to roles in anti-apoptosis, cell migration and tumor progression. AKT1 is mostly expressed in epidermal cells. Activation of AKT1 can be through phosphate addition by the Phosphatidylinositol-3 kinase protein (Faes & Dormond, 2015). Growth factor receptor kinases such as EGFR, activate PI3K, then phosphatidylinositol-4,5-bisphosphate (PIP2) becomes converted to phosphatidylinositol-3,4,5-triphosphate (PIP3). Aggregation of PIP3 causes AKT1 activation which prompts the activation of MTORs which results in increased protein translation involved in cell survival and proliferation (Bahrami *et al.,* 2017a, 2018).

A study by Faried *et al*. (2008) showed that both Akt1 and its effector m-TOR protein levels are overexpressed in cervical cancer and conventional therapies like cisplatin which act by inducing apoptosis through activating caspases and inhibiting the PI3K/Akt/mTOR pathway.

2.9.1.4 Tumor suppressor protein 53 (TP53)

Tumor suppressor protein 53 is activated by the cells in response to stress including damage to

the DNA, hypoxia or dysregulation of oncogenes. It promotes vigilance in cell cycle checkpoints, DNA repair, normal cellular aging as well as apoptosis. It is usually highly inactivated or mutated tumor suppressor protein in cancer including cervical cancer (K *et al*., 2024). The main activity of tumor protein p53 in proliferation is through apoptosis. TP53 affects apoptosis through; transcriptional influence of the Bcl-2 family of proteins by increasing the ratio of the pro-to anti-apoptotic proteins in favor of apoptosis, thereby increasing cellular levels of Bax and BH3 family proteins (Puma, Noxa and Bid) and decreasing cellular levels of Bcl-2 and Bcl-xL proteins, increasing apoptotic machinery such as Apaf-1, which activates caspase 9 which then initiates the caspase cascade and targeting survival signaling such as members of the PI3K/Akt family (Fridman & Lowe, 2003; Hemann & Lowe, 2006).

TP53 is designated as the "keeper of the genome" because it maintains the integrity of the cell cycle. Functional and numeric loss of tumor protein 53 leads to a decreased efficiency in the apoptotic process. The E6 protein of the human papilloma virus binds to the cellular tumor suppressor protein 53 forming protein-protein dimers marking it for degradation and proteasome inactivation. The E6-p53 dimers, even if not degraded, are functionally inactive. Mutations induced by cervical cancer tumors also lead to inactive forms of p53 (Haensgen *et al*., 2001).

2.9.1.5 Caspases

Caspases are the executors of apoptosis. Deregulation of caspases is a common occurrence in cancer. Caspases exist as a family of 10 namely caspase 1-10, the most common and tested in this study being caspase 3. Effector caspase 3 activates key proteins through mitigating their cleavage by proteolysis including PARP (poly (ADP-ribose) polymerase). Caspase 9 on the other hand induces the catalytic cleavage of other caspases including caspase 3 activating them

(Chowdhury *et al*., 2008).

Lu *et al*. (2010) showed that the expression levels of caspase 3 in cervical cancer tumors were significantly reduced when compared to non-cancerous cells which may be a way in which cervical cancer cells evade apoptosis.

2.9.1.6 B cell Lymphoma-2 family of proteins

The complex group of related proteins known as Bcl-2 and family regulates the mitochondrial membrane (outer) by either increasing its permeability to allow soluble apoptotic proteins, including cytochrome c, to diffuse into the cytosol or by causing cytochrome c to engage APAF-1 (apoptotic protease activating factor -1) to transform into the apoptosome, to which caspase 9 binds, starting the apoptotic program by subsequently activating the effector caspases 3 and 7. Inversely, the other class of Bcl-2 proteins, limit the porosity of the mitochondrial outer membrane thereby effectively inhibiting apoptosis. Both sets of Bcl-2 family of proteins exist in the cells and form an equilibrium unless factors such as cell stress or DNA damage potentiate the expression of one over the other (Riedl & Salvesen, 2007; Chipuk *et al*., 2010).

The cytosol, the endoplasmic reticulum, or the outer mitochondrial membrane contain the pro-survival Bcl-2 family of proteins (Bcl-2, Myeloid cell leukemia 1 (Mcl-1), Bcl-xl, Bcl-related gene A1, and Bcl-w). These proteins are designated survival proteins. The two types of anti-survival Bcl-2 proteins are BH-3 only effector proteins (Bcl-2 antagonist killer 1; BAK), and Bcl-2 associated protein x (BAX) (Kvansakul *et al*., 2008; Chipuk *et al*., 2010).

Bcl-2 has been found to be deregulated in cervical cancer tumors and has been used as markers

for tumor progression and disease prognosis (Manusirivithaya *et al*., 2006).

2.9.1.7 Nuclear factor kappa B

This cytosolic transcription-influencing protein crosses to the nucleus to alter the expression of several other proteins, including as cell cycle proteins, adhesion molecules, cyclooxygenases, tumor necrosis factor, cytokines, and angiogenic factors. The primary function of nuclear factor kappa B in cancer is through the regulation of programmed cell death. It is activated early during apoptosis or right before apoptosis is induced and it is believed to be an inhibitor of apoptosis. The method most postulated is through increasing cytosolic levels of pro-survival proteins such as TRAF-1, Bcl-2, Bcl-xL and Bfl-1. Active NF-κB induces the gene transcription for those that code for anti-apoptotic proteins (c-IAP1 and c-IAP-2) which block caspase functions through dimerization or mark them for ubiquitylation or degradation by proteasomes. It can also interfere with the induction of p53. In the cell cycle NF-κB acts by transcribing the gene for cyclin D1 which is implicated in cellular transition from G1/S to G2 thus promoting cell cycle progression (Magné *et al*., 2006; Serasanambati & Chilakapati, 2016).

Mutations in NF-κB are common in cervical cancer tumors leading to its overexpression (Tilborghs *et al*., 2017).Given its central role in apoptosis inhibition and immune evasion, NF-κB remains a prime candidate for targeting with natural inhibitors from bioactive plant extracts.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design and experiment grounds

This study adopted an *in vitro* and in silico experimental design to investigate the phytochemical composition and anti-cervical cancer potential of *R. prinoides* and *G. villosa* plant extracts against cervical cancer cell lines.The experiments were conducted at the Center for Traditional Medicine and Drug Research in the Kenya Medical Research Institute except for the GC/MS analysis which was carried out at Jomo Kenyatta University for Agriculture and Technology.

3.2 Plant sample collection and preparation

Rhamnus prinoides was collected from Mount Kenya region in the coordinates 0° 07′ 15.60″ N, 37° 20′ 7.20″ E while *Grewia villosa* was collected from Mbeere in Embu County, Central Kenya. Mount Kenya and Embu regions were selected due to their rich ethnobotanical diversity and traditional usage of the target plants. The plants were identified, collected and their voucher specimens, Mwitari/Gathiuru/RM/001/2022 (*R. prinoides)* and NSN20 (*G. villosa)*, were preserved at the East African Herbarium, Kenya National Museums. The root barks of both plants were wrapped up individually and shipped to the Kenya Medical Research Institute at the centre for traditional medicine and drug research for processing. A taxonomist was engaged to aid in the process of identification as well as collection. The root barks were air dried at room temperature and a laboratory mill (Wood Rolfe Road Tolles Bury Essex, UK) was used to grind the root barks into a fine powder after they had been split into smaller pieces and dried. After which, the powders were placed in airtight bags until they were needed.

3.3 Cold solvent extraction and solvent partitioning

The method described by Okpako *et al* (2023) was followed (Okpako et al., 2023). The independent variables were the concentrations of plant extracts while the dependent variables were the level of cervical cancer cell inhibition, gene expression changes and wound healing capacity. 800 grams of the root bark of *R. prinoides* that had been previously ground and stored and 500 grams of the ground root bark of *G. villosa* were each weighed separately and soaked separately in 2 liters DCM and Methanol (1:1) mixture for 72 hours. Methanol is a highly polar solvent while DCM is only slightly polar, therefore, mixing the two solvents allowed for the total extraction of highly polar and less polar extracts.

Sequential cold solvent extraction was used whereby the gound plant extracts were first soaked in a mixture of DCM: Methanol then the concentrate (crude extract) was soaked in hexane (to dissolve the non-polar compounds), the remnant of which was soaked in a mixture of ethyl acetate and water (to dissolve the mid-polar and polar compounds respectively). A vacuum rotary evaporator (Buchi Switzerland) at 55 ℃ was used to concentrate the filtrate after they had been filtered through Whatman No. 1 filter paper. A partition of the crude extract was stored, and the remaining extract was transferred to a separating funnel where 400mL of hexane was sequentially added in 200mL portions as shown in Figure 3.1. The n-hexane partition was evaporated by use of a vacuum rotary evaporator at 59 ℃ and the extract dried and stored at -20 ℃ until use. After adding 400 mL of water and ethyl acetate in a 1:1 ratio to the residual extract, the mixture was allowed to form two distinct layers by being left overnight in a decanting funnel. While the ethyl acetate extract was concentrated in a vacuum rotary evaporator set to 67 °C, the water extract was lyophilised in a freeze dryer (Modulyo Edwards high vacuum, Crawley, England, Britain, Serial No. 2261) as shown in Figure 3.1. For thorough extraction, the residue

was repeatedly soaked with fresh solvent at 72-hour intervals using the recovered mixture of DCM and methanol, and a discernible colour shift was noted. The extracts were stored at -20 °C.

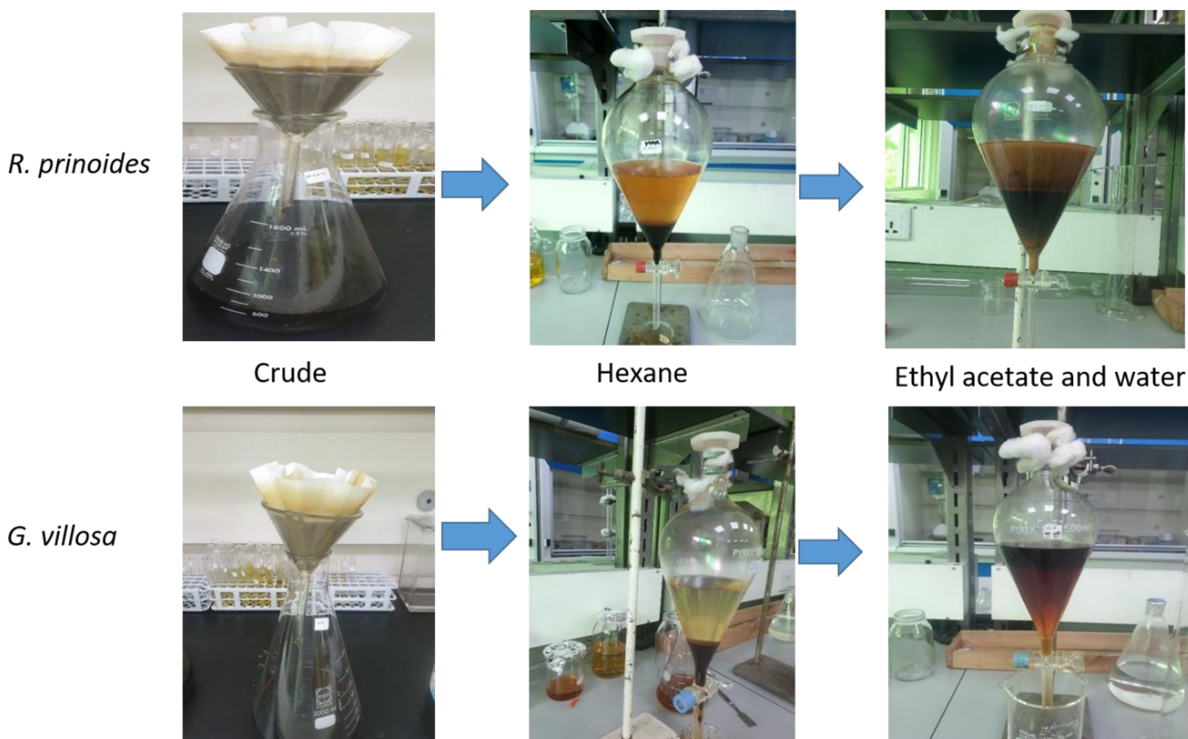

Figure 0.3 Extraction and partitioning of the extracts of *R. prinoides* and *G. villosa* root bark extracts

3.4 Gas Chromatography-Mass Spectrometric analysis

The compounds in the organic extracts of the root barks of *G. villosa* and *R. prinoides* were elucidated using the method described by Okpako *et al*. 2023 with slight modifications (Okpako *et al*., 2023). Helium was used as the carrier drug. Using a GC/MS QP-2010SE instrument (Serial No: 02Q53497QQ5Q, Shimadzu, Kyoto, Japan) fitted with a BPX5 capillary column—which has a low polarity and measures 30 m x 0.25 mm x 0.25 µm film in thickness—the chemicals found in the plant extracts were identified. Firstly, the oven was set to 50 °C for one minute, after which a temperature increase of 10 °C per minute was set to reach an isothermal temperature of 280 °C, which was maintained for 15 minutes and 30 seconds. At a rate of 1.08

millilitres per minute, the temperature of the injector was kept at 200 °C. The carrier gas was helium. An AS30000 auto-sampler was coupled to a gas chromatograph (GC) that was in split mode and had a 10:1 split ratio. After a 4-minute hold, it automatically injected 1 µl of the diluted sample in solvent. The interface temperature and ion source were kept at 200 °C and 250 °C, respectively. At an electron energy of 70 eV, Electron Ionisation (EI) mass spectra were obtained in the m/z range of 35–550 in full scan mode. The National Institute of Standards and Technology's (NIST) mass spectrum database was used for the identification the chemical components. GC-MS instrument calibration was verified using known standards from NIST database to ensure accuracy and reliability.

3.5 Ethical considerations

Every safety regulation in the research site was followed, and every precaution was taken to ensure that SOPs were rigorously followed. Before commencing, approval was obtained from the National Commission for Science, Technology, and Innovation (NACOSTI/P/23/27715) as shown in appendix IX and the Scientific Ethics and Review Unit of the Kenya Medical Research Institute (KEMRI/SERU/CTMDR/104/4466).

3.6 Determination of anti-proliferation activities

3.6.1 Preparation of Extracts

An electric balance was used to weigh 100mg of the plant extracts which were dissolved in 1 ml of dimethyl sulfoxide (DMSO) solvent. The extracts were then stored at -20°C until use.

3.6.2 Preparation of cells

Cervical cancer cell line (HeLa) as well as cells of the African Green Monkey kidney (Vero-CCL 81), sourced from the American Type Culture Collection (ATCC) (Rockville, USA) were used for the study. HeLa cells were chosen due to their representativeness of cervical cancer phenotype, while Vero cells served as a normal control to assess selectivity. The Vero-CCL 81

was used as a non-cancerous control cell. Growth media Minimum Essential Media (MEM) (GIBCO, USA) with 10% Fetal Bovine Serum (FBS), 1% HEPES buffer (GIBCO, USA), 1% streptomycin-penicillin (Solarbio, China) and 1% L-glutamine (Solarbio, China) was used to grow the cells which were incubated in an incubator with humidity (Thermo Fisher Scientific, USA) and 5% CO_2 at 37 °C.

3.6.3 Cell culture

The experiment was carried out as described by Mosmann (1983) with minor adjustments. The cells which were stored in liquid nitrogen were gently warmed in a water bath that was set at 37°C. After centrifuging the cells in the vial, the supernatant was discarded and added to a tissue culture flask containing growth media until the cells attained 70-80% confluence. Harvesting of the cells was carried out as follows; Phosphate Buffered Saline (PBS) solution was used to rinse the cells in the flask which had already adhered to the surface of the flask, then trypsin was used to suspend the cells and the flask was incubated in a 5% carbon (IV) oxide incubator for 3-5 minutes at 37°C after which 1mL of growth media was added. The trypan blue exclusion method was used for the determination of the number of viable cells and the cell density was calculated using a hemocytometer. All consequent cell culture assays were conducted at these conditions.

3.6.3.1 Cell viability assay

The method outlined in Mbugua *et al*, (2019) with minor adjustments was used. Strict adherence to biosafety level II practices was maintained during all cell line experiments. Researchers were trained in human tissue handling protocols. 100μL of 1.0×10^5 cells/ml of the suspended cells in media was seeded in a 96-well plate which was incubated at 37ºC for 24 hours. HeLa cells were treated with a fixed concentration of the extracts at 200 µg/ml to determine which extracts were active. Both HeLa and Vero-CCL 81 cells were treated with the active extracts at varying

concentrations to determine the inhibitory and cytotoxic concentrations that killed 50% of the cancer cells for 48 hours. The controls used were cells treated with negative control (0.4% DMSO), growth media (no cells or blanks) and doxorubicin hydrochloride as the positive control. After 48 hours, the MTT assay method was carried out to determine the percentage of live cells. The MTT assay is a colorimetric laboratory test which quantifies the live cells through a purple color gradient generated by a mitochondrial enzyme that reduces MTT to formazan. MTT dye, which is yellow in color, is converted to purple formazan crystals in living cells (Mossman, 1983). 10 µl of MTT dissolved in PBS was incorporated into each well and the 96-well plates were incubated for 2-4 hours until a purple precipitate forms. The media with the MTT dye was discarded, 100% DMSO (100 µl) added and the absorbance was then taken for each well in a microplate reader (Thermo Scientific, USA) between 570 nm and 720 nm (Mbugua *et al*., 2019).

The percentage of cell viability as outlined in Mukavi *et al*., (Mukavi *et al*., 2020) was calculated as :

$$\%\ Cell\ viability = \frac{Absorbance\ of\ extract\ treated\ cells}{Absorbance\ of\ control\ cells} \times 100$$

Selectivity index (SI) is a measure of the selectivity of the extracts for toxicity against cervical cancer cells when compared to non-cancerous cells. A selectivity index ≥ 2 indicates that an extract is selective. It is calculated as:

$$Selectivity\ index = \frac{CC50}{IC50}$$

Where;

CC_{50} – Concentration of extract that exerted cytotoxic effect to 50% of the normal cells

IC_{50}– Concentration of extract that inhibited the growth of cancer cells by 50%, (Somaida *et al*., 2020; Khunoana *et al*., 2022).

3.7 Morphological analysis

Three distinct extract concentrations (IC_{50}, IC_{25} and $IC_{12.5}$) of the active extracts of *G. villosa* and *R. prinoides*, positive control, doxorubicin hydrochloride at IC_{50} concentration, and the negative control were used to treat HeLa cells at 100,000 cells/ml of growth media for 48 hours, in order to determine their phenotypic effects on cervical cancer cells (HeLa). The EVOS XL Microscope (Thermo Fisher, Scientific) was used to record the morphological changes that resulted. Observed variables included cell shrinkage, membrane blebbing and chromatin condensation.

3.8 Wound healing analysis

The ethyl acetate extracts of *R. prinoides* and *G. villosa* being the most active extracts from the cell viability analysis, were utilized in this experiment. HeLa cells were seeded in 24-well plates at a density of 100,000 cells per ml in growth media for 24 hours. A line perpendicular to the scratch was made using a ruler and a fine tip permanent marker. Following attachment and formation of a confluent monolayer, the cells were rinsed with PBS and a scratch was made using a ruler and a 200µL tip. Fresh media containing the ethyl acetate extracts at IC_{50}, IC_{25}, $IC_{12.5}$ was added and the images taken at 0-, 24- and 48-hour intervals. Image J was utilised to measure the change in wound closure. % Wound closure was calculated using the following formula;

:

$$\%\ Wound\ closure = \frac{\mathrm{A}\ (0)\ -\ \mathrm{A}\ (\mathrm{t})/}{\mathrm{A}\ (0)} \times 100$$

The (A) is the area at time zero (0) and the area after incubation time (t) were used to calculate the percentage closure of the wound (Potikanond *et al*., 2017).

3.9.0 *In silico* evaluation of cervical cancer targets using the active extracts of *R. prinoides* and *G. villosa*

3.9.1 Network pharmacology

3.9.1.1 Drug screening

Canonical Simplified Molecular Input Line Entry System (SMILES) were extracted from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The SMILES were then used to determine the ADME (Absorption, Distribution, Metabolism and Excretion) properties of the compounds in the extracts using the Swiss ADME online database (http://www.swissadme.ch/index.php). The toxicity of the compounds was also elucidated using the pkCSM tool (http://structure.bioc.cam.ac.uk/pkcsm). Swiss ADME and pkCSM tools used in the study are widely validated and computational platforms with established predictive accuracy in drug-likeness and toxicity profiling. The topological polar area (TPSA), bridging the blood-brain barrier, Lipinski's rule of five, the lipophilicity calculated by the logarithm of the partition coefficient between n-octanol and water (Log P O/W), which identifies compounds that interact with the central nervous system (CNS), the oral bioavailability, the molecular weight in Daltons, the number of rotatable bonds, the number of hydrogen donors and hydrogen acceptors, and the interaction with cytochromes were all taken into consideration when predicting the drug-like properties of the compounds.. The compounds that passed the selection criteria were selected (Kamau *et al*., 2024).

3.9.1.2 Identification of the targets

Genes related to cervical cancer were extracted from GeneCards (https://www.genecards.org/), Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org/) and National Center for

Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/gene). The keyword that was used for the search was "cervical cancer", all the results were compiled into one singular column and the duplicates expelled. Targets from the ethyl acetate extracts were generated from Swiss Target Prediction database (http://www.swisstargetprediction.ch/), Similarity Ensemble Approach (SEA) database (https://sea.bkslab.org/), Super-PRED database (https://prediction.charite.de/subpages/target_prediction.php) and Binding DB database (https://www.bindingdb.org/rwd/bind/index.jsp). The Universal Protein Resource (Uniprot), (https://www.uniprot.org/) was used to obtain the gene ids. The results from all the databases were merged into a single column and similar genes were removed. An online tool (https://bioinformatics.psb.ugent.be/webtools/Venn/) was used to locate and extract common genes between the targets from the compounds and the cervical cancer genes.

3.9.1.3 Generation of the protein-protein interaction (PPI) network

The STRING 12.0 database (https://string-db.org/) was used to analyze the common genes, with a minimum threshold of 0.4. The topology was examined using Cytoscape software (version 3.9.1) according to centralities, and the top 30 genes were identified using the Cytohubba plug-in.

3.9.1.4 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment analysis

ShinyGO version 0.77 (http://bioinformatics.sdstate.edu/go/), was used to analyze the common genes on the basis of biological processes, molecular functions, cellular components as well as highlight the networks they interact with through the KEGG analysis. The parameters were set as; species (Human), false discovery rate (FDR) cut-off was set at 0.05 and the top 20 pathways were displayed.

3.9.2 Molecular Docking

The 3D format of the compounds in Structured Data File (SDF) format selected after ADME parameters were analyzed from PubChem database and the PDB database (https://www.rcsb.org/), was used to extract the structures of the proteins of the top 30 genes. The filters used were, X-ray diffraction was the experimental method; the resolution ranged from 0.5 to 3.0 Å; the structure determination methodology was experimental; and the species was human. All co-crystallized ligands were eliminated, polar hydrogen was added, Gasteiger charges were added, and water molecules were eliminated in order to create the targeted proteins using Discovery Studio 2021 (https://discover.3ds.com/discovery-studio-visualizer-download). Docking the ligands to the produced proteins was subsequently done using PyRx software (https://sourceforge.net/projects/pyrx/). The size of the protein was the central value of the three-dimensional grid box. Using Discovery Studio 2021, the compounds with the lowest binding energies were displayed.

3.10.0 Gene Expression Analysis of the effects of the extracts

3.10.1 RNA extraction and purification

HeLa cells were propagated in T-25 culture flasks using growth media. After 24 hours, HeLa cells were then active extracts at their respective IC_{50} values for 48 hours. RNA extraction was done using the Pure Link RNA mini kit (Thermo Scientific, USA) according to manufacturer's instructions. First, cell lysis was conducted for two minutes then the suspension was mixed with a buffer containing 1% 2-mercaptoethanol, precipitated with 70% ethanol, and the contents were moved into a spin cartridge. The spin cartridge was centrifuged, and the RNA (attached to the binding matrix) was washed twice then eluted using RNase free water. A Nanodrop ND-2000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) was used to quantify and assess the quality of the extracted RNA, and the concentrations (ng/µl) obtained

3.10.2 cDNA synthesis

The Solis BioDyne® FIRE Script cDNA synthesis kit following manufacturer's instructions was used to synthesize cDNA using the extracted RNA. Briefly, 1µg of the RNA extracted above was transferred to an RNase free 100 µl tube, then 1µl Oligo (dT) primer (100 M), 0.5 µl of dNTP MIX (20MM), 2µl of 10x RT Reaction Buffer with DTT, 1µl FIRE Script Reverse Transcriptase, 0.5 µl RiboGrip RNase inhibitor (40 U/ l) and 10 µl Nuclease-free water was added to the Eppendorf tube to make a total volume of 20µl per well. The reaction conditions were set at 25 ℃ 10 minutes to allow the primers to anneal, 50 ℃ for 30 minutes for reverse transcription and 85 ℃ for 5 minutes for enzyme activation using the SimpliAmp ™ Thermal Cycler (Thermo Scientific, USA).

3.10.3 Real-time quantitative polymerase chain reaction

The Solis Bio Dyne kit was used for the polymerase chain reaction in line with the manufacturer's guidelines. In brief reaction conditions of amplification were set at 95 ℃ for 12 minutes to allow the activation of the polymerase, 95 ℃ for 15 seconds for denaturation followed by 60-65 ℃ for 20 seconds for annealing and 72 ℃ for 20 seconds for elongation. The process was done in 40 cycles using RT-qPCR Quant Studio V System (Thermo Scientific, USA) was done. The primers used in this study were designed using the NCBI blast tool and the sequences were synthesized by Macrogen (Table 3.1). The comparative threshold (Ct) method was used to analyse the quantitative RT-qPCR data. To normalise the expression of the target genes, internal reference genes GAPDH and β-ACTIN were employed. The standard formula used for analysis was:

$$M\ Ct = CT\ (Target\ gene) - Ct\ \frac{(GAPDH + B - Actin)}{2}$$

For relative expression levels the formula used was:

$$\Delta\Delta\, Ct = \Delta\, Ct\ (Treated) - \Delta\, Ct\ (Control)$$

When set beside the calibrator, n-fold variations were used to indicate the levels of expression of the genes. Using the expression of 2-ΔΔ Ct, the value was utilized to plot the gene expression (Alshatwi *et al*., 2012; Mwitari *et al*., 2013; Kumar *et al*., 2022).

Table 0.1 Primers used in the study

GENES	FORWARD PRIMERS	REVERSE PRIMERS
TP53	CTTCGAGATGTTCCGAGAGC	GACCATGAAGGCAGGATGAG
EGFR	TCTGGAAGTACGCAGACGCC	TGGGAGACTAAAGTCAGACAGTG
ERBB2	TGGGAGCCTGGCATTTCTGC	TGTGCAGAATTCGTCCCCGGATTA
BAX	CAGAGGATGATTGCCGCCG	AAAAGGGCGACAACCCGGCC
BCL2	GGCCTCAGGGAACAGAATGAT	TCCTGTTGCTTTCGTTTCTTTC
AKT1	CCATCTGTCACCAGGGGCTT	ATAGCCACGTCGCTCATGGT
NF-κB	CGCTTAGGAGGGAGAGCCCA	TGCCATTCTGAAGCTGGTGGT
GAPDH	AGACAGCCGCATCTCTTG	TGACTGTGCCGTTGAACTTG
β-ACTIN	GCCAACTTGTCCTTACCCAGA	AGGAACAGAGACCTGACCCC
CASPASE 3	CAAAGAGGAAGCACCAGAACCC	GGACTTGGGAAGCATAAGCGA

Key: TP53- Tumor protein 53, EGFR- Epidermal Growth Factor Receptor, ERBB2-Erb-B2 receptor tyrosine kinase 2, Bax-Bcl-2 Associated X protein, Bcl2-B-Cell Lymphoma protein 2, AKT1-AKT- Serine/Threonine Kinase 1, NF-κB-Nuclear Factor kappa B, GAPDH-Glyceraldehyde-3-phosphate dehydrogenase, β-ACTIN-beta Actin

3.11 Data management and statistical analysis

Data was analyzed using a quantitative approach, gene expression and cytotoxicity values were analyzed using parametric statistical tests. All project-related activities were documented in a lab notebook. An Excel sheet was created using the raw and processed data. Excel data sheets, tables, and bar charts were used for statistical analysis to present the data in an understandable manner. A computer program was used to compute the doses needed to inhibit 50% of the cells (IC_{50}). Quant Studio TM Design and Analysis software (Thermo Scientific, USA) was utilized for analysis of the Real Time PCR results. The mean ± SEM was used to express the data. All statistical analyses were conducted using GraphPad Prism 8.0 (La Jolla, CA). Levels of significance were shown by ns- no significance * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$;**** $p<0.0001$ vs negative control, between the cells treated with the negative control and those treated with the extracts of *R. prinoides* and *G. villosa*

CHAPTER FOUR

RESULTS

4.1 Quantitative analysis using Gas chromatography/Mass Spectrometry

The results from the qualitative phytochemical screening are shown in Appendix I while those from Gas Chromatography are shown in the respective chromatograms found in Appendices II (crude), III (hexane) and IV (ethyl acetate). The results revealed that 7 compounds and 13 compounds were identified from the crude (DCM:Methanol) extracts of the *R. prinoides* and *G. villosa* respectively. The compounds were categorized into various classes. In *R. prinoides,* sugars, anthraquinones, terpenoids and hydrocarbons were identified while in *G. villosa*, hydrocarbons, phenols, fatty acid methyl esters, terpenoids, and alkaloids were identified (Table 4.1). On the basis of the percentage area, in *R. prinoides*, 6-Propyl-7H-benz[de]anthracen-7-one was the most abundant compound with 62% area followed by Methyl beta-D- glucopyranoside with 12% area while in *G. Villosa* (Table 4.2), the most abundant compounds by percentage area were Eicosane with 25% total area, 1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1-m with 19% and Diisooctyl phthalate with 18% respectively. The predominance of anthraquinones and triterpenoids suggests potential anti-cancer activity, consistent with previous findings (Kumar et al., 2022).

Table 0.2 Table 4.1 Phytochemical composition of the crude extract (DCM: Methanol) of *R. prinoides*.

Compound class	Retention time	% Area	Compound Name	Molecular Formulae
Hydrocarbon	5.038	5.99	Undecane	$C_{11}H_{24}$
Sugar	8.44	3.23	D-Allose	$C_6H_{12}O_6$
Methylated Sugars	8.934	12.73	Methyl beta-D-glucopyranoside	$C_7H_{14}O_6$
	9.631	7.14	Methyl alpha-D-mannopyranoside	$C_7H_{14}O_6$
Triterpenoids	15.808	6.4	Lupeol	$C_{30}H_{50}O$
	20.428	2.12	Squalene	$C_{30}H_{50}$
Anthraquinone	16.897	62.39	6-Propyl-7H-benz[de]anthracen-7-one	$C_{20}H_{16}O$

Table 0.3 Phytochemical composition of the crude extract (DCM: Methanol) of *G. villosa*.

Compound Class	Retention time	% Area	Compound Name	Molecular Formulae
Hydrocarbons	7.4	4.19	Tetradecane	$C_{14}H_{30}$
	8.843	8.56	Eicosane, 10-methyl-	$C_{21}H_{44}$
	10.761	8.1	Eicosane, 10-methyl-	$C_{21}H_{44}$
	12.875	5.81	1-Heptadecene	$C_{17}H_{34}$
	12.916	8.35	Eicosane	$C_{20}H_{42}$
Fatty acid Methyl esters	14.086	3.47	6-Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$
	14.406	4.76	l-Norvaline, N-(2-methoxyethoxycarbonyl)-,	$C_{15}H_{29}NO_5$
	12.204	4.12	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
Fatty acid ester	20.3	7.61	Decanedioic acid, bis(2-ethylhexyl) ester	$C_{26}H_{50}O_4$
Terpenoid	16.228	3.96	(4R,4aR)-4,4a-Dimethyl-6-(prop-1-en-2-yl)-1,2,3,	$C_{15}H_{22}$
Phthalate ester	18.185	18.33	Diisooctyl phthalate	$C_{24}H_{38}O_4$
Phenol	8.303	3.51	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$
Alkaloid	12.806	19.22	1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1-m	$C_{12}H_{14}N_2$

In the *G. villosa* and *R. prinoides* hexane extracts 21 compounds and 8 compounds were identified respectively (Appendix II). In *R. prinoides*, there were two fatty acid methyl esters (Methyl 14-methylpentadecanoate and 1, 54-Dibromotetrapentacontane), a hydrocarbon (Eicosane), a methylated hydrocarbon (2-methylectacosane), a triterpernoid (Squalene) and a pentacyclic steroid (9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate, (3.beta.,23E)-). The most abundant compound by percentage area was squalene with 81% (Table 4.3).

For *G. villosa*, hydrocarbons (Eicosane, Tetradecane, Pentadecane, hexadecane, methylated hexadecane and 2-methylhexacosane), fatty alcohols (heptadecanol-1 and pentadecanol), fatty acid methyl esters (Hexadecanoic acid methyl ester and 9-octadecanoic acid methyl ester),a fatty acid ester (isopropanol palmitate), an alkaloid (1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1), a coumarin (8-(1,1-Dimethylallyl)-5,7-dimethoxycoumarin), a phthalate ester (Diisooctyl phthalate) and a triterpenoids (Squalene) and a terpenoid ((7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol) were identified. The most abundant compound was Diisooctyl phthalate with 23% (Table 4.4).

Table 0.4 Phytochemical composition of the hexane extract of *R. prinoides*.

Compound class	Retention time	% Area	Compound Name	Molecular Formulae
Fatty acid methyl esters	16.577	2.19	methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$
	21.8	2.41	1,54-Dibromotetrapentacontane	$C_{54}H_{108}Br_2$
Hydrocarbons	25.219	1.36	Eicosane, 7-hexyl-	$C_{26}H_{54}$
	23.381	1.44	Eicosane, 7-hexyl-	$C_{26}H_{54}$
Methylated hydrocarbon	24.239	1.44	2-methyloctacosane	$C_{29}H_{60}$
Triterpenoid	24.473	81.48	Squalene	$C_{30}H_{50}$
Pentacyclic steroid	29.333	6.54	9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate, (3.beta.,23E)-	$C_{32}H_{52}O_3$

Table 0.5 Phyto-chemical composition of the hexane extract of *G. villosa*.

Compound class	Retention time	% Area (Relative abundance)	Compound Name	Molecular Formulae
Hydrocarbons	7.4	5.84	Tetradecane	$C_{14}H_{30}$
	8.101	0.34	Pentadecane	$C_{15}H_{32}$
	8.802	1.98	9-Eicosene, (E)-	$C_{20}H_{40}$
	8.842	11.3	Hexadecane	$C_{16}H_{34}$
	9.369	1.13	Hexadecane, 2-methyl-	$C_{17}H_{36}$
	10.759	9.49	Eicosane, 10-methyl-	$C_{21}H_{44}$
	14.967	3.76	Eicosane	$C_{20}H_{42}$
	16.836	2.95	Eicosane	$C_{20}H_{42}$
	18.526	1.95	2-Methylhexacosane	$C_{27}H_{56}$
	12.915	6.89	Eicosane	$C_{20}H_{42}$
Fatty alcohols	10.709	2.38	n-Heptadecanol-1	$C_{17}H_{36}O$
	12.87	3.21	n-Heptadecanol-1	$C_{17}H_{36}O$
	14.932	1.62	n-Pentadecanol	$C_{15}H_{32}O$
Fatty acid methyl esters	12.204	7.83	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$
	14.088	7.2	9-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$
Alkaloid	12.812	2.43	1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1	$C_{12}H_{14}N_2$
Fatty acid ester	13.196	1.39	Isopropyl palmitate	$C_{19}H_{38}O_2$
Phenol	16.898	2.15	8-(1,1-Dimethylallyl)-5,7-dimethoxycoumarin	$C_{16}H_{18}O_4$
Phthalate ester	18.184	23.11	Diisooctyl phthalate	$C_{24}H_{38}O_4$
Terpenoid	8.302	1.16	(7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol	$C_{15}H_{26}O$
Triterpenoid	20.422	1.87	Squalene	$C_{30}H_{50}$

In the ethyl acetate extracts, 13 compounds from *R. prinoides* and 10 compounds in *G. villosa* were identified (Appendix III). In *G. villosa*, majority of the compounds were hydrocarbons

(Eicosane, Tricosane, Docosane, Tetradecane-2,6,10-trimethyl, Octacosane and Tetratetracontane), fatty acid esters (Glutaric acid, di(1-phenylpropyl) ester and Decanedioic acid, bis(2-ethylhexyl) ester), an aromatic hydrocarbon (Benzene, (1-methylundecyl)-) and a phenol (Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl)-) as shown in Table 4.5. The most abundant compound was Eicosane with 24.84% area followed by tetratetracintane with 21.95% area.

In *R. prinoides*, amongst the phytochemicals present were a benzaldehyde (3-Ethoxy-4-methoxybenzaldehyde), phenols (4-tert-Butylphenyl acetate and 2',4'-Dihydroxypropiophenone), an alkaloid (2-(3-Phenyl-piperidin-1-yl)-ethylamine), hydrocarbons (Eicosane and Pentadecane-8-hexyl), Fatty acid derivative (3-Amino-4-(4-ethoxy-phenylamino)-benzoic acid), methylated hydrocarbon (Methyloctacosane and Nonadecane, 9-methyl-), triterpenoids (Squalene and Olean-12-en-3-ol, acetate, (3.beta.)-) and a pyrimidine derivative (3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene). The most abundant compound by percentage area was (3-Amino-4-(4-ethoxy-phenylamino)-benzoic acid) with 59% (Table 4.6).

Table 0.6 Phytochemical composition of the ethyl acetate extract of *G. villosa*.

Compound class	Retention time	% Area (Relative abundance)	Compound Name	Molecular Formulae
Fatty acid ester	10.587	6.73	Glutaric acid, di(1-phenylpropyl) ester	$C_{23}H_{28}O_4$
	20.305	3.43	Decanedioic acid, bis(2-ethylhexyl) ester	$C_{26}H_{50}O_4$
Phenol	10.693	3.57	2-methyl-4-(2,4,4-trimethylpentan-2-yl)phenol	$C_{15}H_{24}O$
Aromatic Hydrocarbon	11.028	3.16	Benzene, (1-methylundecyl)-	$C_{18}H_{30}$
Hydrocarbons	12.917	1.58	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$

13.963	3.83	Eicosane	$C_{20}H_{42}$
14.966	7.92	Docosane	$C_{22}H_{46}$
15.926	10.23	Tricosane	$C_{23}H_{48}$
16.837	11.59	Eicosane	$C_{20}H_{42}$
17.703	11.85	Octacosane	$C_{28}H_{58}$
18.526	11.11	Tetratetracontane	$C_{44}H_{90}$
19.329	10.84	Tetratetracontane	$C_{44}H_{90}$
20.197	9.42	Eicosane	$C_{20}H_{42}$
21.182	4.74	Eicosane	$C_{20}H_{42}$

Table 0.7 Phytochemical composition of the ethyl acetate extract of *R. prinoides*.

	Retention time	% AREA	Compound Name	Molecular Formulae
Aldehyde	15.687	10.38	3-Ethoxy-4-methoxybenzaldehyde	$C_{10}H_{12}O_3$
Phenols	15.815	1.49	4-tert-Butylphenyl acetate	$C_{12}H_{16}O_2$
	17.174	5.32	2',4'-Dihydroxy Propiophenone	$C_9H_{10}O_3$
Alkaloid	20.166	1.88	2-(3-Phenyl-piperidin-1-yl)-ethylamine	$C_{13}H_{20}N_2$
Hydrocarbons	20.697	1.62	Eicosane	$C_{26}H_{54}$
	21.601	1.6	Pentadecane, 8-hexyl-	$C_{21}H_{44}$
	23.301	2.44	Eicosane	$C_{26}H_{54}$
Phenol	22.547	58.74	3-Amino-4-(4-ethoxy-phenylamino)-benzoic acid	$C_{15}H_{16}N_2O_3$
Methylated hydrocarbons	24.101	1.67	Nonadecane, 9-methyl-	$C_{20}H_{42}$
	24.95	1.63	2-methyloctacosane	$C_{29}H_{60}$
Triterpenoid	26.165	2.86	Squalene	$C_{30}H_{50}$
	27.731	3.89	Olean-12-en-3-ol, acetate, (3.beta.)-	$C_{32}H_{52}O_2$
Pyrimidine derivative	29.721	6.49	3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene	$C_{16}H_{26}$

4.2 Anti-proliferative effects of the extracts of *G.villosa* and *R. prinoides* on cervical cancer cells and non-cancerous cells

4.2.1 Initial screening of *G. villosa* and *R. prinoides* extracts at a fixed concentration

HeLa were treated with crude, hexane, water and ethyl acetate extracts from *R. prinoides* and *G. villosa* at 200 µg/ml to determine the active ones. The hexane, ethyl acetate, and crude extracts of *R. prinoides* and *G. villosa* had the highest inhibition of the growth of HeLa cells whereas the water extract showed the least inhibition compared to the negative control. The ethyl acetate extracts of the two studied plants were the most active extracts in both plants followed by the hexane extracts whereas the crude showed the least activity. The water extract was excluded from further screening as there was no significant variation between the activity of the water and that of the negative control (Figure 4.1). The inhibitory concentration of doxorubicin hydrochloride was significantly higher than all the extracts.

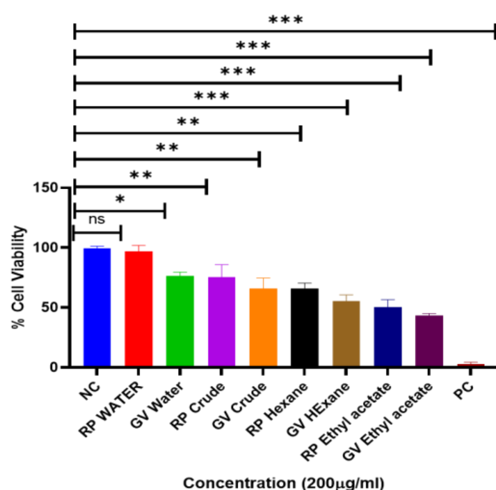

Figure 0.4 Anti-proliferative effects of the extracts of *R. prinoides* and *G. villosa* against HeLa cells *in vitro* at 200 µg/ml.

NC-negative control (0.4% DMSO) and PC-Positive control (doxorubicin hydrochloride). Data is represented as Mean ± SEM. *ns- no significance * p< 0.05; ** p< 0.01 ; *** p< 0.001;**** p<0.0001 vs negative control*

4.2.2 *In vitro* anti-proliferative effects of the extracts of *G. villosa* and *R. prinoides* against cervical cancer cells

HeLa cells were exposed to different doses of the crude, hexane and ethyl acetate extracts of *R. prinoides* and *G. villosa*. In *R. prinoides*, a notable variation between the cells treated with negative control and those treated with different concentrations of the extracts was shown (Figure 4.2A) except for the crude (120-15 µg/ml) and hexane extracts (30-15 µg/ml). In *G. villosa*, there was a notable variation between the HeLa cells treated with varying concentrations of the extracts and those treated with negative control except for crude extract at 12.5 and 6.25 µg/ml (Figure 4.2B). The plant extracts varied in their *in vitro* inhibitory effects. However, all the extracts limited the proliferation of HeLa cells with the potency increasing with the concentration if the extracts. The ethyl acetate extracts of both plants exhibited the highest inhibitory activity on HeLa cells followed by the hexane extracts whereas the least active extracts in both *R. prinoides* and *G. villosa* were the crude extracts. The positive control was more active in its inhibitory effects when compared to the extracts (Figure 4.2).

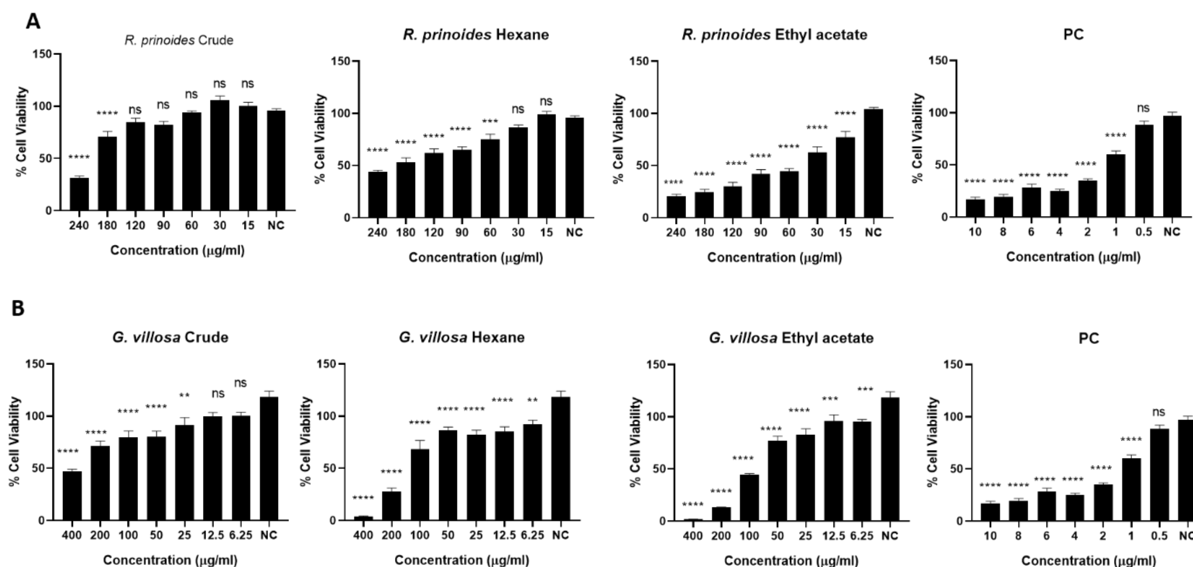

Figure 0.5 ***In vitro*** **anti-proliferative effects of the extracts of *R. prinoides* and *G. villosa* against HeLa cells.**

Data is represented as Mean ± SEM.

PC- positive control. *ns- no significance * p<0.05; ** p< 0.01 ; *** p< 0.001;**** p<0.0001 vs negative control*

4.2.3 *In vitro* Effects of the effects of R. prinoides and G. villosa extracts on non-cancerous cells (Vero-CCL 81 from the kidney of African Green Monkeys)

Vero-CCL 81 cells were exposed to varying concentrations of the crude, hexane and ethyl acetate extracts from *R. prinoides* and *G. villosa*. The inhibitory effects of the extracts on Vero-CCL 81 cells were found to increase as the concentrations increased as shown in Figure 4.3, evidenced by higher viability of Vero CCL-81 cells in lower concentrations of the extracts. The extracts with the least inhibitory effects on the viability of Vero-CCL 81 cells were the crude extracts in both *R. prinoides* (Figure 4.3A) and *G. villosa* (Figure 4.3B). The level of significance between the Vero-CCL 81 cells treated with various concentrations of *R. prinoides* and *G. villosa* crude, hexane and ethyl acetate extracts decreased with decreasing concentrations of the extracts which indicated that the cytotoxicity of the extracts decreased with decreasing concentrations of the extracts. Doxorubicin hydrochloride displayed the most cytotoxic effect on Vero-CCL 81 cells when compared to the extracts of *R. prinoides* and *G. villosa* (Figure 4.3).

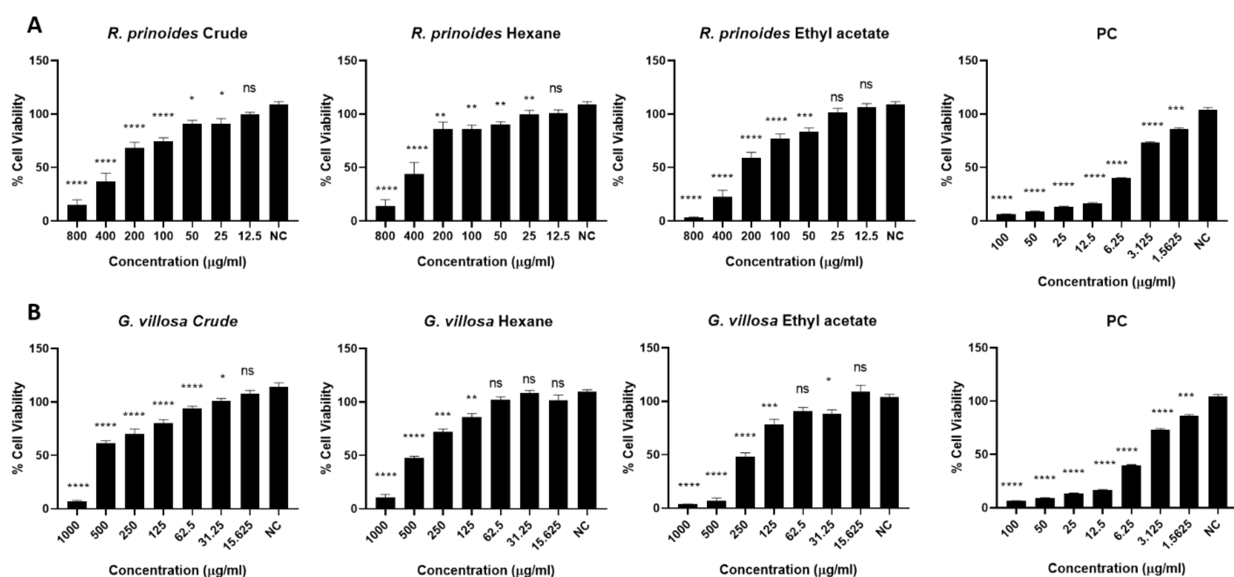

Figure 0.6 Cytotoxic effects of the extracts of *R. prinoides* and *G. villosa* against Vero-CCL 81 cells.

Data is represented as Mean ± SEM.
PC- positive control. *ns- no significance *p<0.05; ** p< 0.01 ; *** p< 0.001;**** p<0.0001 vs negative control*

4.2.4 Selectivity indices of the extracts

The median inhibitory concentrations of the crude, hexane, and ethyl acetate extracts of *R. prinoides* and *G. villosa* on HeLa cells (IC_{50}) and Vero-ccl-81 (CC_{50}) were used to compute the selectivity indices. In *R. prinoides,* the ethyl acetate extract showed the highest selectivity index of 4.40 (IC_{50} of 77.87 µg/ml and CC_{50} 342.62 µg/ml), followed by the crude extract (IC_{50} 204.53 µg/ml and CC_{50} 626.33 µg/ml) with a selectivity index of 3.06 whereas the hexane extract (IC_{50} 210.39 µg/ml and CC_{50} 550.81 µg/ml) showed the least selectivity with a selectivity index of 2.62. In *G. villosa,* the ethyl acetate extract displayed the highest selectivity index of 2.38 (IC_{50} 100.70 µg/ml and CC_{50} 240.67 µg/ml) followed by the hexane extract with a selectivity index of 2.28 (IC_{50} 193.45 µg/ml and CC_{50} 441.15 µg/ml). The crude extract of *G. villosa* showed the least selectivity of all extracts from both plants with a selectivity index of 1.69 (IC_{50} 381.92 µg/ml and CC_{50} 648.20 µg/ml). The positive control has a selectivity index of 3.74 (IC_{50} 1.45 µg/ml and CC_{50} 4.52 µg/ml) as shown in Figure 4.4. These results are in line with prior studies demonstrating high selectivity indices of ethyl acetate extracts in the HeLa cell line model (Bahrami *et al*., 2018)

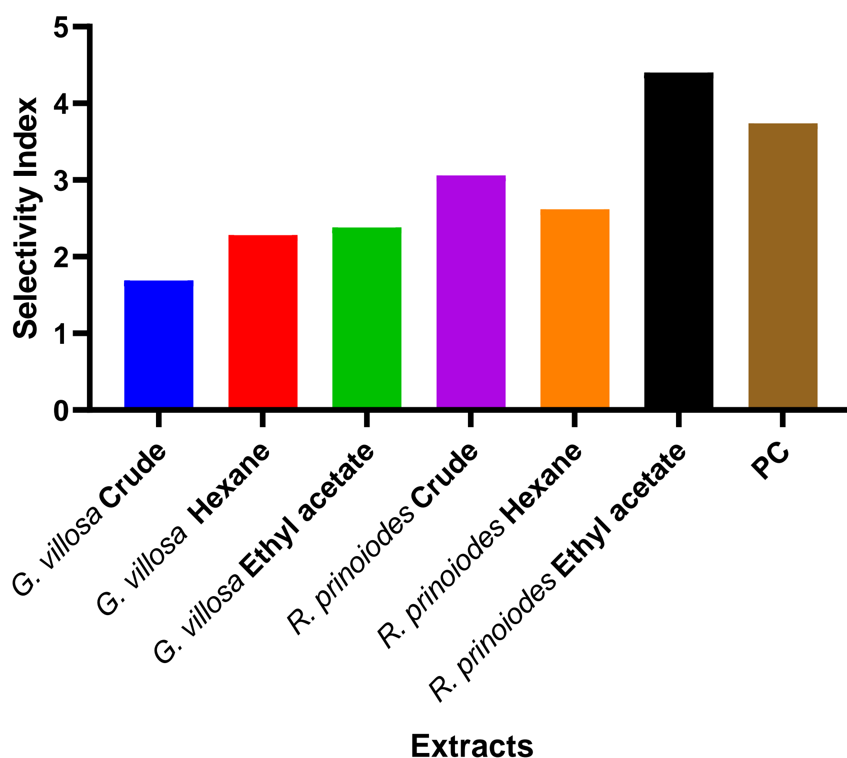

Figure 0.7 Selectivity indices of the crude, hexane and ethyl acetate extracts of *G. villosa* and *R. prinoides*.

4.3 Morphological analysis of the most active extracts of *R. prinoides* and *G. villosa* on HeLa cells.

The ethyl acetate extracts of *R. prinoides* and *G. villosa* which were the most active and the most selective were selected for morphological analysis at three inhibitory concentrations, the IC_{50}, IC_{25} and $IC_{12.5}$. The cytotoxic effects of both plants were dependent on the dose with the IC_{50} concentrations and the IC_{25} concentrations of both plants having the highest cytotoxic effects, however, the positive control had the highest cytotoxic effects on HeLa cells. Cell shrinkage, membrane blebbing and detachment were pronounced at IC_{50} and IC_{25} concentrations as shown in Figure 4.5. HeLa cells were least affected by both plants at $IC_{12.5}$ concentrations. For the two plants, the $IC_{12.5}$ concentrations were regarded as ineffective dosages (Figure 4.5).

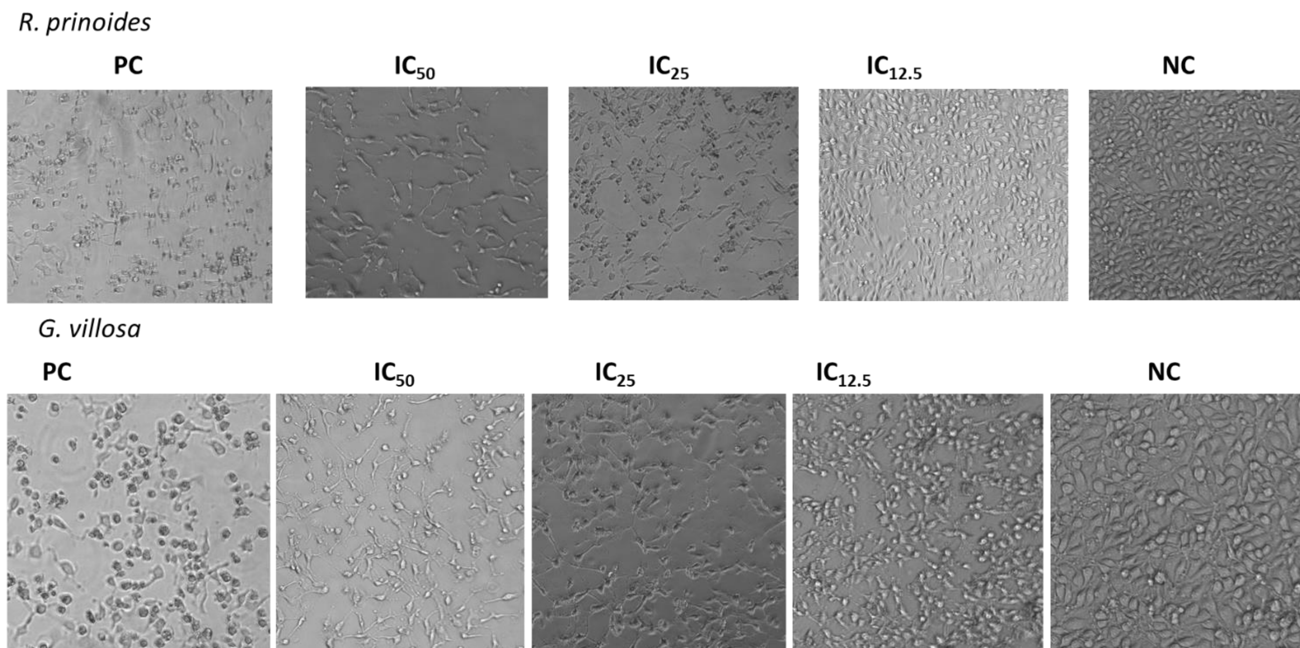

Figure 0.8 Morphological analysis of the effects of the ethyl acetate extracts of *R. prinoides* and *G. villosa* on HeLa cells.

PC- positive control and NC- negative control.

4.4 *In vitro* wound healing analysis of HeLa cells treated with ethyl acetate extracts

The active extracts of *R. prinoides* and *G. villosa* plants were evaluated for their ability to limit cell migration at different inhibitory concentrations, after an artificial wound was made on the confluent monolayer, the rate of wound closure was observed over 48 hours.

In *R. prinoides*, the effects on limitation of cell migration were observed to be both time and dose dependent (Figure 4.6). At both 24 and 48 hours, the IC_{50} concentration had the most inhibitory effect on migration, whereas the IC_{25} concentration had the lowest. In HeLa cells treated with the negative control, the wound had nearly healed after 48 hours, but in the cells exposed to the IC_{50} and IC_{25} concentrations, the wound was still clearly visible.

The IC_{50} concentration had the highest inhibitory effect on migration. The percentage wound closure was the least at both 24 and 48 hours followed by the IC_{25} concentration. After 48 hours, the wound was almost completely closed in the cervical cancer cells treated with negative control while in HeLa cells treated with the IC_{50} and IC_{25} concentrations there was still visibly a wound. The cells treated with the IC_{50} and IC_{25} concentrations showed significant variation in the percentage of wound closure, however the cells treated with the $IC_{12.5}$ concentration showed little distinction from the cells treated with the negative control.

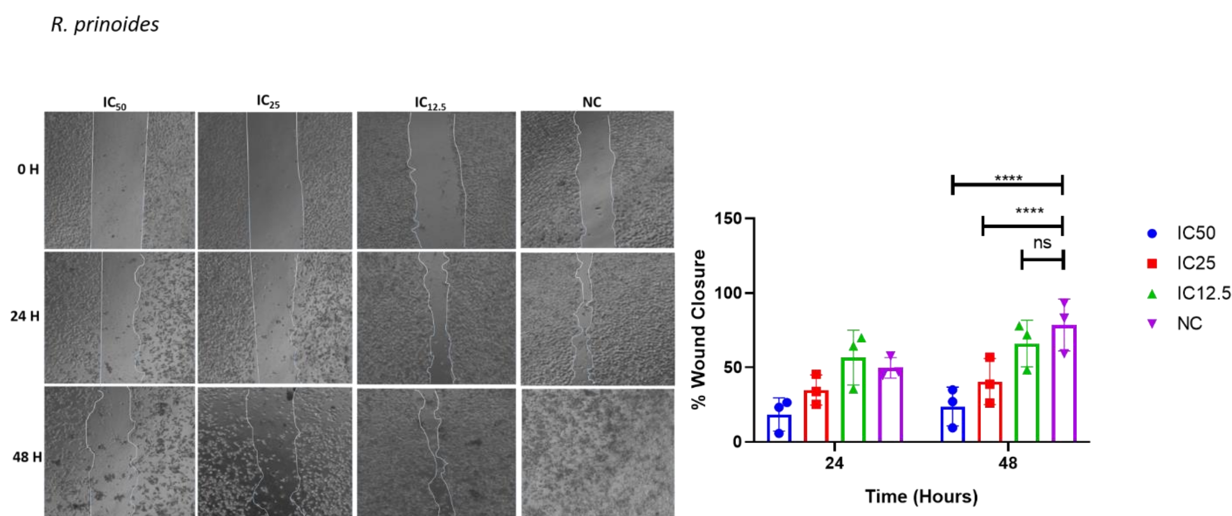

Figure 0.9 Effects of limitation of migration of HeLa cells after treatment with the ethyl acetate extract of *R. prinoides* using *in vitro* wound healing analysis. Data is presented as Mean ± SEM. *ns- no significance * p< 0.05; ** p< 0.01 ; *** p< 0.001;**** p<0.0001 vs negative control.*

In *G. villosa*, similar results were observed on cervical cancer cells as those in *R. prinoides*. The effect on limiting migration of HeLa cells was both time and dose dependent. The IC_{50} and IC_{25} concentrations had the highest inhibitory effects while the $IC_{12.5}$ concentration has the least inhibitory effect. The rate of wound closure was observed to be higher as the dose decreased and vice versa (Figure 4.7). After 48 hours, the cells treated with the $IC_{12.5}$ concentration and those treated with the negative control did not significantly differ in terms of wound closure

percentage, while those treated with the IC_{50} and IC_{25} concentrations of *G. villosa* did. IC_{50}-treated cells had a wound closure of only 18% compared to 95% in the cells treated with negative control.

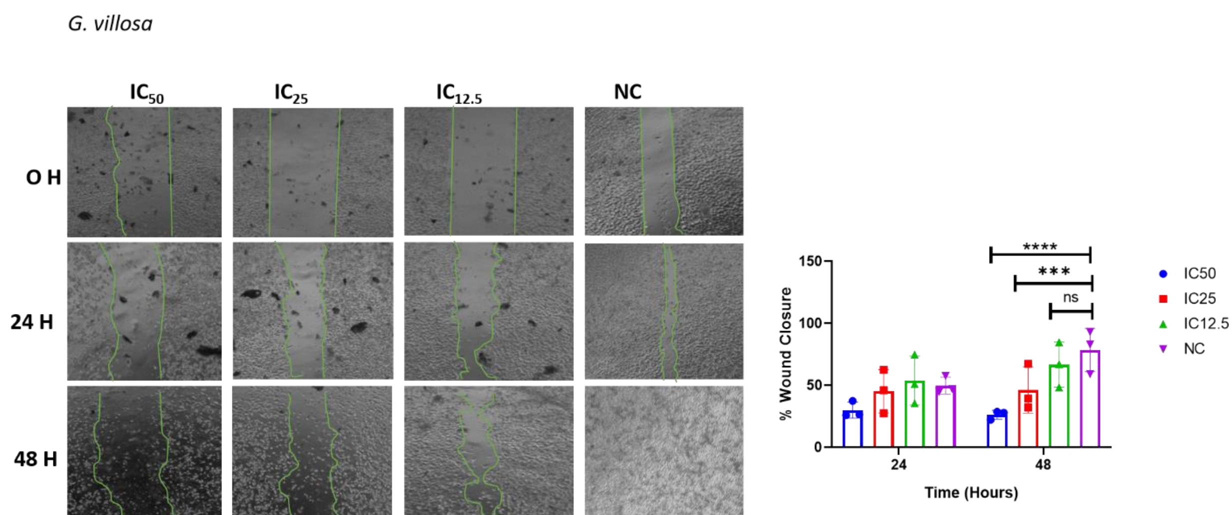

Figure 0.10 Effects of limitation of migration of HeLa cells after treatment with the ethyl acetate extract of *R. prinoides* using *in vitro* wound healing analysis
Data is represented as Mean ± SEM. *ns- no significance * p< 0.05; ** p< 0.01 ; *** p< 0.001;**** p<0.0001 vs negative control*

4.5 *In silico* evaluation of the effects of the extracts of *R. pinoides* and *G. villosa* compounds

4.5.1 Drug screening of the ethyl acetate extracts of *R. prinoides* and *G. villosa*

Out of the 13 compounds identified in *R. prinoides* ethyl acetate extract only 7 met the drug like criteria which included not violating not more than two of Lipinski's rules, lacking the ability to bridge the blood brain barrier and not inhibiting cytochromes. Their ADMET profiles are highlighted in Table 4.7. The compounds that were selected were 8-Hexylpentadecane, 7-Hexylicosane, 9-methylnonadecane, 2-methyloctacosane, Squalene, Olean-12-en-3.beta.-ol, acetate and 3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene.

In *G. villosa*, out of the 14 compounds identified, only 6 compounds met the criteria. The compounds selected were Eicosane, Octacosane, Docosane, Tricosane, Benzene, (1-methylundecyl)- and 2,6,10-trimethyltetradecane. The ADMET profiles are highlighted in Table 4.8 for *G. villosa*.

Table 0.8 The ADMET properties of the compounds in the ethyl acetate extract of *R. prinoides*.

		Absorption			**Distribution**			**Metabolism**			**Excretion**		**Toxicity**					
Compounds	**Lipinski's Rules violations**	**Water solubility**	**Caco2 permeability**	**Intestinal absorption (human)**	**BBB**		**CNS permeability**	**CYP2C9 inhibitior**	**CYP2D6 inhibitior**	**CYP3A4 inhibitior**	**Total Clearance**	**Renal OCT2 substrate**	**AMES toxicity**	**Max. tolerated dose (human)**	**hERG I inhibitor**	**hERG II inhibitor**	**Hepatotoxicity**
1	Yes,0	-1.669	1.753	96.318	Yes	-1.669	-2.251	Yes	No	No	0.703	No	No	1.146	No	No	No
2	Yes,0	-3.777	1.551	94.366	Yes	0.18	-1.602	Yes	No	No	0.33	No	No	0.955	No	No	No
3	Yes,0	-1.947	1.182	92.822	Yes	0.21	-2.886	No	No	No	0.334	No	No	0	No	No	No
4	Yes,0	-1.611	1.479	93.736	Yes	0.257	-1.485	No	No	No	0.912	No	No	0.146	No	No	Yes
5	Yes,1	-7.835	1.133	89.164	No	1.111	-1.051	Yes	No	No	2.045	No	No	-0.307	No	Yes	No
6	Yes,1	-8.602	1.38	90.889	No	1.017	-1.331	Yes	No	No	2.037	No	No	-0.104	No	Yes	No
7	Yes,1	-8.66	1.386	90.383	No	1	-1.306	Yes	No	No	1.905	No	No	0.103	No	Yes	No
8	Yes,1	-6.691	1.129	87.013	No	1.181	-0.501	No	No	No	1.948	No	No	-0.34	No	Yes	No
9	Yes,1	-8.517	1.216	90.341	No	0.981	-0.955	No	No	No	1.791	No	No	-0.393	No	Yes	No
10	Yes,1	-6.649	1.317	97.342	No	0.634	-2.016	26.3	No	No	-0.134	No	No	-0.165	No	Yes	No

EARP11	Yes,1	-6.327	1.4	95.067	No	0.809	-1.681	0	No	No	1.856	No	No	-0.103	No	Yes	No

Legend 1 ; BBB (Blood Brain Barrier), CYP2D6 (Cytochrome P450 2D6), CYP3A4 (Cytochrome P450 3A4), TPSA (Topological Surface Area $Å^2$), 1 (3-ethoxy-4-methoxybenzaldehyde), 2 (4-tert-Butylphenyl acetate), 3 (1-(2,4-dihydroxyphenyl)propan-1-one), 4 (2-(3-phenylpiperidin-1-yl)ethanamine), 5 (Eicosane), 6 (8-Hexylpentadecane), 7 (9-methylnonadecane), 8 (2-methyloctacosane), 9 (Squalene),10 (Olean-12-en-3.beta.-ol, acetate), 11 (3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene).

Table 0.9 The ADMET properties of the compounds in the ethyl acetate extract of *G. villosa.*

		Absorption			**Distribution**			**Metabolism**			**Excretion**		**Toxicity**				
Compounds	**LP**	**Water solubility**	**Caco2 permeability**	**Intestinal absorption (human)**	**BBB**		**CNS permeability**	**CYP2C9 inhibitior**	**CYP2D6 inhibitior**	**CYP3A4 inhibitior**	**Total Clearance**	**Renal OCT2 substrate**	**AMES toxicity**	**Max. tolerated dose (human)**	**hERG I inhibitor**	**hERG II inhibitor**	**Hepatotoxicity**
1	Yes,1	-4.898	1.649	90.819	Yes	0.46	-1.59	No	No	No	0.779	No	No	0.754	No	No	No
2	Yes,0	-7.566	1.495	92.756	Yes	0.88	-1.158	No	No	No	1.727	No	No	0.874	No	Yes	No

3	Yes,1	-8.166	1.413	92.811	No	0.925	-1.383	No	No	No	1.575	No	No	0.238	No	No	No
4	Yes,1	-8.473	1.369	88.984	No	1.052	-1.089	No	No	No	2.07	No	No	-0.101	No	Yes	No
5	Yes,1	-8.338	1.137	88.64	No	1.071	-1.035	No	No	No	2.105	No	No	-0.144	No	Yes	No
6	Yes,1	-7.085	1.114	86.922	No	1.166	-0.762	No	No	No	2.119	No	No	-0.284	No	Yes	No
7	Yes,1	-3.232	1.043	81.424	No	1.468	0.111	No	No	No	2.482	No	No	0.32	No	Yes	No
8	No,2	-8.59	1.371	89.671	No	1.014	-1.199	No	No	No	1.998	No	No	-0.014	No	Yes	No
9	Yes,1	-5.453	1.352	91.099	No	-0.202	-2.595	No	No	No	1.916	No	No	0.628	No	No	No

Legend 2 BBB (Blood Brain Barrier), CYP2D6 (Cytochrome P450 2D6), CYP3A4 (Cytochrome P450 3A4), TPSA (Topological Surface Area $\AA^2$), LR (Lipinski's rule of five violations), CNS (Central Nervous System), 1- (bis(1-phenylpropyl) pentanedioate), 2-(2-methyl-4-(2,4,4-trimethylpentan-2-yl)phenol), 3-(Benzene, (1-methylundecyl)-), 4-(2,6,10-trimethyltetradecane), 5-(Docosane), 6-(Tricosane), 7-(Eicosane), 8-(Octacosane) and 9-(Tetratetracontane).

4.5.2 Generation of predicted targets against cervical cancer

A total of 636 genes for *R. prinoides* and 439 genes from *G. villosa* were gotten from online databases while cervical cancer genes totaled 6934 targets genes. The cervical cancer targets and the targets of *G. villosa* and *R. prinoides*, were fed to an online Venn diagram generator from which common genes were generated. A total of 246 targets were common for both cervical cancer and *R. prinoides* (Figure 4.9) and a total of 172 gene targets were common for both cervical cancer and *G. villosa* (Figure 4.8) were assembled. The PPI network interactive diagram is found in Appendix V. As shown in Figures 4.8 and 4.9, overlapping genes (172 in *G. villosa* and 246 in *R. prinoides*), were identified highlighting potential therapeutic targets.

Figure 0.11 Common gene targets between cervical cancer and the compounds in the ethyl acetate extract of *G. villosa.*

GV- *Grewia villosa*, CC- cervical cancer.

Figure 0.12 Common gene targets between cervical cancer and the compounds in the ethyl acetate extract of *R. prinoides*.

RP-*Rhamnus prinoides*, CC- cervical cancer.

4.5.3 Generation of protein-protein interaction networks of the common genes

For *R. prinoides*, a network consisting of 246 nodes and 3179 number of edges with a median node degree of 25.7 and a median local clustering coefficient of 0.518 was generated. The network that was generated from the common genes in *G. villosa* comprised of 247 nodes, 3179 edges with a median node degree of 25.7 degrees, the network had a median local clustering coefficient of 0.518 with the expected number of edges being 1472 and a PPI enrichment p-value of <1.0e-16 (Appendix V). The protein network displayed interconnections between individual genes and the whole network which indicated a strong biological interconnection as a group for both plant extracts. The genes were selected based on five topological analyses methods: degree, eccentricity, betweenness, closeness and maximal clique centrality (MCC) (Figure 4.10).

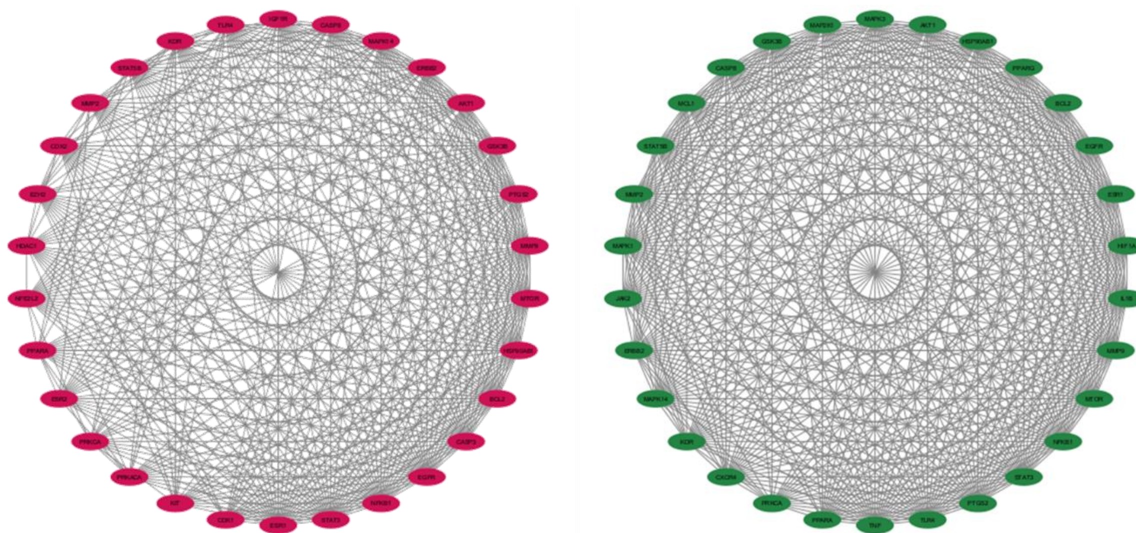

Figure 0.13 The top 30 hub genes from the PPI networks.
On the left are the top 30 genes from *G. villosa* and on the right are the top 30 genes from *R. prinoides*

4.5.4 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Enrichment Analysis

The ethyl acetate extract of *R. prinoides* featured 246 key genes that were enriched in 1921 GO (Gene Ontology) terms; 1637 of these GO-terms were significantly enriched in biological processes (BP), 160 in molecular functions (MF), and 124 in cellular components (CC) ($p<0.05$). The ethyl acetate extract of *G. villosa* featured 172 nodes enriched in 1927 GO terms, 1637 in biological processes, 160 in molecular functions and 124 in cellular components. In the top 20 most noteworthy GO-terms in BP, MF and CC, respectively, in both plant extracts are displayed in dot plot charts in Figure 4.11. The number of enrichments is represented by the x-axis, and the enriched categories within each group are represented by the y-axis. The ranking is based on –log10 (False Discovery Rate) and is in ascending order of relevance. According to GO analysis, the major genes involved in biological processes are involved in reactions to oxidative stress, chemical stress, and oxidative stress, respectively, with respect of the number of genes. The membrane region was the primary site of enrichment in cellular components, whereas nuclear receptor activity, ligand-activated transcriptional factor property, and transmembrane receptor

protein tyrosine kinase activity were the key areas of enrichment in molecular activities. Figure 4.11 shows the top 20 routes (-LogP) out of the 196 pathways that were significantly enriched in the KEGG pathways. The genes were involved in various pathways key to cancer such as central carbon metabolism in cancer, apoptosis, HIF-1 signaling pathway, Ras and PI3K-Akt signaling pathways (Figure 4.12). These enriched pathways, especially PI3K-Akt and HIF-1, are commonly implicated in cervical carcinogenesis (Bahrami *et al.*, 2017b)

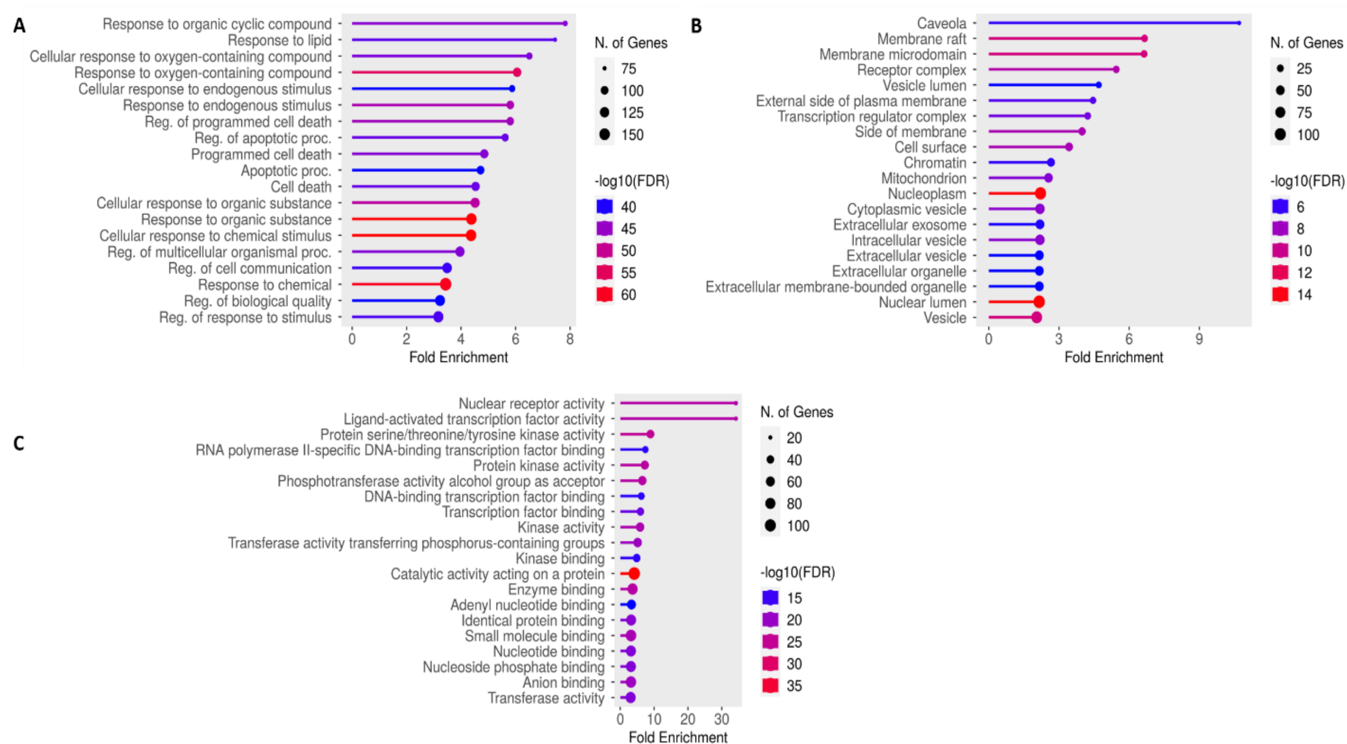

Figure 0.14 Gene ontologies of the compounds in the ethyl acetate extracts of *R. prinoides* and *G. villosa.*

A- Enriched GO terms in cellular components. B- Enriched GO terms in molecular functions. C- Enriched GO terms in biological processes. The x-axis shows the number of genes associated with each GO term, and the y-axis shows the enriched GO terms. The color of the dots represents the significance of enrichment (-log10 (FDR)).

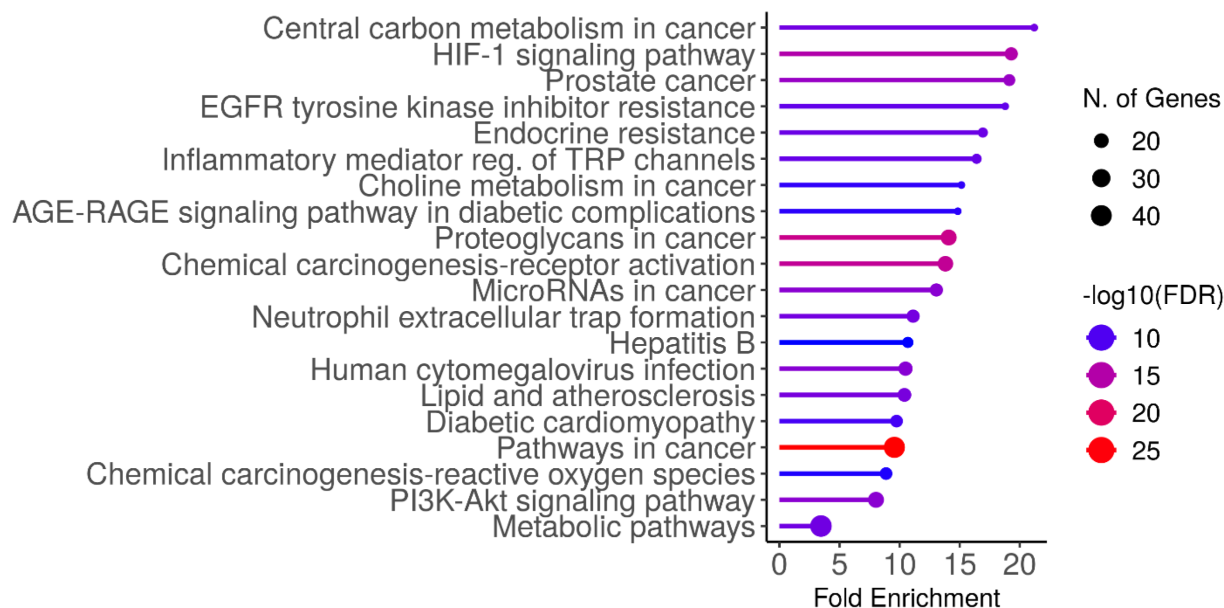

Figure 0.15 KEGG pathway analysis of the compounds in the ethyl acetate extracts of *R. prinoides* and *G. villosa*.
The color shows the adjusted P-value for the pathway analysis, with the more red the bubble the less the FDR and vice versa.

4.6 Molecular Docking

To identify the binding energy of the selected chemical compounds in the ethyl acetate extracts of *R. prinoides* and *G. villosa*, the top 30 genes were selected. In the context of molecular docking, compounds were ligands and the genes were receptors. Because the other compounds were too flexible, only three compounds for *R. prinoides* and two for *G. villosa* were docked. The interactions of the proteins with the compound ligands are shown in Figure 4.15. Figures 4.13 and 4.14 show a heat map of the docking scores of the compounds to the target protarens for *G. villosa* and *R. prinoides* respectively. Doxorubicin hydrochloride had the highest binding scores as shown in Figures 4.13 and 4.14 Squalene from *R. prinoides* had the highest binding affinity of -8.9 with ERBB2, -3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene also had a very good binding affinity of -8.4 with ESR1 from *R. prinoides.* For *G. villosa* the compounds with the highest binding affinities were 2,6,10 –

trimethyltetradecane and Benzene_ (1-methylundecyl) with EGFR and MMP2 respectively. However, all the compounds had a binding affinity ≤ -4 as shown in Appendix VI which makes them good candidates. The genes and their PDB IDs and corresponding docking scores are found in the appendices (Appendix VI).

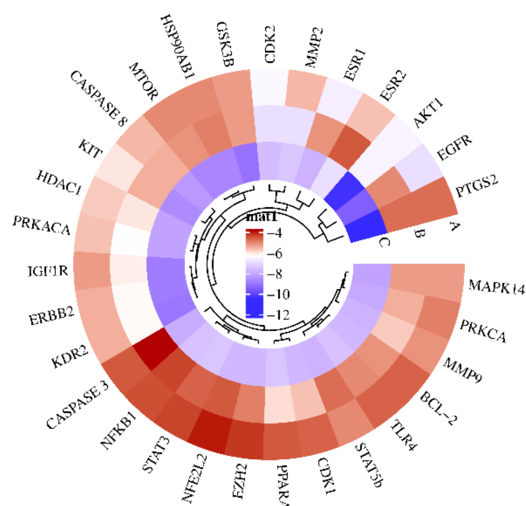

Figure 0.16 Heat map of the compounds from the ethyl acetate extract of *G. villosa in kcal/mol.*

A-2,6,10- trimethyl, tetradecane, B- Benzene(1-methylundecyl)-, C-Doxorubicin hydrochloride.

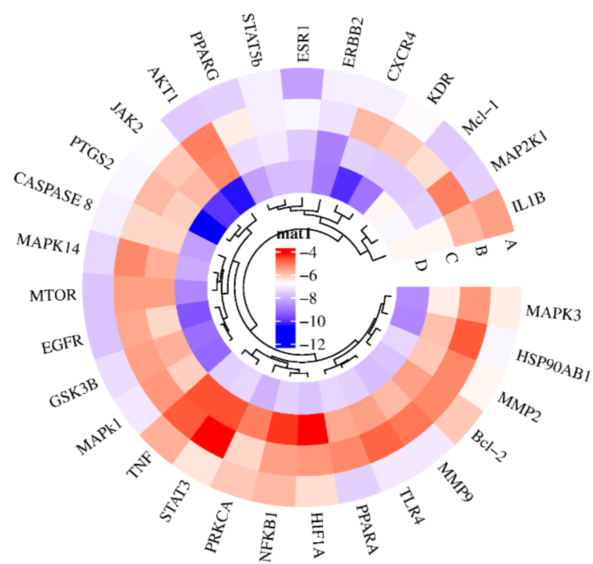

Figure 0.17 Heat map from the ethyl acetate extract of *R. prinoides in kcal.mol.*

A-squalene, B-olean-12-ene-3.beta-0l, C- 3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-c yclopenta[i]indene and D- Doxorubicin hydrochloride**.**

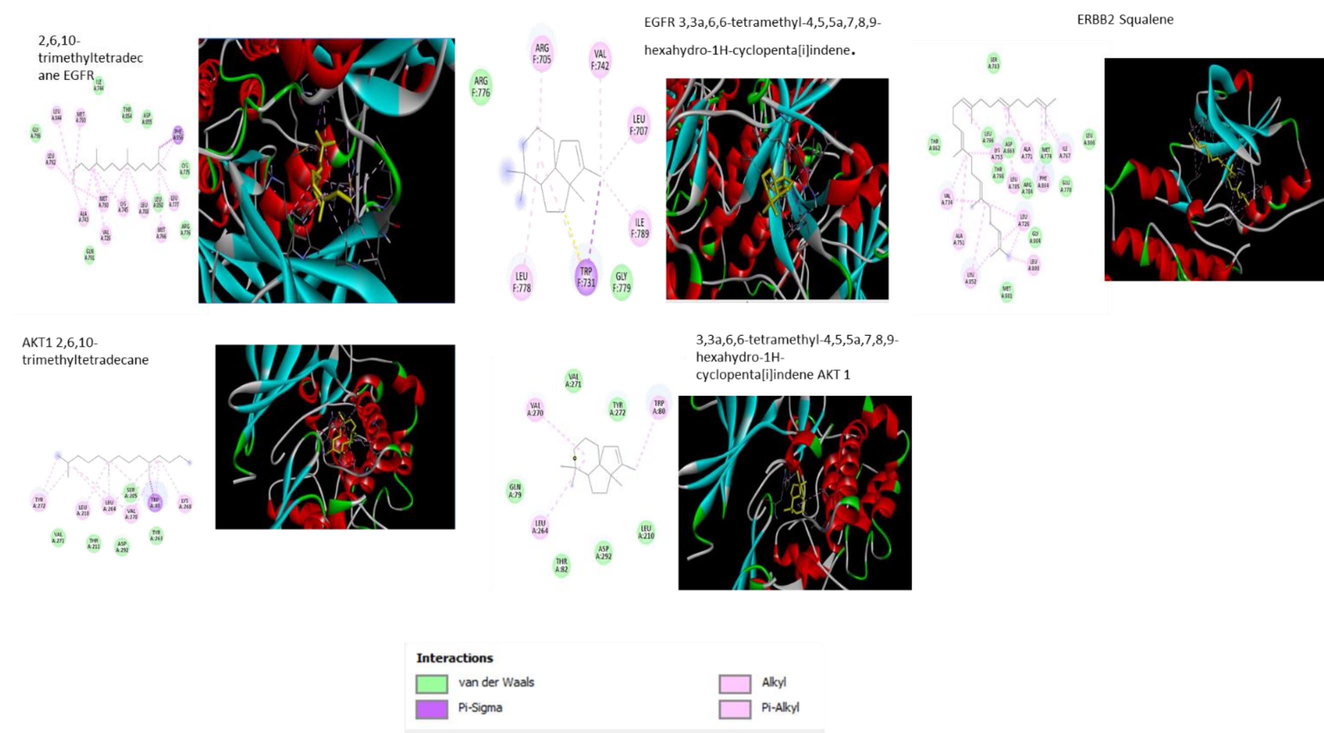

Figure 0.18 2D and 3D representations of the interactions of some of the compounds of the ethyl acetate extracts of *R. prinoides* and *G. villosa* with the gene proteins.

4.7 Validation of predicted targets through gene expression

The study investigated the changes in the gene expression for various genes. The RNA was extracted, quantified and the purity validated using the absorbance ratio of 260/280 and using a Nano-drop (Table 4.9 and Appendix VIII respectively) and gel electrophoresis using agarose (Appendix VII). For *R. prinoides*, EGFR, ERBB2, AKT1, BCL-2 and NF-κB were all down-regulated while p53 and Bax were upregulated (Figure 4.16). P53 and Bax had the highest fold change of 3.73 and 3.29 respectively. In *G. villosa*, EGFR and AKT1 were downregulated while caspase 3 and p53 were upregulated (Figure 4.16). Caspase 3 and p53 had the highest fold change of 2.78 and 2.58 respectively.

Table 0.10 Quantity and 260/280 ratio of extracted RNA from the ethyl acetate extracts of *G. villosa* and *R. prinoides.*

Sample Name	Nucleic Acid (ng/μL)	Absorbance (A260/A280)
G. villosa IC_{50}	410.992	2.091
R. prinoides IC_{50}	1051.844	2.086
Negative control (0.4% DMSO)	589.04	2.066

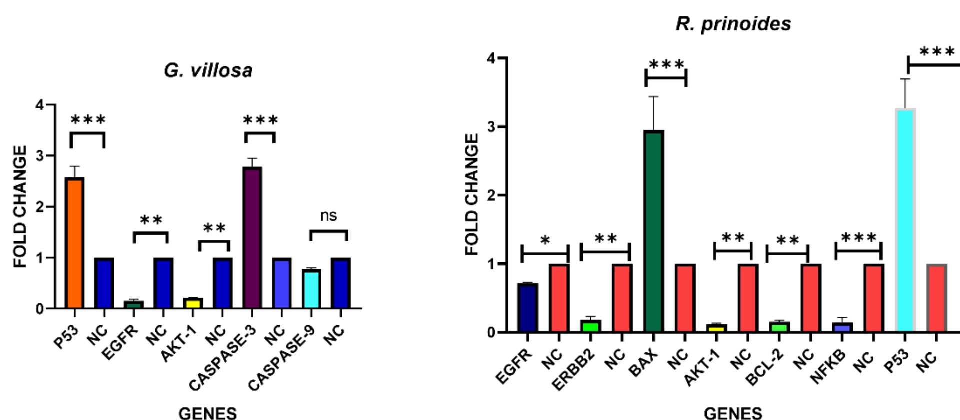

Figure 0.19 Relative fold change of genes exposed to *G. villosa* and *R. prinoides* ethyl acetate extracts.

Error bars represent Mean ± SEM for three replicates. NC-Negative control. ns- no significance * p< 0.05; ** p< 0.01 ; *** p< 0.001;**** p<0.0001 vs negative control

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

This investigation evaluated the anti-cervical cancer properties of *G. villosa* and *R. prinoides* plant extracts through *in vitro* and *in silico* methods in cervical cancer cells. The focus was on anti-proliferative, anti-migration effects in relation to the study objectives. The compounds in the extracts were also identified and used for *in silico* studies in cervical cancer.

The anti-proliferative activities of the crude, hexane and ethyl acetate extracts of *R. prinoides* and *G. villosa* plants were evaluated in this study against cervical cancer cells. The extracts generally prevented cervical cancer cells from proliferating, and the effect was measured according to the median inhibitory concentration (IC_{50}). As stated by NCI in the United States of America, an extract is considered inactive if its IC_{50} value is greater than 200 µg/ml, moderately anti-proliferative if it is between 30 µg/ml and 200 µg/ml, and highly anti-proliferative if it is less than 20 µg/ml for botanicals and less than 4 µg/ml for compounds (Canga *et al*., 2022). With IC_{50} values of 77.87 µg/ml and 100.70 µg/ml, the ethyl acetate extracts of *R. prinoides and G. villosa* respectively showed moderate anti-proliferative activity which is stronger than *Rhamnus alaternus* extracts reported by Ammar *et al* (2007)) (Ammar et al., 2007). *In vitro*, the other extracts had no notable effect on cervical cancer cells, according to NCI.

The anti-proliferative effects of the *Rhamnus* genus in different cancer cell lines has been previously tested and recorded (Nigussie *et al*., 2021). The anti-proliferative effects of *Rhamnus alaternus* against human and murine leukemic cell lines (K562 and L1210 respectively) were

investigated by Ammar *et al*., (2007). According to the findings the ethyl acetate, methanol and aqueous extracts had IC_{50} values of 232, 298 and 606 µg/ml in the human cell line (K562) and 176, 767 and 560 µg/ml for the murine cell line (L1210) (Ammar *et al*., 2007). Chen *et al*., (2016) study results of the effects of bark of *Rhamnus davurica* against the human colon cancer cell line (HT-29) and a human gastric cancer cell line (SGC-7901) showed that the methanol extracts were shown to have anti-proliferative activity with IC_{50} values of 24.96 and 89.53 µg/ml in the HT-29 and SGC-7901 cancer cells, respectively. The effects were however selective as the extracts had a higher IC_{50} value for the non-cancerous liver cells (L-02) of 229.12 µg/ml (Chen *et al*., 2016; 2018).

Diverse investigations have demonstrated the anti-proliferative properties of plant extracts from the *Grewia* genus (Singh, *et al*., 2022). With findings of IC_{50} values of 199.5 µg/ml, 177.8 µg/ml, 53.70 µg/ml, and 54.90 µg/ml, respectively, the methanol extract of *Grewia asiatica* had moderate to low anti-proliferative activity against human breast cancer cells (MCF-7), human cervical cancer cells (HeLa), human acute myeloblastic leukaemia cells (HL-60), and chronic myelogenic leukaemia cells (K-562). However, another study that used hydro-methanolic extracts shown inhibitory activity against Hep-2 (larypharyngeal cancer cells) with an IC_{50} value of 80.4 µg/ml, NCI-H522 (human lung cancer adenocarcinoma cells) with an IC_{50} value of 73 µg/ml, and MCF-7 cancer cells (34.9 µg/ml) (Qamar *et al*., 2020). Results of investigations on the aqueous extracts of *Grewia asiatica* fruits revealed that they showed anti-proliferative action against MCF-7 (IC_{50} 61.23 µg/ml) and lung cancer cell line (IC_{50} 50.37 µg/ml) (Marya *et al*., 2011). With an IC_{50} value of 345 µg/ml, the methanol extract of *Grewia tiliaefolia* demonstrated low inhibitory action against Hep-2 (Ramshankar *et al*., 2008).

The selectivity index was computed to determine how well the extracts limited the proliferation of cervical cancer cells while sparing non-cancerous cells. High selectivity is indicated by a high selectivity index. Medicinal plants are regarded as extremely selective if their selectivity index is greater than two, and less selective if it is less than two (Badisa *et al*., 2009).

All the extracts from *R. prinoides* demonstrated great selectivity for cervical cancer cells with selectivity indices >2 with the ethyl acetate extract of *R. prinoides* showed the highest selectivity for cervical cancer cells while sparing the non-cancerous cells with a selectivity index of 4.40. The extracts from *G. villosa* demonstrated good selectivity for cervical cancer cells *in vitro* (Selectivity indices >2) except for the crude extract of *G. villosa* which showed the least selectivity for cervical cancer with a selectivity index of 1.69 which is less than 2. However, all the extracts demonstrated limiting effects in HeLa cells in a trend of increasing activity with increasing concentration, making them viable options for further studies on anti-cancer therapy.

This study examined how ethyl acetate extracts affected the suppression of cell migration, a crucial characteristic of cancer cells. In contrast to the HeLa cells treated with the negative control, the results demonstrated that the ethyl acetate extracts of *R. prinoides* and *G. villlosa* hindered HeLa cells' migration at both 24 and 48 hours, and that the inhibition increased as the treatment time increased and as the dose increased.

At 24 hours, the ethyl acetate extracts of *R. prinoides* and *G. villosa* exhibited inhibition at the IC_{50} and IC_{25} concentrations with 18.438 and 29.958 % wound closure for the IC_{50} and 24.81 and 45.9% wound closure for the IC_{25} respectively compared to the negative control which had

49.74% wound closure and for 48 hours at IC_{50} and IC_{25} concentrations *R. prinoides* ethyl acetate extract had 23.80 and 40.51% wound closure while *G. villosa* had 26.27 and 46.24 % wound closure for the IC_{50} and IC_{25} concentrations respectively which were far less than the negative control which had 78.48% wound closure. However, there was no discernible variation when the effects of the negative control and the ethyl acetate extracts of *G. villosa* and *R. prinoides* at $IC_{12.5}$ values were compared. According to these findings, the extracts demonstrated anti-migratory effects on cervical cancer, making them promising candidates for treatments that target the disease.

These results are concurrent with the results of an evaluation of the rhizome extract of *Kaempferia parviflora* on cervical cancer cells whereby they limited the *in vitro* migration of cervical cancer cells with the highest inhibition being at 40 hours with over 80% inhibition of migration being recorded (Potikanond *et al*., 2017). According to another study that examined the effects of *Plumbago zeylanica* ethanoic root extracts, they inhibited cervical cancer cell migration *in vitro* in a manner that increased as the dose of the extract increased. The highest inhibition was observed at 48 hours, with less than 20% wound closure at 20 µg/ml (Mohapatra *et al*., 2022).

The extracts' proven anti-cervical cancer properties are partially due to the compounds they contain. GC/MS analysis as well as qualitative screening were carried out to identify the classes as well as specific compounds in the organic extracts.

Various phenols were identified in this study including 4-tert-Butylphenyl acetate, 3-Amino-4-(4-ethoxy-phenylamino)-benzoic acid and 2',4'-Dihydroxy Propiophenone in the ethyl acetate extract of *R. prinoides*, 2-methyl-4-(2,4,4-trimethylpentan-2-yl)phenol in the hexane extract of *R. prinoides*, 2,4-Di-tert-butylphenol identified in the ethyl acetate extract of *G. villosa* and 8-(1,1-Dimethylallyl)-5,7-dimethoxycoumarin identified in the hexane extract of *G. villosa*.

In vitro experiments employing HeLa cells have demonstrated the effectiveness of phenols on cervical cancer, including flavanols (Quercitin (Vidya Priyadarsini *et al*., 2010) and Kaempferol (Xu *et al*., 2008)) and flavones (Apigenin (Zheng *et al*., 2005) and Luteolin (Yan *et al*., 2012)).
Various alkaloids were recognized in the extracts of *R. prinoides* and *G. villosa* . These include 2-(3-Phenyl-piperidin-1-yl)-ethylamine in the ethyl acetate extract of *R. prinoides*, 1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1-m in the crude and hexane extracts of *G. villosa*. Alkaloids are nitrogenated, pharmacologically active secondary metabolites. They contain a ring and a nitrogen atom. The carbon skeleton is used for their classification. Some alkaloids are steroidal, indole, aporphine, tropane and isoquinoline (Fernandes *et al*., 2015; Jadhao *et al*., 2024) .Their use in phyto-medicine has been around since time immemorial.

Notable alkaloids that have been used against cervical cancer are vincristine and vinblastine compounds isolated from the plant *Catharanthus rosea* from Madagascar (Dhyani *et al*., 2022), paclitaxel from *Taxus brevifolia* (Corte *et al*., 2020) and camptothecin from *Camptotheca acuminata* (Martino *et al*., 2017).

Various alkaloids which have shown activity against HeLa cells *in vitro* include briofilin, a steroidal alkaloid isolated from *Bryophyllum pinnata* and naucleficine and naucleactonine which are indole alkaloids isolated form *Nauclea oreantalis* (Fernandes *et al*., 2015).

Some of the triterpenoids identified in the extracts are squalene and Olean-12-en-3-ol, acetate, (3.beta.)- in the ethyl acetate extract of *R. prinoides.* Triterpenoids are secondary metabolites with an isopentenyl pyrophosphate backbone. They exist as triterpenoids, saponins which are triterpenic glycosides or phytosterols.

By modifying pro-inflammatory mediators including cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), and inducible forms of nitric oxide synthase (iNOS), numerous research has evaluated and documented the anti-inflammatory effect of triterpenes (M.R. Patlolla & V. Rao, 2012; Rao, 2004). Boswellic acid from the plant *Bosweillia serrata* has previously reported to suppress the activity of NF-κB and 5-LOX (Poeckel & Werz, 2006) while oleanic acid and betulinic acids block the formation of iNOS (Yun *et al*., 2003;Suh *et al*., 2007). Additionally, they have been demonstrated to elicit anti-proliferative activity through blocking the cell cycle progression as shown by the inhibition of the G1 and G2/M phase by the Japanese apricot extracts which are rich in oleanic acid and ursolic acid (Nakagawa *et al*., 2007), initiating apoptosis by inducing the caspase cascade as shown by studies in betulinic acid (Mullauer *et al*., 2010), betulinic acid and acetyl 11-keto boswellic acid have been demonstrated to limit angiogenic initiation by inhibiting angiogenesis and inhibiting cell migration (Kwon *et al*., 2002; Singh *et al*., 2007).

Using molecular docking, this work investigated the binding affinities of several proteins involved in cervical cancer to determine the possible mode of action of the ethyl acetate extracts of *R. prinoides* and *G. villosa*. A binding energy of less than zero signifies spontaneous binding between a ligand and a receptor, a binding affinity of less than -5.0 is indicative of average binding affinity, and a binding affinity of less than -7.0 is indicative of very strong binding affinity as well as more stable ligand complex's structures (Tu *et al.,* 2021).

Key compounds from *R. prinoides* showed strong binging;3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene with binding energy affinities in kcal/mol as :-7.1 for ERBB2, -8.4 for ESR1, -7.7 for AKT1, -7.8 for EGFR, -7.0 for PTGS2, -7.8 for mTOR, -7.2 for TLR4, -7.2 for MMP2, -7 for HSP90AB1, -7.4 for GSK3B, -7.1 for CXCR4, -7.1 for STAT5B, -7.6 for PPARA, -7.5 for MAPK14, -7.1 for CASPASE 8, -7.2 for MAPK1, -7.7 for MCL-1 and -7.6 for PPARG. Olean-12-en-3-l_acetate (3.beta.) compound in the ethyl acetate extract of *R. prinoides* also displayed good binding affinity energies with various protein targets as: -7.1 for ESR1, -7.3 for ERBB2 and -7.1 for STAT5B. Squalene in the ethyl acetate extract of *R. prinoides* also had good binding affinity energy as: -8.9 for ERBB2, -7.7 for ESR1, -7.7 for KDR2, -7.2 for STAT5B, -7.7 for MCL-1 and -7.4 for PPARG. Benzene_(1-methylundecyl) compound in the ethyl acetate extract of *G. villosa* had good binding affinity energies in kcal/mol with two protein targets: -7.0 for CDK2 and -7 for MMP2. All the compounds, however, had binding affinity energies of ≤ -4 which validates their anti-proliferation activity through the interactions with various genes.

An investigation led by He *et al*. (2023), evaluating anti-cancer activities of flavonoids in *Artemisia absinthium* found that the elicited activity of the flavonoids against HeLa cells *in vitro* was through the targeting signaling proteins such as EGFR, STAT3 and ESR1 quite like the compounds isolated in this study which could lead to the postulation that the interaction of the compounds with various signaling molecules could play a part in the efficacy of the ethyl acetate extracts of *R. prinoides* and *G. villosa* (He *et al*., 2023).

Results from a separate investigation led by Yunos *et al*. (2023) showed that the anti-cancer properties of Eurycomanone and Eurycomalactoe, which were isolated from *Eurycoma longifolia* roots, could be ascribed to their interaction with DHFR and TNF-α, with associated binding affinity energies of -8.05 and -8.87 kcal/mol and -8.83 and -7.51 kcal/mol, respectively (Yunos *et al*., 2023). This emphasises even more how crucial it is to confirm how compounds interact with significant cervical cancer protein targets.

In this investigation, the gene expression effects of *R. prinoides* and *G. villosa* extracts on the proteins-compound interactions with the lowest binding affinity energies and some important apoptotic genes were examined as a potential mechanism of action for the extracts' *in vitro* suppression of cervical cancer cells. The findings display that the ethyl acetate extracts of *R. prinoides* and *G. villosa* induce apoptosis and downregulated signaling molecules involved in cancer growth, proliferation and metastasis. The ethyl acetate extracts of *R. prinoides* and *G. villosa* upregulated the expression of TP53 in HeLa cells by a fold change of 3.27 and 2.58 respectively. The Bax: Bcl-2 ratio was 11.61 in favour of Bax after the ethyl acetate extract of *R. prinoides* also enhanced the expression of Bax by a factor change of 3.29 and decreased the

expression of Bcl-2 by a fold change of 0.28. A Bax/Bcl-2 ratio of 11.61 signifies a pro-apoptotic shift, while TP53 upregulation further validates activation of tumor suppressor pathways. In cervical cancer cells treated with *G. villosa* ethyl acetate extracts, a fold change of 2.78 was observed for caspase 3, whereas a fold change of 0.216 and 0.157 was observed for AKT1 and EGFR, respectively. Additionally, HeLa cells exposed to the ethyl acetate extract of *R. prinoides* at IC_{50} concentration showed a downregulation of EGFR, ERBB2, AKT1, and NF-κB, with fold changes of 0.58, 0.28, 0.28, and 0.144, respectively. The bioactivity shown can be linked to the phytochemicals and compounds identified.

Numerous research has demonstrated how extracts and isolated phyto-compounds affect various genes and proteins, and they have connected these effects to the plants' ability against cervical cancer. The emodin extract of *Rhamnus sphaerosperma* var *pubescens* was shown to decrease the levels of AKT and prevent AKT activation *in vitro* as well as increase the levels of Bax and decrease the levels of Bcl-2 after treatment of cervical cancer cells (Moreira *et al*., 2018). Findings from a study by Liu *et al*. (2016) revealed that theanine and its tea-derived derivative downregulated the expression of EGFR and NF-κB in cervical cancer cells *in vitro*. This allowed them to block the EGFR/Met-Akt/NF-κB signalling pathway and stop the cervical cancer cells from migrating (Liu *et al*., 2016). In a similar fashion, a study by Potikanond *et al*. (2017) revealed that *Kaempferia parviflora* rhizomes prevented cervical cancer cells from proliferating *in vitro* by blocking MAPK and PI3K/AKT signalling proteins such AKT, PI3K, ERK1/2, and Elk1. (Potikanond *et al*., 2017). Findings from a 2009 study by Peng *et al*., revealed that phenolic fractions of *Duchesnea indica* treated cervical cancer cells were able to raise the expression levels of the Bax/Bcl-2 ratio by raising Bax expression levels and lowering Bcl-2 expression

levels *in vitro* (Peng *et al*., 2009). An *in vitro* study on the effects of methanolic neem extracts on cervical cancer cells found that treatment with the extract at various dosages was able to decrease the mRNA levels of Bcl-2 and caspase-3 protein. (Kumar et al., 2022).

In this study, it was shown that tumour protein 53(TP53), a crucial protein in the body that helps suppress tumours, was up regulated. These results coincide with other investigations on the anti-proliferation effects of other plant extracts via TP53 elevated levels.

According to a study by Mahfudh *et al*. (2008), eurycomanone that was isolated from the roots of *Eurycoma longifolia* upregulated the expression of TP53, Bax, and Bcl-2 genes (Mahfudh & Pihie, 2008). Another study by Rezai *et al*. (2019) found that TP53 mRNA expression was downregulated after being treated with hydro-alcoholic extracts from *Achillea wilhelmsii* (Rezai *et al*., 2019). When used on cervical cancer cells *in vitro*, xanthirrhizol, a sesquitepernoid molecule derived from *Curcuma xanthorrhiza*, was demonstrated to increase p53 expression (Ismail *et al*., 2005). Leaf extracts of Excoe*caria agallocha* were shown to increase mRNA levels of p53 after treatment of cervical cancer cells *in vitro* (Sultana *et al*., 2022) and hexane extracts of *Tulbaghia violacea* were also able to raise the mRNA expression of TP53 (Motadi *et al*., 2020).

The results of this investigation lend credence to the historic usage of these botanicals in cervical cancer treatment, and it has empirically demonstrated that the plants contain compounds that can be studied further for drug discovery of potent compounds in cervical cancer therapy. The

findings reveal that plant extracts disrupt cancer progression through selective cytotoxicity, inhibition of migration and modulation of gene expression relevant to apoptosis and growth.

5.2 CONCLUSIONS

i. The plant extracts under study demonstrated selective *in vitro* anti-proliferative activity in a dose dependent manner.

ii. Various compounds including phenols, hydrocarbons, alkaloids and triterpenoids were identified in the crude, hexane and ethyl acetate extracts of *R. prinoides* and *G. villosa*.

iii. Selected compounds from *R. prinoides* and *G. villosa* ethyl acetate extracts were found to interact with various cervical cancer proteins and targets with high binding affinity energies, notably, with EGFR and AKT1

iv. Treatment with the ethyl acetate extracts of *R. prinoides* and *G. villosa* led to the upregulation of p 53 and Bax genes with concurrent downregulation of EGFR, ERBB2, AKT1, BCL-2, NF-κB and CASPASE 3 mRNA levels.

5.3 RECOMMENDATIONS

The suggestions that follow can be made from this research:

i. Plant extracts from *R. prinoides* and *G. villosa* may be utilized as viable options for drug discovery in cervical cancer therapy.

ii. Induction of apoptosis, inhibition of signaling molecules could be used as potential mechanisms of action of *R. prinoides* and *G. villosa* extracts of cervical cancer cells (HeLa).

5.4 SUGGESTIONS FOR FURTHER RESEARCH

i. While this study provides sound evidence of the anti-proliferative effects of *R. prinoides* and *G. villosa* extracts *in vitro*, the effects of the extracts in vivo are yet to be determined.Animal investigations can be conducted to evaluate the anti-cancer potential of *R. prinoides* and *G. villosa* plants in animal models.

ii. Gas chromatography/ Mass Spectrometry is an excellent tool; however, it can only identify volatile compounds therefore Liquid chromatography/ Mass Spectrometry can be explored to exhaustively identify the polar compounds present in the plants.

iii. Further in-depth studies including nano formulation and 3D culture spheroids can be considered as alternatives to increase the selectivity and efficacy of the plant extracts that are active.

iv. Combinatorial therapy can be considered to evaluate whether the extracts can have synergistic effects together that are less toxic and more efficacious.

5.5 STUDY LIMITATIONS

This study would have been cemented by carrying out experiments in more than one cell line for cervical cancer. However, due to financial constraints in procuring a new cell line, it had to be carried out using the HeLa cell line only.

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APPENDICES

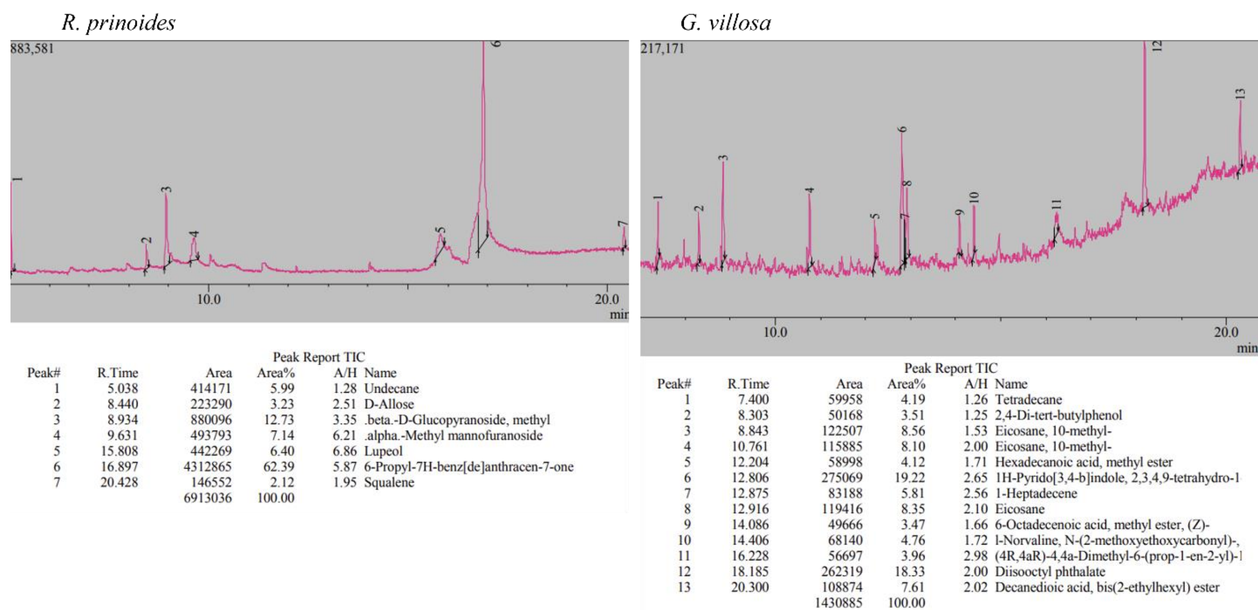
Appendix I Qualitative phytochemical profile of extracts of *R. prinoides* and *G. villosa*

	Rhamnus prinoides				*Grewia villosa*			
Extracts	**Crude**	**Hexane**	**Ethyl acetate**	**Water**	**Crude**	**Hexane**	**Ethyl acetate**	**Water**
Alkaloids	++	+++	++	-	++	++	+++	-
Saponins	+++	++	-	++++	+	-	++	-
Flavonoids	-	-	-	-	-	-	-	-
Terpenoids	+++	-	++	+++	++	-	-	++
Glycosides	+	+++	++++	++	-	-	-	-
Tannins	+++	++	++++	++++	+++	+++	+++	++++
Phenols	++++	++	++++	+++	+++	++	++++	+++

Key: ++++= **highly abundant,**+++= **abundant,** ++= **present,**+= **detectable and - = undetectable**

The quantities of the flavonoids in the extracts were not detectable using the color-based method.

Appendix II Chromatograms of the crude extracts of *R. prinoides* and *G. villosa*



Peak Report TIC

Peak#	R.Time	Area	Area%	A/H	Name
1	5.038	414171	5.99	1.28	Undecane
2	8.440	223290	3.23	2.51	D-Allose
3	8.934	880096	12.73	3.35	.beta.-D-Glucopyranoside, methyl
4	9.631	493793	7.14	6.21	.alpha.-Methyl mannofuranoside
5	15.808	442269	6.40	6.86	Lupeol
6	16.897	4312865	62.39	5.87	6-Propyl-7H-benz[de]anthracen-7-one
7	20.428	146552	2.12	1.95	Squalene
		6913036	100.00		

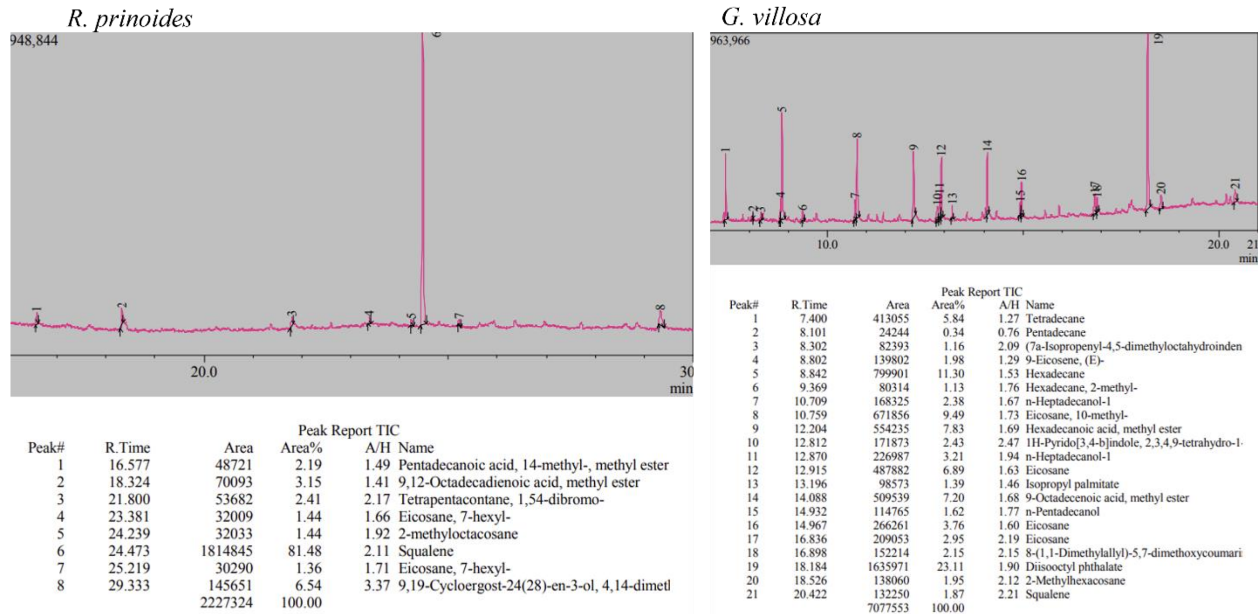
Peak Report TIC

Peak#	R.Time	Area	Area%	A/H	Name
1	7.400	59958	4.19	1.26	Tetradecane
2	8.303	50168	3.51	1.25	2,4-Di-tert-butylphenol
3	8.843	122507	8.56	1.53	Eicosane, 10-methyl-
4	10.761	115885	8.10	2.00	Eicosane, 10-methyl-
5	12.204	58998	4.12	1.71	Hexadecanoic acid, methyl ester
6	12.806	275069	19.22	2.65	1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1
7	12.875	83188	5.81	2.56	1-Heptadecene
8	12.916	119416	8.35	2.10	Eicosane
9	14.086	49666	3.47	1.66	6-Octadecenoic acid, methyl ester, (Z)-
10	14.406	68140	4.76	1.72	l-Norvaline, N-(2-methoxyethoxycarbonyl)-,
11	16.228	56697	3.96	2.98	(4R,4aR)-4,4a-Dimethyl-6-(prop-1-en-2-yl)-1
12	18.185	262319	18.33	2.00	Diisooctyl phthalate
13	20.300	108874	7.61	2.02	Decanedioic acid, bis(2-ethylhexyl) ester
		1430885	100.00		

Figure 1A Figures of the chromatograms of the compounds in the crude extracts of *R. prinoides* and *G. villosa*.

7 compounds and 13 compounds were identified from *R. prinoides* and *G. villosa* respectively

Appendix III Chromatograms of the hexane extracts of *R. prinoides* and *G. villosa*



R. prinoides — Peak Report TIC

Peak#	R.Time	Area	Area%	A/H	Name
1	16.577	48721	2.19	1.49	Pentadecanoic acid, 14-methyl-, methyl ester
2	18.324	70093	3.15	1.41	9,12-Octadecadienoic acid, methyl ester
3	21.800	53682	2.41	2.17	Tetrapentacontane, 1,54-dibromo-
4	23.381	32009	1.44	1.66	Eicosane, 7-hexyl-
5	24.239	32033	1.44	1.92	2-methyloctacosane
6	24.473	1814845	81.48	2.11	Squalene
7	25.219	30290	1.36	1.71	Eicosane, 7-hexyl-
8	29.333	145651	6.54	3.37	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimetl
		2227324	100.00		

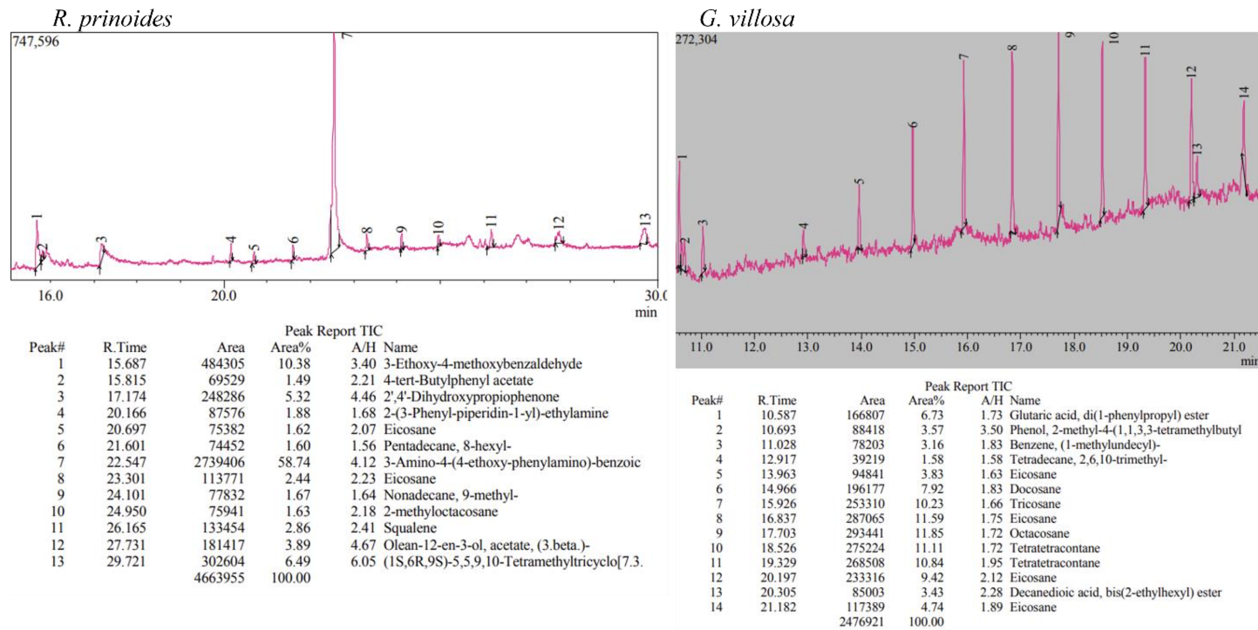
G. villosa — Peak Report TIC

Peak#	R.Time	Area	Area%	A/H	Name
1	7.400	413055	5.84	1.27	Tetradecane
2	8.101	24244	0.34	0.76	Pentadecane
3	8.302	82393	1.16	2.09	(7a-Isopropenyl-4,5-dimethyloctahydroinden
4	8.802	139802	1.98	1.29	9-Eicosene, (E)-
5	8.842	799901	11.30	1.53	Hexadecane
6	9.369	80314	1.13	1.76	Hexadecane, 2-methyl-
7	10.709	168325	2.38	1.67	n-Heptadecanol-1
8	10.759	671856	9.49	1.73	Eicosane, 10-methyl-
9	12.204	554235	7.83	1.69	Hexadecanoic acid, methyl ester
10	12.812	171873	2.43	2.47	1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1-
11	12.870	226987	3.21	1.94	n-Heptadecanol-1
12	12.915	487882	6.89	1.63	Eicosane
13	13.196	98573	1.39	1.46	Isopropyl palmitate
14	14.088	509539	7.20	1.68	9-Octadecenoic acid, methyl ester
15	14.932	114765	1.62	1.77	n-Pentadecanol
16	14.967	266261	3.76	1.60	Eicosane
17	16.836	209053	2.95	2.19	Eicosane
18	16.898	152214	2.15	2.15	8-(1,1-Dimethylallyl)-5,7-dimethoxycoumari
19	18.184	1635971	23.11	1.90	Diisooctyl phthalate
20	18.526	138060	1.95	2.12	2-Methylhexacosane
21	20.422	132250	1.87	2.21	Squalene
		7077553	100.00		

Figure 1B Figures of the chromatograms of the compounds in the hexane extracts of *R. prinoides* and *G. villosa* respectively.

8 compounds from *R. prinoides* and 21 compounds from *G. villosa* were identified.

Appendix IV Chromatograms of the ethyl acetate extracts of *R. prinoides* and *G. villosa*



Peak Report TIC (*R. prinoides*)

Peak#	R.Time	Area	Area%	A/H	Name
1	15.687	484305	10.38	3.40	3-Ethoxy-4-methoxybenzaldehyde
2	15.815	69529	1.49	2.21	4-tert-Butylphenyl acetate
3	17.174	248286	5.32	4.46	2',4'-Dihydroxypropiophenone
4	20.166	87576	1.88	1.68	2-(3-Phenyl-piperidin-1-yl)-ethylamine
5	20.697	75382	1.62	2.07	Eicosane
6	21.601	74452	1.60	1.56	Pentadecane, 8-hexyl-
7	22.547	2739406	58.74	4.12	3-Amino-4-(4-ethoxy-phenylamino)-benzoic
8	23.301	113771	2.44	2.23	Eicosane
9	24.101	77832	1.67	1.64	Nonadecane, 9-methyl-
10	24.950	75941	1.63	2.18	2-methyloctacosane
11	26.165	133454	2.86	2.41	Squalene
12	27.731	181417	3.89	4.67	Olean-12-en-3-ol, acetate, (3.beta.)-
13	29.721	302604	6.49	6.05	(1S,6R,9S)-5,5,9,10-Tetramethyltricyclo[7.3.
		4663955	100.00		

Peak Report TIC (*G. villosa*)

Peak#	R.Time	Area	Area%	A/H	Name
1	10.587	166807	6.73	1.73	Glutaric acid, di(1-phenylpropyl) ester
2	10.693	88418	3.57	3.50	Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl
3	11.028	78203	3.16	1.83	Benzene, (1-methylundecyl)-
4	12.917	39219	1.58	1.58	Tetradecane, 2,6,10-trimethyl-
5	13.963	94841	3.83	1.63	Eicosane
6	14.966	196177	7.92	1.83	Docosane
7	15.926	253310	10.23	1.66	Tricosane
8	16.837	287065	11.59	1.75	Eicosane
9	17.703	293441	11.85	1.72	Octacosane
10	18.526	275224	11.11	1.72	Tetratetracontane
11	19.329	268508	10.84	1.95	Tetratetracontane
12	20.197	233316	9.42	2.12	Eicosane
13	20.305	85003	3.43	2.28	Decanedioic acid, bis(2-ethylhexyl) ester
14	21.182	117389	4.74	1.89	Eicosane
		2476921	100.00		

Figure 1C Figures of the chromatograms of the compounds in the ethyl acetate extracts of *R. prinoides* and *G. villosa* respectively.

13 compounds from *R. prinoides* and 14 compounds from *G. villosa* were identified.

Appendix V Protein-protein network of the interaction between the common protein targets between the ethyl acetate extracts of R. *prinoides* and *G. villosa* and cervical cancer

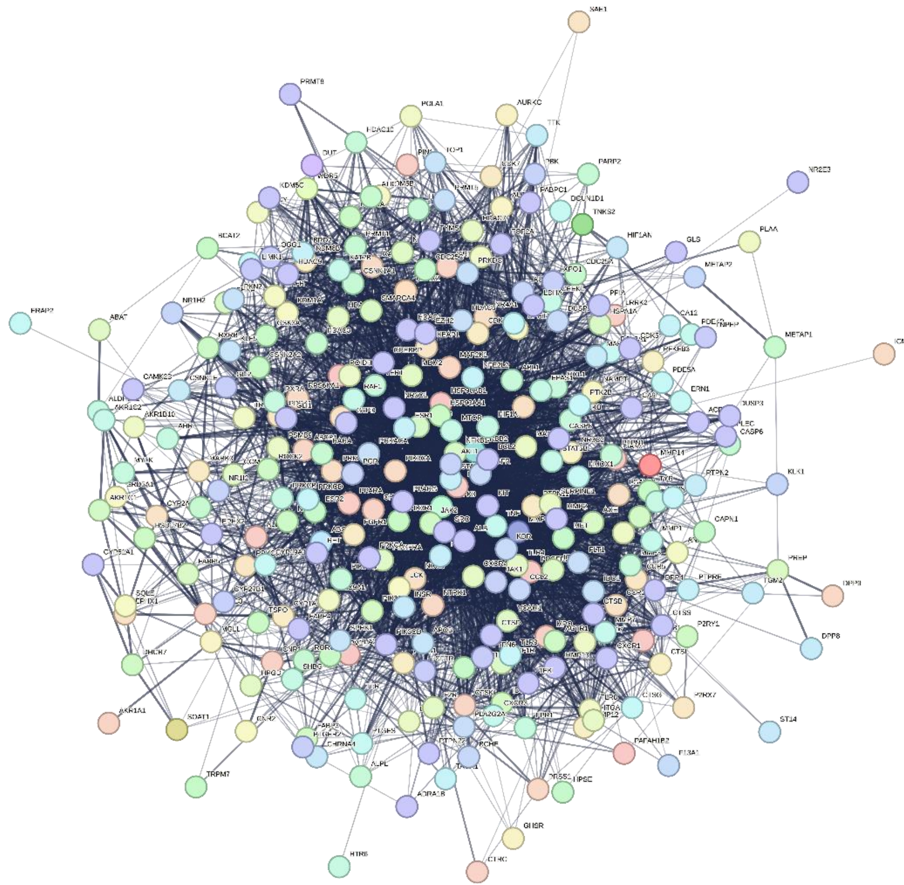


Figure 1D Figure of PPI network of the interactions between the targets from the plant extracts and cervical cancer gene targets.

String database was used to generate the protein-protein interaction diagram.

Appendix VI Molecular docking scores of the compounds identified in the ethyl acetate extracts of *R. prinoides* and *G. villosa*

Compound	**KIT**	**CDK2**	**ESR1**	**MMP2**	**AKT1**	**EGFR**	**KDR2**	**IGF1R**	**PTGS2**	**mTOR**	**TLR4**	**STAT3**	**MMP9**	**HSP90AB1**	**GSK3B**	**HDAC1**	**EZH2**	**ERBB2**	**CDK1**	**CXCR4**
A	-6.1	-6.5	-6.7	-5.6	-6.6	**-7**	-5.5	-5.3	-4.8	-5.1	-4.7	-4.4	-5.2	-5.1	-5.3	-5.8	-4.3	-5.5	-4.7	N/A
B	-5.5	-7	-5.2	-7	-6.6	-5.1	-6.3	-6.2	-4.8	-5.2	-5.1	-4.7	-5.8	-5	-5.3	-6.1	-5	-6.3	-5.7	N/A
C	N/A	N/A	**-8.4**	-6.7	**-7.7**	**-7.8**	-6.9	N/A	**-7**	**-7.8**	-7.2	-6.5	**-7.2**	-7	-7.4	N/A	N/A	**-7.1**	N/A	**-7.1**
D	N/A	N/A	-7	-5.3	-5.2	-5.7	-6.1	N/A	-5.9	-5.6	-4.9	-4.3	-5.1	-4.8	-5.6	N/A	N/A	**-7.3**	N/A	-5.9
E	N/A	N/A	**-7.7**	-6.1	-5.3	-6.3	**-7.7**	N/A	-6.2	-5.6	-5.6	-4.7	-5.9	-6	-5.8	N/A	N/A	**-8.9**	N/A	**-7.6**
PC	-8.7	-7.7	-7.9	-7.5	-10.5	-9.6	-9.1	-9	-10.6	-8.8	-7.6	-7.5	-7.9	-8.8	-9.2	-8.2	-7.8	-9	-7.8	-10.2
Compound	**PRKA**	**STAT5b**	**PPARA**	**PRKCA**	**MAPK14**	**ESR2**	**CASPASE8**	**NFE2L2**	**BCL-2**	**NF-KB1**	**CASPASE3**	**MAP2K1**	**JAK2**	**IL1B**	**MAPK3**	**MAPk1**	**Mcl-1**	**HIF1A**	**TNF**	**PPARG**

	CA																			
A	-5.7	-5.1	-4.6	-5	-5.3	-5.7	-5.6	-4.1	-4.7	-4.5	-4.6	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
B	-6.4	-4.8	-6	-5.5	-5.3	-4.6	-5.5	-4.6	-5.2	-4.4	-4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
C	-6.1	**-7.1**	**-7.6**	N/A	**-7.5**	N/A	-7.1	N/A	-6.1	-5.9	N/A	-7.6	-6.9	-5.6	-6.6	**-7.2**	**-7.7**	-6.4	-5.8	-7.6
D	-6.3	**-7.1**	-5.3	N/A	-5.3	N/A	-6.3	N/A	-5.3	-5.6	N/A	-5.2	-6.1	-5.9	-5.5	-5.5	-6.4	-5.5	-4.8	-6.6
E	-5.2	**-7.2**	-5.7	N/A	-5.8	N/A	-6.3	N/A	-5.6	-4.5	N/A	**-7.6**	-5.9	-6.7	-6.6	-6.2	**-7.7**	-4.3	-4.7	**-7.4**
PC	-8.2	-7.9	-7.5	-8.1	-8.2	-7	-8.4	-7.8	-7.8	-7.6	-8	-9.3	-10.1	-6.7	-8.7	-8.5	-6.7	-7.8	-7.6	-8.4

1 Legend: Compound A- 2,6,10 –trimethyltetradecane, compound B- Benzene_(1-methylundecyl), compound C-3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene - ,compound D- Olean-12-en-3-ol_acetate_(3.beta.), compound E- Squalene and the PC- Doxorubicin hydrochloride. N/A indicates that docking was not performed for that protein using the compound (ligand)

Appendix VII RNA Quantification images

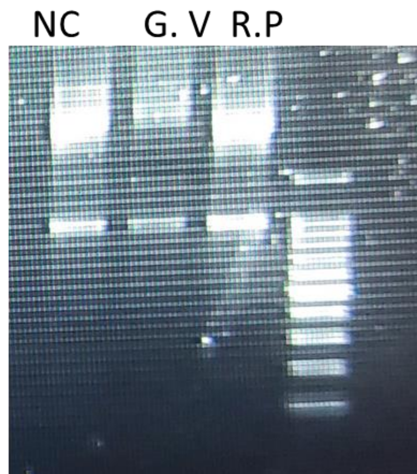


Figure 1E Image of the gel electrophoresis of the RNA extracted from cervical cancer cells treated with *R. prinoides* and *G. villosa* ethyl acetate extracts.

Appendix VIII RNA quality images from Nano-drop

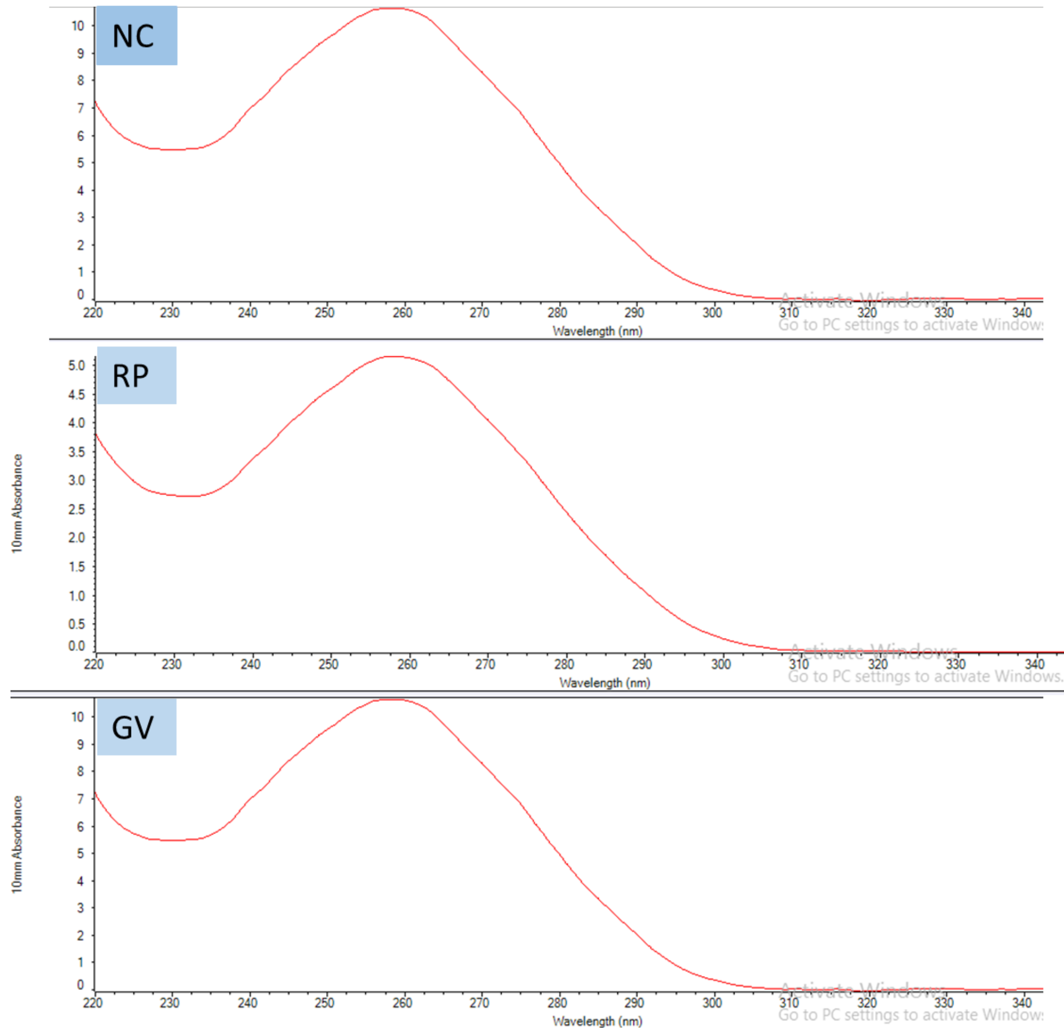
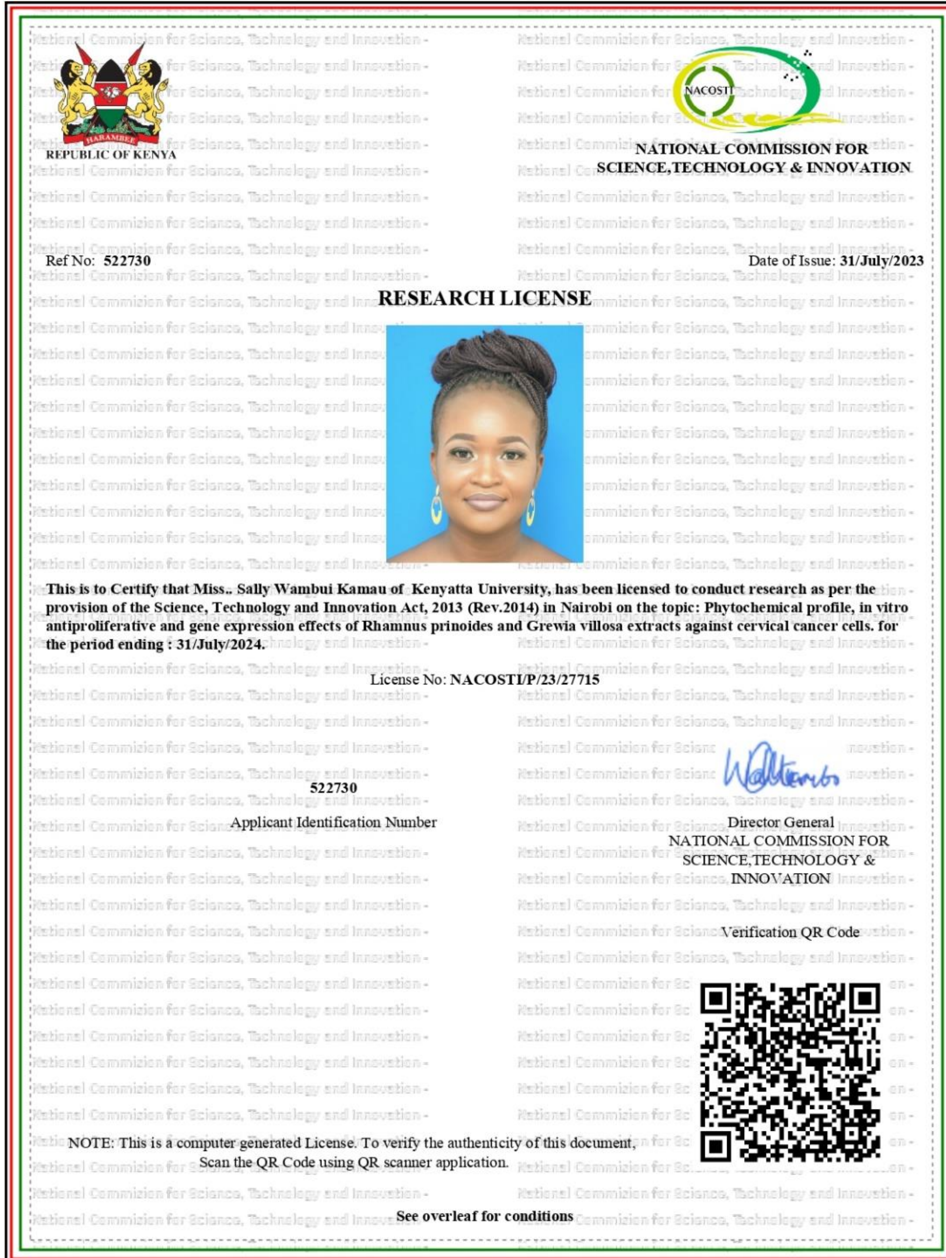


Figure 1F Nano-drop images of RNA extracted from cervical cancer cells treated with ethyl acetate extracts of *R. prinoides (RP), G. villosa (GV)* and the negative control (0.4% DMSO) ascertaining the quality of the purified RNA.

Appendix IX NACOSTI approval for research authorization



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Date of Issue: **31/July/2023**

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Figure 1E Ethical approval certificate for research from the National Council of Science and Technology (NACOSTI), received in July 2023 before the study commenced.

Appendix X Image of publication from research

Heliyon 10 (2024) e37324

Research article

Network pharmacology, molecular docking and experimental approaches of the anti-proliferative effects of *Rhamnus prinoides* ethyl-acetate extract in cervical cancer cells

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ABSTRACT

Background: Cervical cancer, one of the lethal cancers among women, is a challenging disease to treat. The current therapies often come with severe side effects and the risk of resistance development. Traditional herbal medicine, with its potential to offer effective and less toxic options, is a promising avenue. This study was undertaken to investigate the potential of *Rhamnus prinoides* (*R. prinoides*) root bark extracts in selectively inhibiting the proliferation of cervical cancer cells, using the HeLa cell line as an *in vitro* model.
Methods: R. prinoides plant extracts were first screened at a fixed concentration of 200 μg/ml to determine the active extract. The selective anti-proliferative activity of the active extract was evaluated in a concentration dilution assay using the (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide) MTT assay on cancerous (HeLa) cells and non-cancerous (Vero) cells to determine the half-maximal inhibitory (IC_{50}) and half-cytotoxic concentrations (CC_{50}), respectively. Functional assays on cell morphology (by microscopy), cell migration (wound healing assay) and cell cycle (by flow cytometry) were also conducted. The active extract was analyzed using Gas Chromatography/Mass Spectrometry (GC/MS) to determine any compounds it contained. Following identification of possible gene targets by network pharmacology, the genes were validated by molecular docking and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR).
Results: The ethyl acetate extract of *R. prinoides* (EARP), the most active extract, selectively inhibited the growth of cervical cancer cells, their migration and induced cell cycle arrest at the S phase. *In silico* analysis revealed that squalene, 3,3a,6,6-tetramethyl-4,5,5a,7,8,9-hexahydro-1H-cyclopenta[i]indene and Olean-12-en-3.beta.-ol, acetate showed acceptable drug-like characteristics and may be partly attributed to the bioactivity demonstrated and the deregulation of the mRNA expression of AKT1, NF-κB, p53, Bax, Bcl-2, and Er-b-B2.
Conclusion: This study, for the first time, demonstrates the anti-proliferation effects of EARP and forms a firm foundation for further drug development studies.

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Figure 1F Publication from results from the study in *Heliyon* journal (Elsevier) published in September 2024. https://doi.org/10.1016/j.heliyon.2024.e37324