THE USE OF A 2D CELL CULTURE MODEL TO INVESTIGATE THE EFFECTS OF SELECTED PLANTS ON TUBULARIZATION OF CERVICAL CANCER CELL LINES

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BLOEMFONTEIN

by

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STATEMENT REGARDING INDEPENDENT WORK

I, Kgaogelo Vincent Napo, identity number _____ and student number _____, do hereby declare that this research project submitted to the Central University of Technology, Free State for the Degree MASTER OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY, is my own independent work; and complies with the code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment of the requirements for the attainment of any qualification.

22 June 2024

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“I am not saying I’m gonna change the world, but I guarantee you that I will spark the brain that will change the world”

-Tupac Shakur

List of Abbreviations and Acronyms

NCR	National Cancer Registry
HPV	Human papillomavirus
2-D	Two dimensional
TLC	Thin layer chromatography
CANSA	Cancer Association of South Africa
ECM	Extracellular matrix
bFGF	Basic fibroblast growth factor
MMPs	Matrix metalloproteinases
VEGF	Vascular endothelial growth factor
IC50	Half maximal inhibitory concentration
O_2	Oxygen
FGF	Fibroblast growth factor
CO_2	Carbon dioxide
DCM	Dichloromethane
DMEM	Dulbecco's modified eagle media
FBS	Foetal bovine serum
MeOH	Methanol
DOX	Doxorubicin
$DMSO_4$	Dimethyl sulfoxide
	3 - (4,5-dimethylthiazol-2-yl) -2, 5 - diphenyltetrazolium bromide
v/v	volume to volume
Rf	Retention factor
UV	Ultraviolet
HP	Hewitt Packard
pH	Potential hydrogen

ODS	Octadecylsilane
HCL	Hydrochloric acid
H2SO4	Sulphuric acid

RESEARCH OUTPUTS

PUBLICATIONS

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ABSTRACT

Background

The current available cell-based *in vitro* assays for preliminary screening of potential chemotherapeutic agents focus on cell viability and apoptosis of cancer cells. There are no validated high throughput methods that focus on vascularization and tubularization, the two biological processes critical for the process of metastasis of cancer. Malignancy of cancer cells is the cause of mortality in cancer patients. It is projected that halting metastasis in cervical cancer, for example, can reduce the death rates by more than half. The process of metastasis is highly dependent on unregulated over-proliferation, tubularization and vascularization of cancer cells, which all promote localized benign tumors to malignancy.

This project investigated tubularization of cervical cancer cell lines, and the anti-tubularization activity of two medicinal plants; *Boscia albitrunica* and *Steganotaenia araliacea* hochst, cited to have anti-cancer properties. Without proliferation, differentiation, tubularization and vascularization, both angiogenesis and metastasis cannot occur, which means neoplasms remain localized, and metastasis halted.

Methods

Phytochemical composition of both plant extracts was investigated. *Boscia albitrunica* and *Steganotaenia araliacea* hochst organic and water extracts were investigated for their growth inhibition potential of HeLa and C33A cervical cancer cell lines monolayer using MTT assay. Tubular-like structure formation ability of cells was investigated through 2-D cell culture model using Matrigel to investigate the most active fraction extract. The 2-D Matrigel technology was employed to grow cells on and through the Matrigel. Long distance objective light microscope was used to view the formation or non-formation of cellular tubular-like structures after cancer cells were treated with plant extracts that showed highest anti-proliferation activity on monolayer cells.

Results

Phytochemical analysis of raw plant materials of both plants showed the presence of secondary metabolites including alkaloids and flavonoids, known to possess various pharmacological activities including anticancer activity. *Steganotaenia araliacea* hochst and *Boscia albitrunca* showed the IC_{50} on both cell lines. *B. albitrunca* MeOH extract displayed a high IC_{50} value of 28.6931 µg/ml. This value indicated very low hazardous cytotoxicity status. *S. araliacea* hochst MeOH extract displayed an IC_{50} value of 14.78 µg/ml and indicated a moderate hazard and potency of cytotoxicity. *Boscia albitrunica* methanol and DCM extracts exhibited below 42% and 30% cell growth inhibition against HeLa and C33A cell lines respectively. *Steganotaenia araliacea* hochst water extract showed over 60% growth inhibition at 10 µg/ml concentration on HeLa cell line and was further fractionated by solid phase extraction (SPE) to concentrate the active constituents. Fraction 87 of the extract showed the most cytotoxic activity on HeLa cell line and was used to investigate vascularization and tubularization on the 2-D Matrigel cell culture model using the HeLa cell line. The 87 SPE fraction of *S. araliacea* hochst water extract displayed anti-

tubularization mechanisms in the 2-D Matrigel cell culture system at 10 ug/ml in positive (Vincristine), negative control (untreated) and treated wells individually.

Conclusion

The advanced 2-D Matrigel sandwich cell culture model used in this study can be used for high throughput screening for potential anti-metastasis, anti-tubularization agents.

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Chapter 3: The chapter details the methodology used to carry out the study.

Chapter 4: The chapter provides the findings of the study.

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Chapter 1

Introduction

1. Introduction

1.1 Introduction to study

Cancer remains a significant health threat as its rate of incidences continue to increase (Siegel *et al*., 2017; Cabasag *et al*., 2022). This is despite the wide scale of increased awareness of the disease, early diagnosis regiments and novel therapeutic interventions (Petignat & Roy, 2007; Luo *et al*., 2022). In 2020, cervical cancer ranked the fourth most common cause of cancer incidence and mortality in women globally since 2008. (Arbyn *et al*., 2020). The late diagnosis, adverse drug reaction concerns, occurrence of drug resistance, and exorbitant costs of treatment are few of the factors that contribute to increasing rates of mortalities (Rawla, 2019 and Oyenihi & Smith, 2019.

There are key processes involved in metastasis. These include proliferation, differentiation, tubularization and vascularization (Devine *et al*., 2019). Proliferation and differentiation relate to the growth and division of cells. The second pillar of metastasis is tubularization which leads to angiogenesis; the growth of capillaries, vascular tubes, and vessels to transport nutrients, oxygen to cells. The dysfunctional regulation of the two of the four pillars (proliferation and tubularization) increases the chances of metastasis in cervical cancer (Martin & Gurevich, 2021). The current study targeted the two pillars: proliferation and tubularization, as mechanisms for inhibition of neoplasm growth and metastasis.

The number of cervical cancer incidences and deaths necessitate an alternative approach to treatment and management of this disease (LaVigne *et al*., 2017). The approach should include the use of a broad base avenue of conventional and non-conventional treatment regiments such as the use of medicinal plants (Gupta *et al*., 2021). Inclusion of the plant-based regimens for cancer management could accelerate reduction of death rates (Ikram *et al*., 2021). This inclusion, however, requires that the inhibitory effects of medicinal plants on the growth of cancer cells be verified. This study observed the effects of selected medicinal plants extracts on proliferation and tubularization of cervical cancer cells, the two processes essential for metastatic progression. The main aim of the study was to assess the effect of selected medicinal plants extracts on the two processes that contribute to metastasis. The study evaluated the antiproliferative effects of the two medicinal plants: *Boscia albitrunica* and *Steganotaenia araliacea* hochst on monolayer cervical

cancer cell lines, and their effects on the formation of tubular structures necessary for the tubularization process, using a 2-D Matrigel technology.

1.2 Background

Cervical cancer is characterized by abnormal and uncontrollable growth of cells resulting in the formation of malignant tumors in the cervix (Gu & Wen., 2021). It is the most common genital cancer, accounting for approximately 12% of all cancers in women, predominantly in less developed countries such as South Africa (Torre *et al*., 2015).

The National Cancer Registry (NCR), which operates within the National Health Laboratory Service (NHLS), is the primary cancer surveillance system in South Africa, and it maintains the largest repository of cancer data in the country (Cassim *et al*., 2021). The NCR reported 6 181 including 15.05% of all cancers active new cases of cervical cancer in the year 2018. The NCR stated 29.07% of all types of cancers are more prevalent in black females than other female ethnics. The age group with the highest frequency of cervical cancer diagnosis is 45-49.

Reported causations of cervical cancer vary. Arbyn *et al*., 2020 reported that infection with oncogenic human papillomavirus (HPV) types, more commonly HPV 16, is the most vital risk factor in its etiology. A study investigating population-level exposure to risk factors in relation to late-stage cervical cancer found that high risk factors including immune system deficiency, age, and smoking were significant predictors (Dunyo *et al*., 2018).

The importance of early detection and education to promote early treatment increases the chances of successful treatment and survival (Islam *et al*., 2017). Thus far, Southern African countries have embarked of several programs of detection of cervical cancer precursors via opportunistic periodic screenings in schools and high learning institutions. In cases where cervical cancer precursors are detected, they can be removed by ablation or excision in a way to prevent progression to invasive cancer (Denny & Kuhn, 2017).

On a clinical level, the current validated treatment methods such as chemotherapy and radiotherapy have a record of undesirable side effects (Chamseddine & Rejniak., 2020). Thus, the need for future studies to look for safer and efficacious chemotherapeutic agents including those that limit metastasis. This study investigated the use of sandwich 2-D Matrigel cell culture model to screen for potential agents that disrupts processes crucial to angiogenesis and metastasis, the two

processes that contribute to the spread of cancer growth within the body, and subsequently to mortality (Hutchinson & Kirk, 2011; Weng *et al*., 2021).

1.3 Research Problem

The high incidence of death among cervical cancer patients in South Africa is two-fold. The first factor is poor access to proper and accurate screening and treatment services mainly due to limited human and financial resources (Lekota, 2018). The second factor is the high incidence of metastasis in cervical cancer. The process of metastasis is highly dependent on unregulated over-proliferation, tubularization and vascularization of cancer cells, which all promote localized begin tumors to malignancy (Novosel *et al*., 2011; Yan *et al*., 2021). Failure to localize cancerous cell growth is the chief causation of morbidity and mortality in cancer patients (Idachaba *et al*., 2019). The five-year survival rate for metastatic cervical cancer is around 16.5% compared to 91.5% for localized cervical cancer (Ferlay *et al*., 2013). The literature shows that research on cancer focuses primarily on cell proliferation while the cell transportation mechanism aspect of angiogenesis and metastasis have been under investigated (Quintero-Fabián *et al*., 2019; Moore *et al*., 2020; Yan *et al*., 2021; Ahuja *et al*., 2021). There is an urgent call to employ methods that mirror these cellular mechanisms *in vitro*.

The standard cancer treatment regiments such as chemotherapy and radiotherapy have been reported to show high levels of cytotoxicity while tumors often develop drug resistance over time (Islam *et al*., 2017). These limitations necessitate efforts for discovery of safer and efficacious alternative treatments for cervical cancer (Chakraborty, 2019; Khatami *et al*., 2020). Medicinal plants have the potential of discovering new efficacious and less toxic drug candidates. furthermore, the use appropriate drug screening *in vitro* model is necessary to assess not only cell viability, but other processes involved in cancer initiation, progression and metastasis, hence the exploration of the below proposed cell culture model.

The study utilized the 2-D sandwich Matrigel cell culture as a model to study cancer cell proliferation and tubularization processes, which are key to cancer metastasis (Smith *et al*., 2012; Trenti *et al*., 2018; Hackethal *et al*., 2021). The use of such technology is important to the broader understanding of how metastatic cervical cancer traits can be halted before an irreversible prognosis. Therefore, this study investigated the cancer cell anti-proliferative activity as well as the anti-tubularization potential of *Boscia albitrunca* and *Steganotaenia araliacea* hochst.

1.4 Research aim

The aim of the study was to develop and validate a 2-D Matrigel cell culture technology, as a model to investigate inhibitory effects of *Boscia albitrunca* and *Steganotaenia araliacea* hochst on proliferation and tubularization of selected cervical cancer cells.

1.5 Research objectives

- Screen *Boscia albitrunca* and *Steganotaenia araliacea* hochst extracts for their cell anti-proliferative effect on the HeLa and C33A cancer cell lines.
- To identify the classes of secondary metabolites, present in the plants through phytochemical analysis.
- To fractionate using chromatographic techniques that showed anti-proliferative activity at 10µg/ml.
- To investigate the anti-tubularization effects of active fractions on selected cervical cancer cells (HeLa or C33A) using 2-dimensional Matrigel cell culture technology

1.6 Hypothesis

One of the most effective ways to halt metastasis is to disrupt tubular formation (tubularization). The selected plants: *Boscia albitrunca* and *Steganotaenia araliacea* hochst, with known anti-cancer properties and suitable phytochemical compositions, were assessed in this study for their anti-proliferation and anti-angiogenic properties looking at their effects on the formation of tubular-like structures on the HeLa and C33A cell lines.

The study hypothesis was that at least one of the two plants exert anti-cancer activity through interruption of formation of tubular-like structures of cancer cells, and cell proliferation: two processes crucial to the metastatic mechanisms of cancerous cells.

1.7 Motivations

The motivation for this study was to explore how the sandwich 2-D Matrigel cell culture technology can be used as an investigative tool and model to observe the inhibitory effects of selected plant extracts on the abovementioned pillars of metastasis. Effective use of this technology may unlock the discovery of hits or lead anti-cancer agents that target and disrupts these key processes involved in metastasis over and beyond research previously done using monolayer cytotoxic assays.

1.8 Scope of the project

Metastasis consists of several important pillars (proliferation, differentiation, angiogenesis, tubularization and vascularization). The study scope only investigated the *in vitro* processes such as proliferation and tubular formation using the considered cell culture model.

Chapter 2

Literature Review

Literature Review

The literature review discusses several processes that bring about metastasis as an important component of malignant cancer. It elucidates cervical cancer causations and different metastatic stages. The two medicinal plants are discussed, as well as the importance of using 2-D sandwich Matrigel cell culture model to observe tubularization process.

2.1 Introduction

Cancer is defined as the abnormal growth of cells with the potential to proliferate in an uncontrollable manner (Yadav & Mohite, 2020). Some of the hallmark causes of cancer include independence of cells from growth signals, poorly controlled replication, sustained angiogenesis, and capacity to penetrate other tissues (Abbas & Rehman, 2018). The above process is known as metastasis.

2.2 Cervical Cancer

Cervical cancer is the cancer of the cervix initiated in the cells lining the cervix as shown in **Figure 1**.

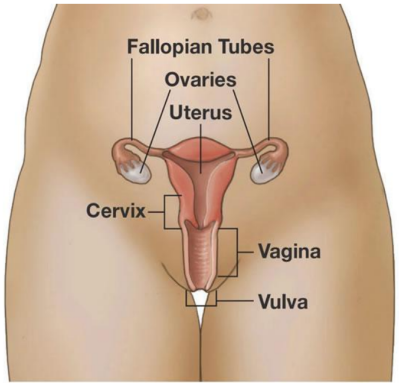

Figure 1: A Pictorial illustration of the female reproductive system (accessed from https://www.cancer.gov/publications/dictionaries/cancer) Denny & Kuhn, 2007.

The disproportional favour in rates of proliferation of cells around the cervix area allows cancerous cells to form tumors (Pillay *et al*., 2017). This rate also allows a faster metastasis of these cancerous cells to other organs in the body (Yang *et al*., 2021).

The disease epidemiology in sub-Saharan African countries show that cervical cancer encompasses 20-30% of all cancers in this region (Simms *et al*., 2019). In South Africa, cervical cancer has the most incidence and is the leading cause of cancer mortality among women of

reproductive age between 15 and 44 years, after breast cancer (Denny & Kuhn, 2017; Akokuwebe *et al*., 2021).

Numerous studies also affirm that referral process for cervical cancer patients is tedious, extremely expensive and weighing on the health systems that is already facing problems with communicable diseases for instance Tuberculosis, Malaria and HIV/AIDS (Lambert *et al*., 2017).

Fig. 2 represents a plot of cervical cancer incidences and mortality across regions in different continents. The latest estimate in the last decade reveal that Southern Africa has very high incidence and mortality rates especially in the early 30s age (Castle *et al*., 2021). The age standardized mortality rate was estimated at 18 per 100 000 women in Southern Africa, compared with 6.8 per 100 000 internationally (Denny & Kuhn, 2017). Most importantly, **Fig. 2** reveals the high incidence and mortality against age-standardized rate per 100,000 than developed regions (Castle *et al*., 2021).

The number of cervical cancer cases in underdeveloped countries continue to increase yearly. Reports from Denny & Kuhn (2017) and Castle *et al*., (2021) stated 4 248 cervical cancer deaths in South Africa that year (2019), with cervical cancer ranking as the first cause of cancer deaths among women of all ages. Current estimates have shown that every year more recently in South Africa, 5 743 women are diagnosed with cervical cancer and 3 027 die from the disease increasingly (Akokuwebe *et al*., 2021).

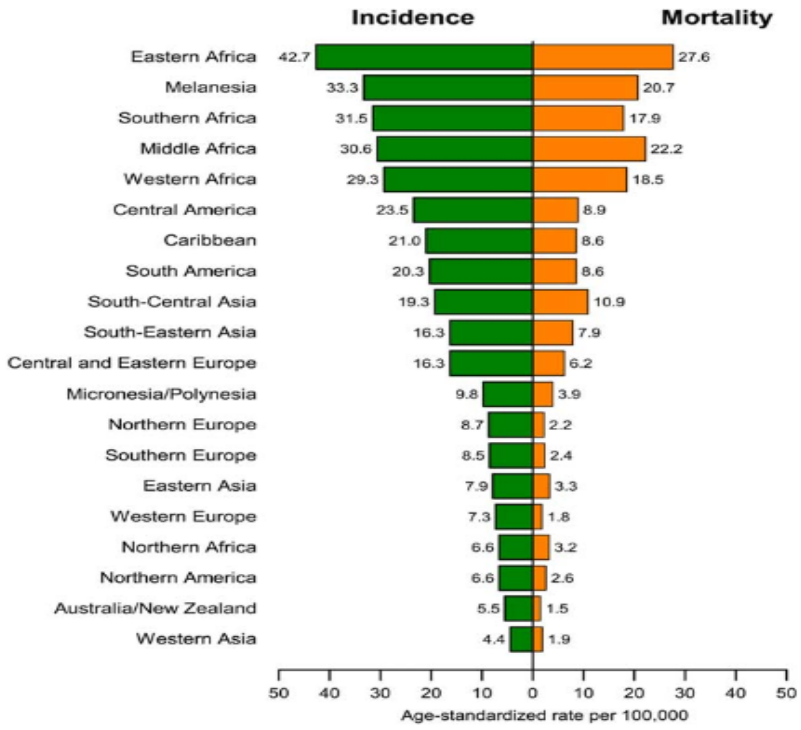

Figure 2: A graphical representation of cervical cancer incidence, and mortality rates across continental regions against age-standardized rate per 100,000 (adapted from Torre *et al*., 2012).

2.3 Causes and detection of cervical cancer

2.3.1 Persistent infections

Human papillomavirus (HPV), particularly types 16, 18 and 45 are recognized as the principal causative agents of cervical cancer and its precursor lesions (Brotherton *et al*., 2017). HPV accounts for about 15% cases of cervical cancers (Potikanond *et al*., 2017). Most infections are asymptomatic and cleared by the immune system within a year (Potikanond *et al*., 2017). However, in up to 10 % of women, the infection can persist, and in a smaller number of women, persistent infection with oncogenic HPV may eventually lead to cervical cancer (Abotchie & Shokar, 2009).

2.3.2 Epigenetic dysregulation and lifestyle behaviors

Genomic instability and tumor suppressor genes silencing contribute to the causation of all cancers including cervical cancer (Chalmers & Wu., 2020; Stahl *et al*., 2016). Several modifiable risk factors for cancers in general include smoking, physical inactivity, obesity, and reproductive behaviors (Jemal *et al*, 2012).

2.3.3 Chronic inflammation

The link between inflammation and cervical cancer is well documented (Bergers & Fendt, 2021). Numerous inflammatory diseases increase the risks of a wide variety of cancers such as cervical cancers (Mantovani *et al*., 2008). Tumors that are epidemiologically unrelated to overt inflammatory conditions often activate oncogenes which orchestrate abnormal production and recruitments of inflammatory cells (Zanconato, *et al*., 2019).

2.3.4 Detection/diagnosis of cervical cancer

Appropriate screening procedures and early detection methodologies for metastatic cervical cancer are therefore necessary. Papanicolaou (Pap) smear test is one of the foremost trusted methods of cervical cancer detection (Koliopoulos *et al*., 2017). This screening procedure is an exfoliative cervicovaginal cytology that investigates the presence of precancerous or cancerous cells on the cervix (Kerkar & Kulkarni, 2006). Lack of use of such screening procedures results in late detection, diagnosis, and poor prognosis. Also, non-frequent screening culture often results in cervical cancer metastasizing rapidly before it can be adequately diagnosed (Lambert *et al*., 2017). Late cervical cancer diagnosis leads to ineffective surgeries, and consequently, the use of other therapies such as chemotherapy and radiation therapy (Petignat & Roy, 2007; Ortiz-Sánchez *et al*., 2016).

2.4 Cervical cancer staging

Cervical cancer progression is measured by allocating stages to state and the extent of the tumor (Devine *et al*., 2019). The staging of cancer gives enlightenment to the size of the cancerous cell and how far the cancer has spread. **Fig. 3** illustrates the four stages of cervical cancer, how chances of survival decrease as the stages increase, and organs first affected by metastasis.

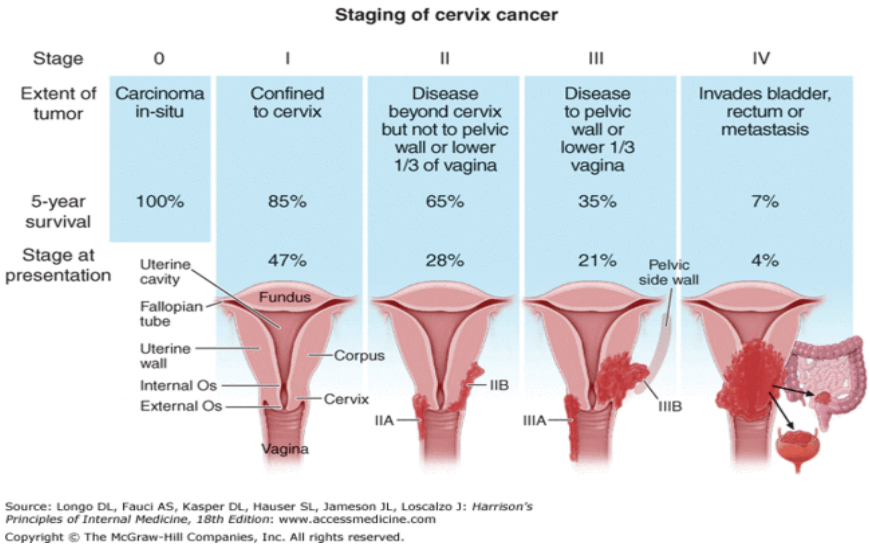

Figure 3: A pictorial representation of the progression of cervical cancer through all 4 stages (Adapted from Longo *et al*., 2013).

Stage 1: Cancer is confined to the cervix and easily treatable if diagnosed early and renders a higher chance of survival (Zhang *et al*., 2019). Stage one can be divided into two parts: 1A and 1B. In stage 1A, the growth can only be observed with a microscope or colposcopy due to its miniature size (Saslow *et al*., 2012). In stage 1B, growth can be seen without a microscope because the cancerous areas are larger, however, the cancer is still only observed in the tissues of the cervix and has not metastasized.

Stage 2: Cancer has not spread to other body organs but is larger in size than stage 1. In stage 2 the cancer spreads to local lymph nodes close to the tumor but is not present in the muscles and ligaments of the pelvic wall or lower vagina. According to Longo *et al*., (2013) at stage two the cancer metastasis extends outside the neck of the womb into the surrounding tissues.

Stage 3: Cervical cancer in stage 3 has metastasized from the cervix and into surrounding structures in the pelvic region (Zhang *et al*., 2019). Stage 3A results from the cancer metastasizing to the lower third of the vagina while Stage 3B occurs when tumor has grown through to the pelvic wall or is blocking one or both tubes that drain the kidneys.

Stage 4: Cervical cancer is highly advanced and has spread to one or more organs in the body outside the cervix (secondary metastasis) (Longo *et al*., 2013). By this stage the cancer has spread to nearby and farther organs such as the bladder, rectum, pancreas, and lungs.

2.5 Metastatic mechanisms

This study focused on evaluating and assessing how the effects of selected plant extracts on proliferation and tubular formation affect metastasis. Any lead compounds that disrupt the two processes individually or collectively are crucial in halting metastatic capabilities. As a result, it is imperative to understand and recognize the mechanisms, mode of action and importance of cell proliferation and tubular formation to metastasis.

The importance of metastasis is signified by how mortality is often related to the metastatic disease rather than primary tumor (Aziz, & Aziz, 2017). It is important to discuss and understand the metastatic process as it significantly impacts prognosis. The mechanisms of metastasis can be categorized into three processes: invasion, intravasation and extravasation (Yang *et al*., 2021). Invasion is the dissociation of malignant tumors from primary tumor mass, allowing the cells to invade the surrounding stroma (Mbele *et al*, 2017; Welch & Hurst, 2019). The invasion process is followed by intravasation, which is the movement of blood vessels within tumor areas to provide a way for the detachment of cells to circulatory system and metastasis to other sites (Mbele *et al*, 2017; Majidpoor & Mortezaee, 2021).

Intravasation is followed by the interaction between the tumor cells and the stroma in the development of angiogenesis (Zuazo-Gaztelu & Casanovas, 2018). Extravasation process is the development of adhesion to endothelial cell monolayer and the basement membrane of the blood vessel endothelium and migrating into the underlying interstitial extracellular matrix. (Peng *et al*., 2017). The settled tumor is then able to proliferate at the secondary location (Mbele *et al*, 2017; Welch & Hurst, 2019). The three processes discussed above play key roles in enabling and sustaining the overall metastatic process.

2.5.1 Proliferation

Cell proliferation of normal cells is the increase in cell numbers resulting from cell division. The increase in cell numbers is important for tissue formation, wound healing, and replenishment of lost/damaged cells (Post & Clevers, 2019). This process has countless advantages, but it requires multipronged levels of regulation.

The process of cell proliferation is tightly controlled to prevent excessive proliferation through sustained proliferative signaling, controlled gene expressions and growth suppressors (Srdanovic

et al., 2022). The tight control of cell growth and division is necessary to maintain cellular homeostasis *in vivo* and *in vitro* (Luo *et al*., 2021). The pathologic consequences of uncontrolled cell growth and proliferation are required for increased tubularization and metastatic properties to be observed (Srdanovic *et al*., 2022).

2.5.2 Tubularization

Tubularization can be best described as the process of the formation of tubes. During the observation of this process under a microscope, a network of tubular like structures can be seen. These tubes allow for oxygen supply and nutrients to flow from one cell to another which then forms a network structure of cells.

Under optimal conditions, (the endothelial cells combined with conditioned media and plated on a basement membrane extract) tubular formation can occur promptly and can be easily quantified.

2.5.3 Angiogenesis

Solid tumors are usually part of normal tissues, and under optimal conditions, can attack neighbouring tissues or pass out through the connective system to colonize distant sites in the body i.e., the cervix (Montaigne, 2020). These secondary tumors (metastases) are the main cause for almost 90% of the deaths caused by cancer (Garner & Visser, 2020). This capability of tumor cells to attack and metastasize is the final of the six hallmarks of cancer: unlimited replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitors, ability to develop blood vessels, evasion of programmed cell death, and tissue invasion and metastasis (Mantovani, 2009; Garner & Visser, 2020).

2.5.4 Metastasis

Although metastasis accounts for most cervical cancer related deaths, this intricate process remains the least understood aspect of cancer biology (Lambert *et al*., 2017). During this complex and highly selective process, malignant tumor cells leave the primary initial site and disseminate by blood or lymph vessels. Not all cancerous cells metastasize as this highly selective process requires specific intrinsic properties in the tumor cells and well-suited tumor microenvironment (Bergers & Fendt, 2021).

Localized cervical cancer is an easier task for treatment due to direct and concise drug delivery mechanisms (Kurmi *et al*., 2020). This study presents the opportunity to understand and carry out

in vitro 2-D Matrigel cell culture model to observe the effects of selected ethno-pharmacology approach extracts on elements essential to metastatic progression.

2.6 Medicinal Plants

The two plants which were identified to have the cytotoxic activity are discussed below. The importance of such medicinal plants in traditional medicine, as well as their origins, description and historical uses are discussed.

2.6.1 Importance of Medicinal Plants

Natural products from medicinal plants provide important sources of potential pharmaceutical "lead" compounds and novel drugs (Berkovich *et al*., 2013). Plant based anti-tumor agents such as Vinblastine and Vincristine are useful in treating cervical cancer patients according to Berkovich *et al*., 2013. The two drugs prevent cell division during mitosis (anaphase) by binding to the microtubular proteins of the mitotic spindle. Some plant-based agents are in high demand due to their low toxicity effects on healthy and cytotoxic effects on cancerous cells (Greenwell & Rahman, 2015).

Plants Investigated

The two medicinal plants (*S. araliacea* hochst and *B. albitrunca*) have cytotoxic activities (Solyomváry *et al*., 2017; Maroyi, 2019). Both plants have been used for anticancer activity however have not been investigated for activity against cervical cancer specifically (Kudamba., *et al*; Maroyi, 2019).

2.6.2.1.1 *Boscia albitrunca*

Boscia albitrunca is commonly referred to as shepherd's tree (English) and Umvithi (IsiZulu). This medium-sized tree reaches heights of 7 - 10 meters and has an attractive dense, round to spreading crown as shown in **Fig. 4**. The trunk is noticeably smooth and white or whitish grey with bare stems (Lotter M, 2002).

Figure 4: A pictorial identification of *Boscia albitrunca* (Umvithi). Image accessed from PlantZAfrica.com.

2.6.2.1.2 Plant distribution

Boscia albitrunca can be found in the drier parts of Southern Africa, in areas of low rainfall such as Botswana, Limpopo, Gauteng, North-West, Swaziland, the Free State, Northern Cape, and KwaZulu-Natal. This distribution range extends into Zambia, Zimbabwe, and Mozambique.

2.6.2.1.3 Medical uses

The leaves and roots are known to be used as herbal medicines for gastrointestinal disorders (stomachaches, diarrhea, and dysentery), respiratory conditions (coughs, bronchitis, and asthma), wound healing (wounds, cuts, and sores), pain relief (headache and body aches), fever and malaria (Maroyi, 2019). In northern Africa, the roots are used to treat Gonorrhea infections (Lotter, 2002).

2.6.2.1.4 Phytochemical composition

Boscia albitrunica is primarily composed of alkaloids, tannins, flavonoids, and phenolic constituents that have been isolated from the plant's anatomy (Pendota *et al*., 2015; Maroyi, 2019).

2.6.2.1.5 Pharmacological activity

The inclusion of this plant is necessary to study how this selected plant extract of known alkaloids reports antiproliferative and cytotoxic actions (Lotter, 2002).

2.6.2.2.1 *Steganotaenia araliacea* hochst

Steganotaenia araliacea hochst is known as carrot tree (English) and Umbonjana (IsiZulu). The carrot tree is a small, aromatic, and deciduous tree with the potential of growing between 2-7 meters tall. The leaves are pinnate with 3-5 pairs of serrate leaflets with a terminal one as shown in **Fig 5**. It produces tiny white flowers in compound umbels in dry seasons. The fruits are small, flat and double winged (Alemika *et al*., 2004).

Figure 5: A pictorial identification of *Steganotaenia araliacea* hochst (Umbonjana). Adapted from Raimondo *et al*, 2009.

2.6.2.2.2 Plant distribution

This plant is found between dry to moist tropics (near sea level to over 1 800 meters). It is a remarkably drought-resistant plant but can also be grown in areas of high rainfall when the soil is well drained. It has been reported in Limpopo, Mpumalanga, Zimbabwe and as far north as Ethiopia.

2.6.2.2.3 Medical uses

The stem-bark contains several dibenzocyclo-octadiene lignans. These have displayed cytotoxic (antimitotic) activity as reported in colchicine on 11 human tumor cell lines (Orwa *et al*., 2009). Orwa *et al*, 2009 has also reported that saponins isolated from the leaves have shown anti-leukemic activity.

The roots are used to treat snake bites, painful chest conditions, sore throat and swelling caused by allergies. The bark is chewed as a treatment for fever and is used in preparing a medication for heart complications (Orwa *et al*., 2009). The plant also has diuretic properties (Kayombo, 2016). A decoction, prepared by boiling the bark for at least one hour, is added to milk and administered orally to adults as a remedy for digestive disorders such as indigestion, flatulence, and abdominal pain.

2.6.2.2.4 Phytochemical composition

Phenols and alkaloids have been isolated from *S. araliacea* hochst in previous research (Taafrout *et al*., 1984; Solyomváry *et al*., 2017).

2.6.2.2.5 Pharmacological activity

Cytotoxicity of *S. araliacea* hochst extract against several cancer cell lines [MDA-MB-231 (breast), PANC - 1 (pancreas) and HT - 29 (colon)] was reported by Capistrano *et al*., 2015. While *Steganotaenia araliacea* hochst has a long history of traditional use, there is limited scientific research available on its pharmacological properties and medicinal benefits.

This was the basis for their selection for this study as previous studies have only screened the plants for growth inhibition of cancer cells using monolayer cytotoxic assays. The selected metastasis process of tabularization can only be studied using 2-dimensional models to allow formation and visualization of tubular-like structures. The study designs and uses the two-dimensional sandwich Matrigel cell culture in *vitro* model to study. The proposed model is discussed below.

2.7 Two-Dimensional Matrigel Cell Culture Model

A typical 2-D cell culture model, the cells adhere to an artificial plastic or glass substrate and are in contact with other cells only at their periphery. Cell cultures provide a suitable *in vitro* model for studying the standard physiology and biochemistry of cells; the effects of drugs and toxic compounds on the cells, carcinogenesis; in drug screening and development (Antoni *et al*., 2015). This model allows for consistent and reproducible results. It is one of the crucial tools used in cellular and molecular biology (Antoni *et al*., 2015).

Two-dimensional cell culture models are important tools for providing some predictions of drug activity, *in vivo* toxicity and drug responses for many targets and pathways and are still very useful in drug discovery (Maltman & Przyborski, 2010). This study used a novel sandwich 2-D Matrigel cell culture model to better emulate a particular aspect of cells, in an *in vivo* environment. Compared to the previous flat and single layer cells grown a single dimension plastic.

The 2-D sandwich Matrigel cell culture model provides artificial environment containing growth medium (Matrigel) that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, gases (O_2 and CO_2) and regulates the physio-chemical environment (pH, osmotic pressure, temperature) in which cells can grow (Antoni *et al*., 2015). Establishment of a sandwich 2-D Matrigel cell culture model will increase natural intercellular contact and communication (Fröhlich, 2018; Foglietta *et al*., 2020).

Innovation of effective and unique viable cell culture dimensions is important for the advancement of cell culture models (Saleh & Genever, 2011). Progression from single monolayer models to sandwich 2-D Matrigel models is motivated by the need to work with cellular models that mimic the functions of living tissues as traditional 2-D cell cultures are used to perform *in vitro* research and the greatest challenge being getting it as close as possible to the *in vivo* state (Antoni *et al*., 2015).

Sandwich 2-D Matrigel model grown cells could prove to be more physiologically relevant and positively assist various biological mechanisms investigating viability, proliferation, differentiation, migration, response to stimuli, and invasion of tumor cells into surrounding tissues, angiogenesis stimulation and general cell function and *in vivo* relevance (Manini *et al*., 2018). The use of the 2-D cell culture model in this study has advanced focus into *in vitro* Matrigel cell cultures research in the future. The aim was to produce an environment that nears towards natural biological bodily functions and processes. Methodology in this research is highly significant to ensure that the standard protocol can be followed again in future. The following chapter will outline and expand on how each experiment was conducted and create a pathway for standardization and reproducibility.

Chapter 3

Materials and methods

3. Materials and methods

This chapter provides methodologies followed for plant collection and extraction, phytochemical analysis, fractionation of an extract of interest to obtain concentrates of active phytochemical constituents, and cytotoxicity evaluation of extracts using assays based on mono-layer cell culture and on the 2-D Matrigel model.

3.1 Samples collection

The two medicinal plants; *Boscia albitrunca* and *Steganotaenia araliacea* hochst, were collected in November 2018 from local market in Durban, Kwa-Zulu Natal (South Africa). Both plants were authenticated by a Botanist at the University of the Free State. Specimen voucher numbers were not issued as plants were not collected from the wild. Upon arrival at the laboratories, the plants were washed, rinsed thoroughly and air dried under dark conditions at room temperature for 2 weeks.

3.2 Extraction and solvent partitioning of crude extract

For extraction, maceration method was used in this study as adapted from Azwanida (2015). The plant stems were ground to fine powder using a grounder. Finely grounded materials were weighed prior to extraction (10 g each), then extracted with water. Another 10 g of plant material was extracted sequentially with organic solvents in their increasing order of polarity, starting with hexane, dichloromethane, ethyl acetate and methanol. The mixtures were left on the orbital shaker for 48 hours, filtered with filter paper, and filtrates were dried under vacuum using rotary evaporator (model: SHIMADZU QR 2005-S). Aqueous filtrates were concentrated in a freeze dryer (Freeze Dryer Tabletop: BK-FD Model). Concentrates were aliquoted in pre-weighed vials and left under a fume hood for further drying. Dry extracts were weighed, and percentage yields were determined.

3.3 Cell culture

The cell culture environment was maintained in a CO_2 incubator at 37°C, 5% CO_2 and 95% relative humidity. The growth medium Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µ/ml penicillin and 10 µg/mL Streptomycin, was used to grow the cells. Doxorubicin was used as the standard drug. The cells were grown until 80% confluence was reached (Freshney, 2015).

3.4 Cell lines

3.4.1 HeLa cell line

HeLa cell line was commercially obtained from Cellonex company, South Africa. HeLa is the first human "cell line" to be established in culture. It has a vast historical use in cervical cancer studies such as the investigation of the molecular mechanism of paclitaxel in regulating proliferation and apoptosis of cervical cancer Hela cells (Gu *et al*., 2017). Additionally, HeLa cell line was successfully used in two cervical cancer studies prior this study, by El-Readi *et al*., 2013 and Yu *et al*, 2007, which support addition of this cell line to study.

3.4.2 C33A cell line

The C33A cell line was obtained from Cellonex, South Africa. This cell line has been immensely useful in the investigation of proliferation and apoptosis of cervical cancer cells studies in previous literature such as in García-Zepeda *et al*, 2013. The C33A cell line has also been used in the investigation of the role of interleukin-6 in cervical cancer tumor growth by Vascular Endothelial Growth Factor (VEGF) dependent angiogenesis (Salven *et al*., 1998; Wei *et al*., 2003; Khan & Bicknell, 2016).

3.4.3 Thawing of cells

Cells were obtained frozen in cryovials. The vials were stored in liquid nitrogen until further use. On the today of use, vials were suspended carefully in a 37˚C water bath for 2 minutes, ensuring the water does not reach the mouth of the cap. The aliquots were transfer to a pre-warmed flask (37˚C) containing thawing media (DMEM + 20% FBS). Flasks were incubated for 24 hours, followed by a change of media every 24 hours until cells reached 80% confluency. At 80% confluency, cells were exposed to trypsin, counted, sub-divided and cultured or used in an experiment.

3.4.4 Cell viability count

Aliquots of 20 µl of cell suspension was mixed with 20 µl of trypan blue staining solution in an Eppendorf (1:1 ratio). From which 20 µl was pipetted onto a microscopic slide and counted using Countess™ 3 Automated Cell Counter (LCD 1280 x 800 pixels). The viable (live) and dead cell concentrations were obtained.

Medium was prepared for a 96 well plate by preparing 10 ml of medium constituting of 9 ml (DMEM) + 1 ml (FBS). Cells were kept at 37 ˚C in a humidified environment in a 5% CO_2

incubator. The cells were sub-cultured at 90% confluency and subsequently observed under a microscope, the cells were washed with PBS and harvested using trypsin. The concentration of viable cells was evaluated using trypan blue dye under an automated cell counter (Countess FI, life technology). A total of $1x10^5$ cells/ml were seeded into each well in a 96-well microtiter plate and incubated under the same conditions for 24 hours.

3.4.5 Addition of drug/Treatment

Dry extracts were weighed (10 mg) and 500 µl of 100% $DMSO_4$ was added and sonicated, to make a stock solution of 2 mg/ml. Three-fold serial dilutions of 100, 10 and 1 µg/ml were prepared in culture media. Following which, 100 µl was added to treatment wells and another 100 µl of fresh complete medium was added. The first row of the 96-well plate was a blank (200 µl medium only), second row was the positive control (100 µl of cell suspension and 100 µl of medium), and the three dilutions of the extracts, and the standard drugs were added from the 3rd column onwards in triplicates. The plate was then incubated for 48 hours.

3.4.5 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Anti-proliferative effect was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Kumar *et al*., 2013). Following a 48-hour incubation of cells exposed to test samples, 25 µl of MTT (5 mg/ml) was added to the wells followed by a 4-hour incubation. Subsequent to this, the supernatant was removed and 100 µl of $DMSO_4$ was added. The crystals at the bottom of the plate were dissolved gently in $DMSO_4$. Absorbance was read at 540 nm using the Multiskan GO Plate Reader to obtain optical density values. Below formular is used to calculate IC_{50}.

$$IC_{50}/K_I = 1 + [S]/K_m$$

3.5 Thin layer chromatography

Thin Layer Chromatography (TLC) is used to separate mixtures (Kumar *et al*., 2013). Phytochemical compositions of extracts were profiled using TLC. TLC Silica gel aluminum plates (8 cm x 10 cm) were used as stationary phase, while for mobile phase, three different solvent systems: Toluene-acetone (8:2) (v/v) for non-polar solvent, Toluene-chloroform-acetone (40:25:35) for semi-polar solvents and n-butanol-glacial acetic acid-water (50:10:40) for polar solvents were used in elution. Different compounds in the sample mixture travelled up the TLC plate at varying rates due to the differences in their attraction to the stationary phase and because

of differences in solubility in the solvent system (mobile phase) used (Mangold & Stahl, 1969). The separation of components is measured by the retention factor (R_f) values (Kumar *et al*., 2013), which the distance compounds travel relative to the top marked distance.

Extracts of *Boscia albitrunca* and *Steganotaenia araliacea* hochst were spotted on a stationary phase, 1 cm from the bottom of the plate. Plates were put in mobile phase tank with only the bottom (less than 1 cm) immersed in mobile phase) and allowed to develop. The plates were taken out when mobile phase reached 1 cm from the top of the plate, air-dried, and then viewed under UV light and fluorescence.

Retention factors were calculated using the following formula (Gyawali *et al*., 2020):

$$R_f \text{ value} = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the mobile phase}}$$

The greater the R_f value of a compound, the longer the distance travelled on the TLC plate. When comparing two dissimilar compounds run under identical chromatographic conditions, the compound with the larger R_f value is less polar as it interacts less strongly with the polar absorbance on the TLC plate. Conversely, if the structures of the compounds in a mixture are known, a predict made that a compound of low polarity will have a larger R_f value than a polar compound run on the same plate.

3.6 Fractionation by column chromatography

A long glass column was attached to a glass column holder to provide stability and support. The tip of the column was tightly packed with cotton wool to act as a filter. For the stationary phase of the column, thirty grams (30 g) of silica gel was mixed with 100 ml hexane in a flask to make a slurry.

The slurry was quickly poured into the glass column and left to settle. The sides of the column were rinsed with the hexane to rinse remnants of silica gel.

Steganotaenia araliacea hochst and *B. albitrunca* were reconstituted separately in 10 ml of hexane and gently mixed. The mixture was poured gently into the column using a glass pipette above the stationary phase, cautiously to avoid unsettling the stationary phase.

The mobile phase was prepared using Chloroform and Methanol (7:3, v/v). Mobile phase was poured gently into the column and consistently checked to prevent it from running dry. An elution rate of 45 drops/min was counted. Twenty milliliters (20 ml) of eluent were collected for each for each test tube. Ninety-four (94) test tubes were collected in total at 10-minute intervals.

Ninety-four eluents were collected from the column chromatography and spotted on TLC plates to identify their purity eluents that displayed a similar separation pattern on TLC were grouped together and combined. A total of 5 fractions were separated and collected.

3.7 Two-Dimensional cell culture model

This model was used to study anti-tubularization patterns in cells when treated with different test samples. The assay was performed according to Swift., *et al* 2010 and Yamasaki., *et al* 2020 with slight modifications. A six well plate was pre-coated with (2 g collagen in 12 ml distilled water, shaken until fully dissolved, 2 ml in each well) and the coated plate placed in sterile hood overnight.

Immediately before use, the fresh DMEM (2 ml) was added to each well to neutralize the collagen. The plate was placed in -20 freezer for 1 hour to ensure ice-cold neutralized collagen solution, then left in sterile hood for 15 minutes to thaw but remain cold.

Three tests were performed: positive control, negative control, and experimental samples (active plant extracts). The positive control, well 1, were untreated cells to observe undisturbed cell proliferation. The negative control, well 2, was treated with a standard drug (Vincristine sulfate) to observe anti - tubularization patterns. Lastly, well 3, was treated with the selected active plant fraction that showed anti-proliferative activity against cell monolayer growth when MTT assay was used (*S. araliacea* hochst active extract).

About 0.5 ml of treatment (standard drug and active extract 10 ug/ml) were administered into wells 2 and 3 respectively. The freshly coated dishes were left in a humidified incubator at 37°C for one hour to allow the test samples to settle well in the coating until mix well.

Corning® Matrigel matrix (100 µl) concentration 10 mg/ml was added to each well and incubated (37°C) for 30 minutes. Cells were then seeded at 1000 cells/well in each well and allowed 12 hours in the incubator at 37°C to attach. After attachment, cells were covered with a second layer of Matrigel, therefore creating a sandwich.

The following day cells were observed to ensure Matrigel sandwich was established and then cultured for 7 days. The cell growth was viewed under Leica microsystems CMS GmbH® microscope at x 20 magnification. The medium (2 ml) was replaced after every 2 days (day 1, 3, 5 and 7) after microscope viewing to maintain nourished environment in each well.

3.8 Phytochemical analysis

Phytochemical analysis is a qualitative method performed to test the presence or absence of secondary plant metabolites (Yadav and Agarwala, 2011). These are classes of chemical compounds responsible for the biological properties such as antioxidant activity, antimicrobial effect, and anti-cancer properties of plants (Pereira *et al*., 2009; Saxena *et al*., 2013).

It is crucial to conduct phytochemical screenings of *S. araliacea* hochst and *B. albitrunica* plant extracts to understand their phytochemical composition and to reveal the active principle by isolation and characterization of their constituents (Saxena *et al*., 2013).

Below are the phytochemical screening tests performed:

Determination of alkaloids

To investigate presence of alkaloids about 2 ml of 1% hydrochloric acid was added to 0.2 g of powdered plant sample. Moreover 1 ml of Meyer's reagent followed by 1 ml of Drangendorff reagent were added to the mixture. An appearance of an organic precipitate indicated the presence of alkaloids.

Determination of anthroquines

Approximately 12 ml of 10% hydrochloric solution was added to the 1 g of powdered plant material then boiled for 5 minutes. After cooling the mixture was filtered. Additionally, 10 ml of chloroform was added to the filtrate. The chloroform layer formed was pipetted into a second test tube. About 10 ml of ammonia solution was added to the second test tube. The mixture was shaken. The formation of a rose-pink colour on the top layer indicated presence of anthroquines.

Determination of flavonoids

In the analysis of flavonoids, 2 ml of 1% hydrochloric acid was added to the 0.2 g powdered plant sample then heated for 3 minutes. After cooling the mixture was filtered. Furthermore, 1ml of dilute ammonia solution was added to 5 ml of the filtrate. A yellow precipitate was formed, indicating presence of flavonoids.

Determination of glycosides

In investigating glycosides, 2 ml of acetic acid was added to the 0.5 g plant sample. The mixture was then treated with 1 drop of 0.1% of ferric chloride. Following that, 1 ml concentrated sulfuric acid cautiously added to the mixture. An appearance of a brown ring was indicative of the presence of deoxy sugars found in glycosides.

Determination of pentose

Approximately 40 ml of distilled water was added to 2 g of powdered plant sample and filtered. The 2 ml filtrate was extracted and added into a second test tube. Then 2 ml of HCL + negligible amount of phloroglucinol was added into the second test tube. The mixture was heated for 5 minutes. The formation of a red color following the heating indicated of the presence of pentose.

Determination of phytosterols

About 10 ml of chloroform was added to the weighed 0.1 g powdered plant sample. Thereafter, 1 ml of concentrated H_2SO_4 was cautiously added from the side of the test tubes to 0.5 ml of the chloroform extract. An appearance of a reddish-brown color in the chloroform layer indicated of the presence of phytosterols.

Determination of saponins

About 5 ml of distilled water was added to 0.5 g plant sample, mixture was boiled then filtered. Around 3 ml of distilled water was added to the filtrate. The mixture was shaken vigorously for a period of 5 minutes. Frosting was indicative of the presence of saponins.

Determination of tannins

Administration of 20 ml distilled water was added to 0.5 g plant sample. The mixture was boiled and filtered while still warm. Three (3) drops of ferric chloride were added to the filtrate. A blue-black precipitate was indicative of the presence of tannins.

Determination of triterpenoids

In determining triterpenoids, 1 ml of chloroform and 3 ml of concentrated sulphuric acid was added to 2 mg of powdered plant material. The formation of an interface with a reddish-brown coloration indicated the presence of triterpenoids.

3.9 Statistical Analysis

The experiments were done in triplicates. The cell growth inhibition activities and cytotoxic effects of the plant extracts reported as IC_{50} values. The mean value and standard deviations were calculated and presented in a graphic format. Additional tests on more dilutions were analyzed

using non-linear regression from Graph-Pad Prism software version 8.0 (Graph-Pad Software, Inc., San Diego, USA). Plant samples that did not undergo full dilution were reported using the Microsoft Excel software, where data was also represented as standard error of mean.

Chapter 4

Results

4.0 Results

This chapter presents all results from the experiments carried out in this study. Sections 4.1-3 outlines outcomes of the extraction process, screening of *Boscia albitrunca* and *Steganotaenia araliacea* hochst extracts on the HeLa and C33A cancer cell lines, and the phytochemical analysis. Extracts with considerable cell culture inhibitory results were profiled and fractionated via the use of chromatographic techniques in preparation for *in vitro* 2-D Matrigel cell culture.

4.1 Percentage yields

Water, and four organic solvents; Hexane, DCM, Methanol and Ethyl acetate, were used in the extraction process: Following extraction, extracts of both plants (*S. araliacea* hochst and *B. albitrunica*) were weighed in grams as shown in **Table 1**, and percentage yields calculated.

Table 1: Percentage yields of *S. araliacea* hochst and *B. albitrunica* from the four organic solvents.

Plant Sample	Dried powder (g)	Solvent	Dried extract (g)	% Yield
Steganotaenia araliacea hochst (Umbonjana)	10.03	Hexane	0.10	1.00
		DCM	0.01	0.10
		MeOH	0.30	3.00
		EtOAc	0.02	0.20
Boscia albitrunca (Umvithi)	9.89	Hexane	0.05	0.51
		DCM	0.03	0.30
		MeOH	1.91	19.31
		EtOAc	0.01	0.10

[1]

Percentage yields were observed in methanol solvent in both plants displaying increased percentage yields of 3.00% and 19.31%, respectively. The percentage yields observed across the two plants varied between 0.1% and the outlier *B. albitrunca* methanol extract of 19.31%.

Table 2: Percentage yield of inorganic (water) extracts of *S. araliacea hochst* and *B. albitrunca*.

[1] DCM - Dichloromethane; MeOH - Methanol; EtOAc – Ethyl Acetate

Plant Sample	Dried powder (g)	Solvent	Dried extract (g)	% Yield
S. A. hochst (Umbonjana)	10.03	Water	0.07	0.70
B. albitrunca (Umvithi)	9.89	Water	0.92	9.30

Table 2 above illustrates the percentage yields recorded for the water plant extracts of *S. araliacea* hochst and *B. albitrunca*. *B. albitrunca* results showed a higher percentage yield of 9.30% while S. *araliacea* hochst showed a lower percentage yield 0.70%.

4.2 Phytochemical analysis

The phytochemical characteristics of the two medicinal plants investigated are summarized in **Table 3**. The images of the phytochemical testing outcomes are shown in Annexures A-C.

Table 3: Tabular summary of phytochemical constituents of both medicinal plants studied: *S araliacea* hochst and *B. albitrunca.*

Phytochemicals	*S. araliacea* hochst	*B. albitrunca*
Alkaloids	+	+
Anthraquinones	+	+
Flavonoids	+	+
Glycosides	+	-
Pentose	+	-
Phytosterols	+	+
Saponins	-	-
Tannins	+	-
Triterpenoids	+	-

[2]

Steganotaenia araliacea hochst showed presence of phytochemicals for all constituents tested except saponins. Only alkaloids, anthraquinones, flavonoids and phytosterols were present in *B. albitrunca*. Interestingly, saponins were not present in either plant differing with recently

[2] The presence of the specified phytochemical in the plants can be identified by the (+ or ++) sign while the absence can be identified by the (-) sign.

published studies showing presence of the constituent in both plant extracts (Bitrus *et al*., 2022; Shatri and Mumbengegwi, 2022).

Phytochemical activity in plant extracts is deeply linked with extracts capacity for cell growth inhibition. The cell growth inhibition capabilities of both plants are demonstrated in the **Fig. 6-14** below which were repeated in triplicates (n=3).

4.3 Cell culture

The below cell growth inhibition experiments were performed in triplicates (n=3) to observe consistency. It is very important to note that 50% inhibition is the benchmark for activity. An extract is considered active if it reaches +50% inhibition.

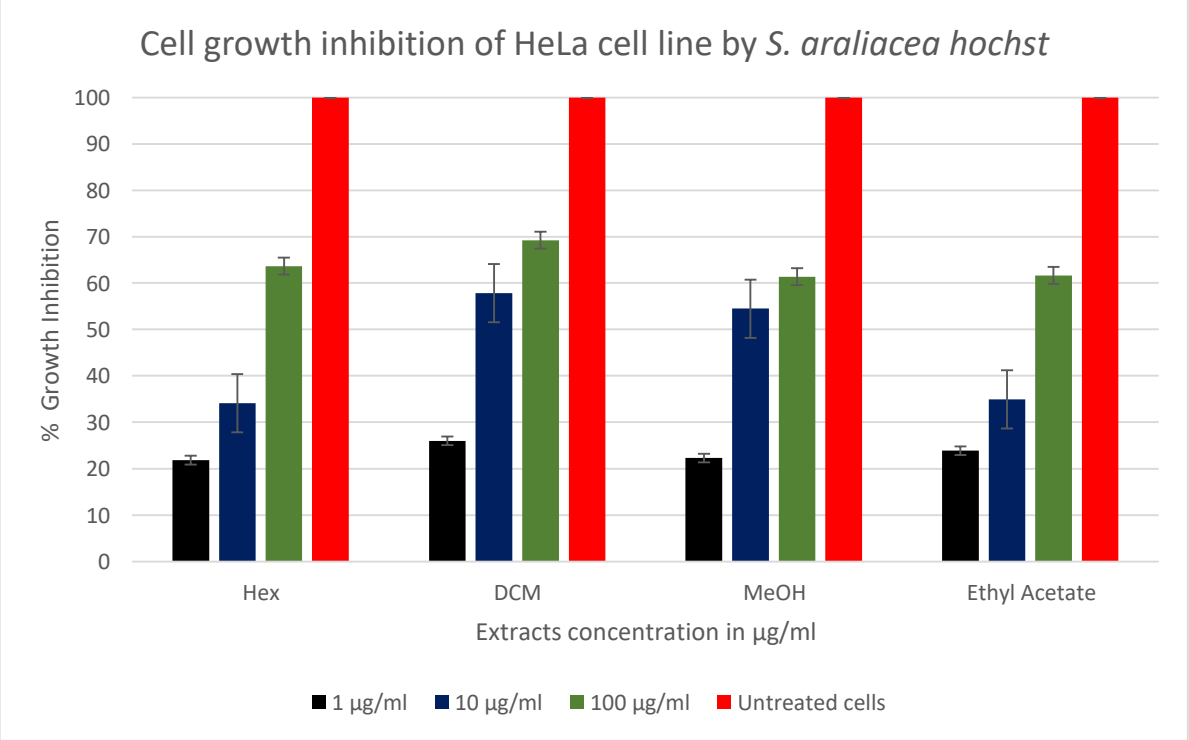

Figure 6: Cell growth inhibition of HeLa cell line by *S. araliacea* hochst extracts at varied concentrations of 1, 10 and 100 µg/ml.

Fig. 6 Results exhibits the cell growth inhibition effects of *S. araliacea* hochst extracts; all 100 µg/ml concentrations (green) showed over 60% cell growth inhibition with the DCM extract reaching the highest inhibition of 69%. DCM and Methanol illustration graphs show that extracts reached over 50% inhibition at 10µg/ml.

It is also evident from figure 6 that 1 µg/ml concentrations showed low activity with below 30% cell growth inhibition at1 µg/ml.

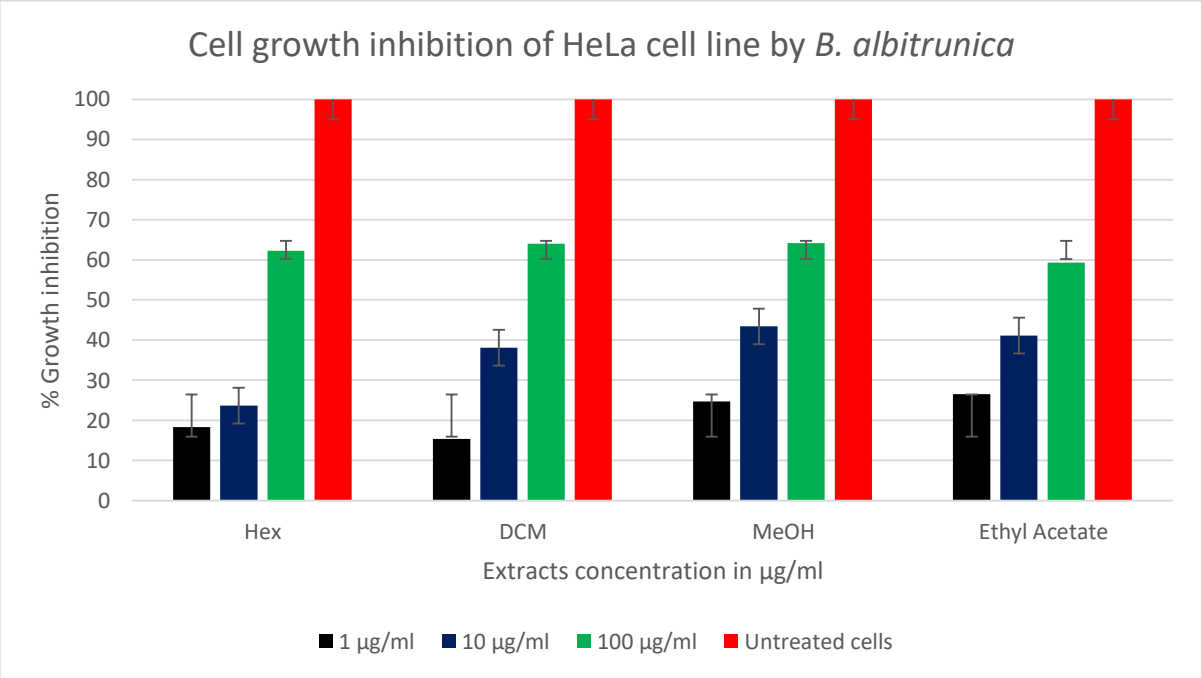

Figure 7: Cell growth inhibition percentage of *B. albitrunca* against a HeLa cell line for the four mentioned solvents: Hexane, DCM, methanol, and ethyl acetate at varied concentrations of 1, 10 and 100 µg/ml.

Fig. 7 above displays 60% cell growth inhibition at 100 µg/ml concentrations by all four extracts. DCM, methanol, and ethyl acetate extracts all demonstrate a similar cell growth inhibition of ~ 40% at 10 µg/ml drug concentration. Evidently all extracts at 1 µg/ml drug concentrations showed less than 30% cell growth inhibition.

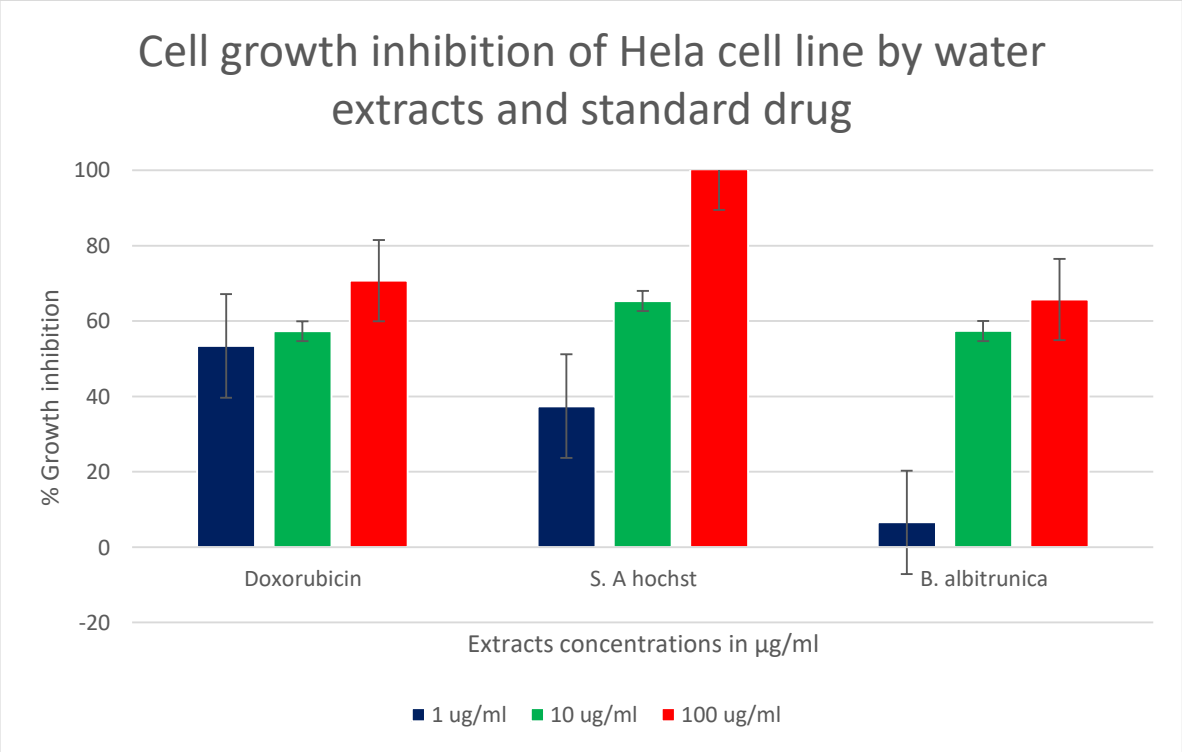

Figure 8: Cell growth inhibition of HeLa cell line by the two water extracts and standard drug (Doxorubicin) at varied concentrations of 1, 10 and 100 µg/ml.

Fig. 8 illustrate that the *S. araliacea* hochst water extract showed cell growth inhibition at concentrations of 10 and 100 µg/ml. Noteworthy, is the above 60% cell growth inhibition of the *S. araliacea* hochst water extract at 10 µg/ml concentration while *B. albitrunca* also showed nearly 60% inhibition at the same concentration.

Second experiments were performed, whereby more dilutions of the test samples were screened (see **Fig. 9** below). This result was in contrast to the results obtained in **Fig. 8** which did not illustrate 100% growth inhibition. In these experiments, the standard drug, Doxorubicin showed a general linear increase of cell growth inhibition percentage between 0.5 to 1.5 Log concentration.

To convert a concentration from logarithmic units to micrograms per milliliter (ug/ml), the below formula can be used: Concentration (ug/ml) = 10^ (log concentration). Therefore 1.5 log concentration is equivalent to 31.62 µg/ml.

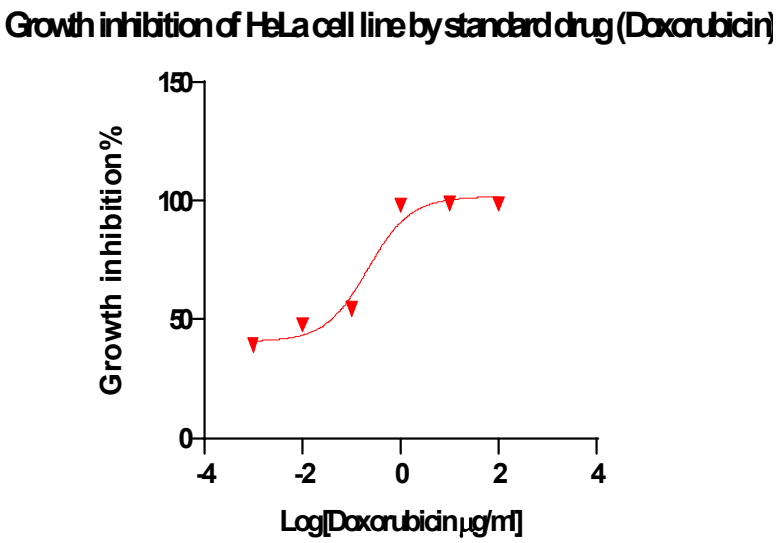

Figure 9: The cell growth inhibition of HeLa cell line by Doxorubicin is recorded showing the LOG of drug concentrations between -4 to 4.

The extracts that showed over 50% inhibition at a concentration of 10 µg/ml were selected, diluted to 6 dilutions, and screened for growth inhibition.

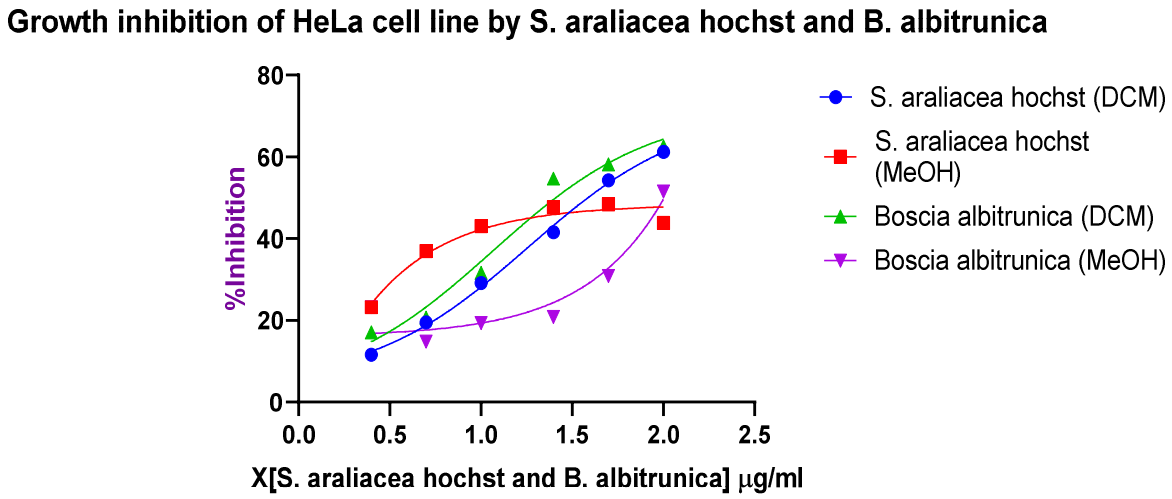

Figure 10: The cell growth inhibition of HeLa cell line by *S. araliacea* hochst and *B. albitrunca* is recorded above using the Prism graph-pad software measured in Log as they compare to fig. 6 and 7.

The results recorded above in **Fig. 10** show a sustained increase in cell growth inhibition of both plants. The *S. araliacea* hochst and *B. albitrunca* DCM extracts showed the highest levels of cytotoxicity. Methanol extracts showed very low cytotoxicity as can be seen by how the graph

pattern of *B. albitrunca* (MeOH) has a downward trend initially with the increase in Log concentration from 0.5 to 1.0 and finally halts below less than 50% cell growth inhibition at 2.0.

The same pattern can be observed with *S. araliacea* hochst with initially increases in cell growth inhibition percentage however reaches a plateau after 1.5 Log concentration.

The below **Fig. 11-14** are singular graphs of the above **Fig 10** to ensure clearer observation and analysis.

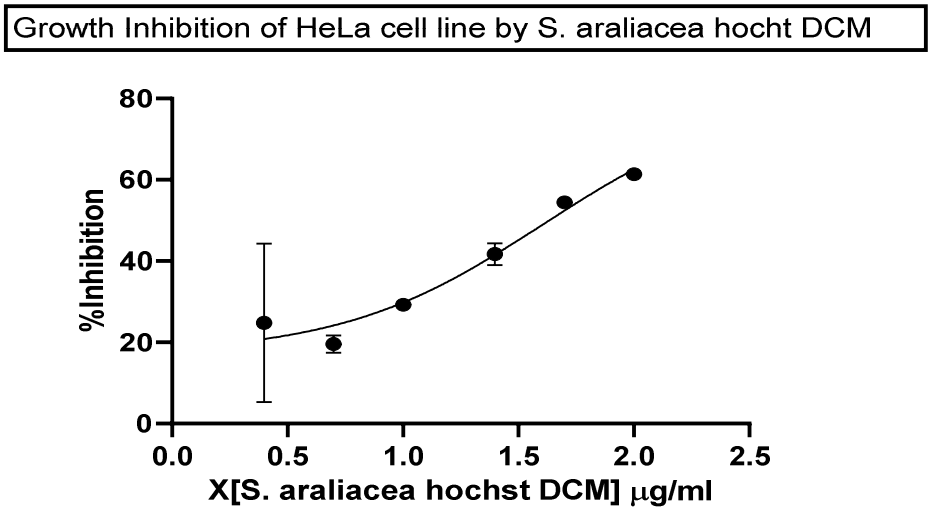

Figure 11: The DCM extract of *S. araliacea* hochst exerted increased cell growth inhibition with increasing concentrations and achieved an IC_{50} at 1.8 Log concentration.

Cell growth inhibition of *S. araliacea* hochst DCM extract is presented alone in **Fig. 11** to provide more clarity on the growth pattern and the IC_{50} as the extract was selected for further studies. The graph above illustrated a linear increase in cell growth inhibition percentage with increasing drug concentrations. The IC_{50} is achieved at 1.8 Log concentration.

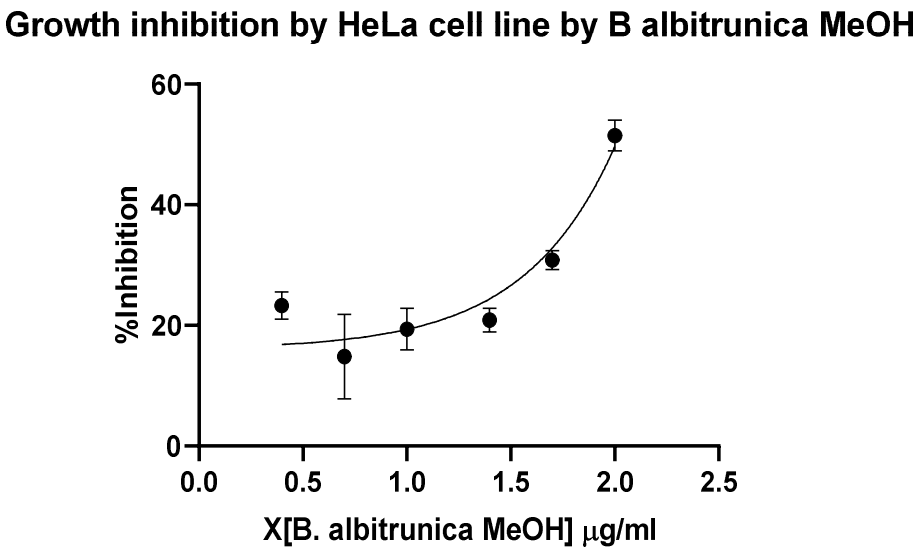

Figure 12: The figure above shows the results of growth inhibition of *B. albitrunca* (MeOH) extract against the HeLa cell line.

Increasing cell growth inhibition in **Fig. 12** with increasing concentration of *B. albitrunca* methanol extract against the HeLa cell line is reported. The highest cell growth inhibition of over 50% is evident at 2.0 Log concentration.

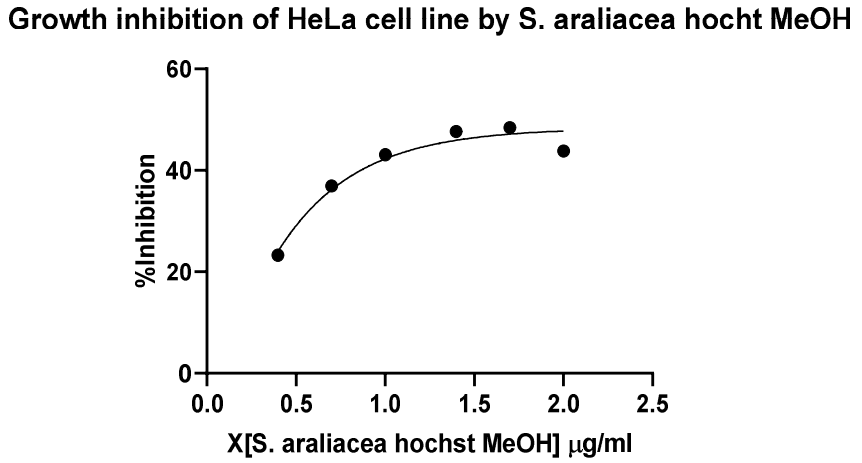

Figure 13: The figure above shows the results of cell growth inhibition of *S. araliacea* hochst (MeOH) extract against the HeLa cell line.

Fig. 13 above shows the plateauing results on the cell growth inhibition of the *S. araliacea* hochst methanol extract against the HeLa cell line. The results display an increasing cell growth inhibition pattern from 0.5 Log concentration while the highest anti-proliferation (50%) is observed between

1.5 and 2.0 Log concentrations. Subsequently the cell growth inhibition percentage plateaus at 50% as the drug concentration increases beyond 2 Log.

The screening of the water extracts yielding the following results:

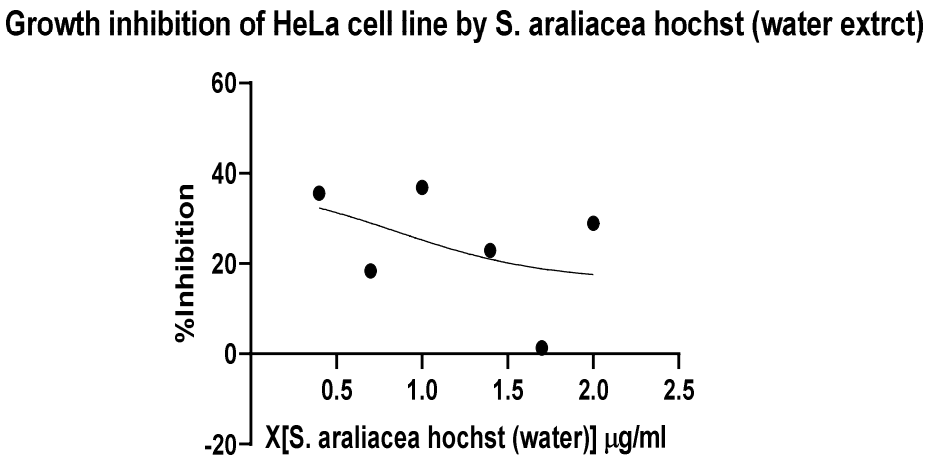

Figure 14: The cell growth inhibition of HeLa cell line by *S. araliacea* hochs*t* water extract is recorded.

Fig. 14 depict a sporadic assemble set of results despite 2 repeats of experiment which have the highest inhibition of 35% and the lowest of 20%. The water extract of *S. araliacea* hochst showed low cell growth inhibition between 0.5 and 2.5 log concentrations.

Table 4: Tabular summary of IC_{50} and R-squared values of *S. araliacea* hochst and *B. albitrunica* extracts in DCM and methanol solvents. Legend: Values with~(sign) are cited as being ambiguous - recorded value is not definite but within perimeter. Kindly refer interpretation to table 6.[3]

96-well plate dilutions				
	S. araliacea hochst (DCM)	*S. araliacea* hochst (MeOH)	*B. albitrunca* (DCM)	*B. albitrunca* (MeOH)
IC_{50} (µg/ml)	18.14 (Moderate Hazard)	14.78 (Moderate Hazard)	11.66 (Moderate Hazard)	28.6931 (Moderate Hazard)
R-squared	0.9893	0.8215	0.9577	0.8677

[3] IC50 – Inhibitory concentration; R-Squared -coefficient of determination

S. araliacea hochst and *B. albitrunca* DCM extracts displayed in **Table 4** above exhibited IC_{50} values 18.14 µg/ml and 11.66 µg/ml respectively. Both extracts displayed relatively moderate hazard and potency in terms of cytotoxicity.

Table 5: Tabular summary reporting the IC_{50} and R-squared results values of *S. araliacea* hochst water extract and Doxorubicin. Kindly refer interpretation to table 6.

dilutions 96-well plate		
	S. araliacea hochst (water extract)	Standard drug (Doxorubicin)
IC_{50} (µg/ml)	14.63 (Moderate Hazard)	2.155 (Hazard)
R-squared	0.400	0.8691

[4]

B. albitrunca MeOH extract displayed a high IC_{50} value of 28.6931 µg/ml. This value indicated very low hazardous cytotoxicity status. *S. araliacea* hochst MeOH extract displayed an IC_{50} value of 14.78 µg/ml and indicated a moderate hazard and potency of cytotoxicity.

The coefficient of determination (r_2) indicates how closely the growth curve generated can be fitted with non-linear regression statistics for the cell lines and IC_{50} values are shown in **Table 4** and **5** (van Tonder *et al*., 2015). **Table 4** presented r-squared values results between the four extracts range from 0.8215 to 0.9893.

Table 5 results exhibit the potency of the anti-cancer activity of *S. araliacea* hochst water extract with an IC_{50} value of 14.63 µg/ml and the standard drug, Doxorubicin recorded 2.155 µg/ml. These values indicate relatively moderate anti-cancer activity and moderately hazardous cytotoxicity properties according to the Council of Scientific and Industrial Research (CSIR) standard criteria (see **Table 6**). R-squared determinations were 0.4 and 0.8691 for *S. araliacea* hochst (water extract) and doxorubicin respectively.

[4] IC50 – Inhibitory concentration; R-Squared -coefficient of determination

Table 6: CSIR Standard criteria for IC_{50} interpretation (Makhoahle and Mashele, 2021)

IC_{50}, µg/ml	Status
> 100 µg/ml	Inactive
< 100 µg/ml > 15 µg/ml	Weak activity
< 15 µg/ml > 6.25 µg/ml	Moderate activity
< 6.25 µg/ml	Potent activity (Hazard)

C33A Cell culture results

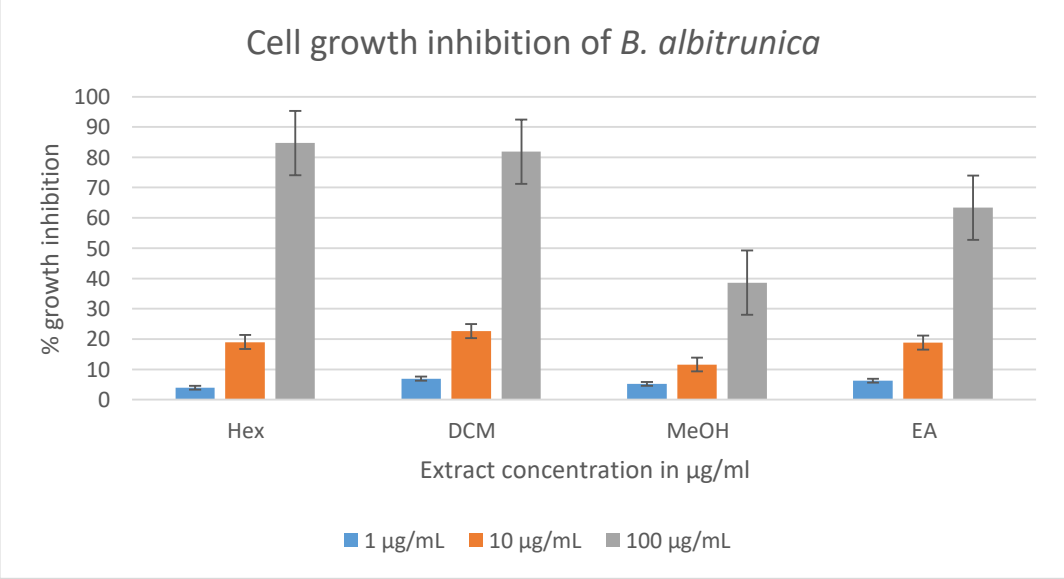

Figure 15: Cell growth inhibition of C33A cell line by *B. albitrunica* extracts at varied concentrations of 1, 10 and 100 µg/ml.

Fig. 15 illustrates the results of *B. albitrunca* against the second cell line in this study, C33A. Results show over-all growth inhibition of above 50% for all 100 µg/ml extracts except Methanol. The 10 and 1 µg/ml extracts showed low growth inhibition activity of 25% and lower. The general trend in the **Fig. 15** results is higher activity in Hexane, DCM, and EA extracts than Methanol extract, at the highest concentration measured.

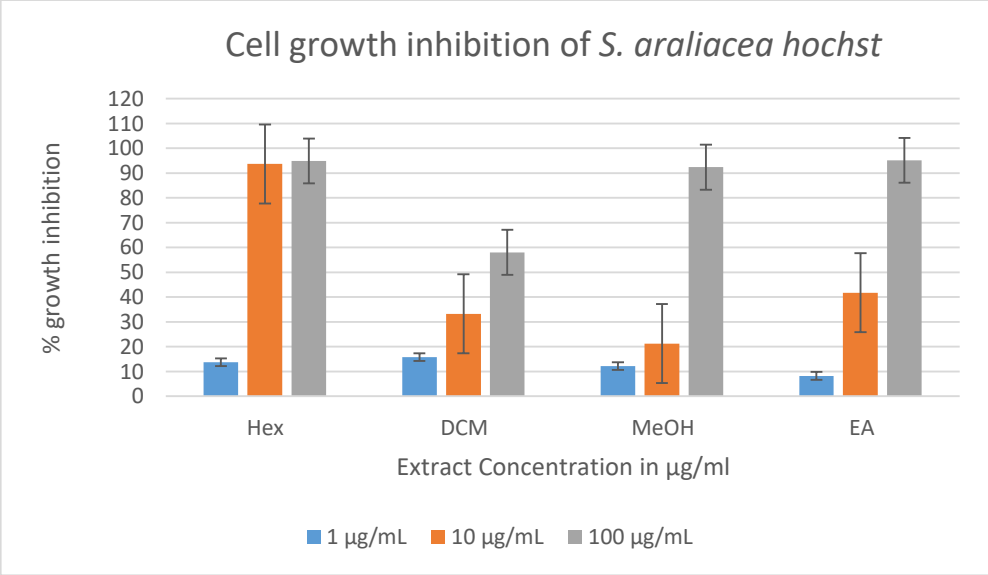

Figure 16: Cell growth inhibition of C33A cell line by *S. araliacea* hochst extracts at varied concentrations of 1, 10 and 100 µg/ml.

It is evident from the results in **Fig. 16** that the plant extracts of *S. araliacea* hochst showed higher growth inhibition activity than *B. albitrunca* in **Fig. 15**. All 100 µg/ml extract concentration displayed >50% growth inhibition, particularly Hexane, Methanol and EA which showed >90% growth inhibition at the highest measured concentration.

Hexane showed the most significant growth inhibition activity of over 93% followed by EA which showed notable activity of above 40% at the 10 µg/ml concentration.

The two moderately polar extracts (DCM and Methanol) showed lower activity especially at the two lower extract concentrations of 1 and 10 µg/ml with growth inhibition activity of lower than 40%.

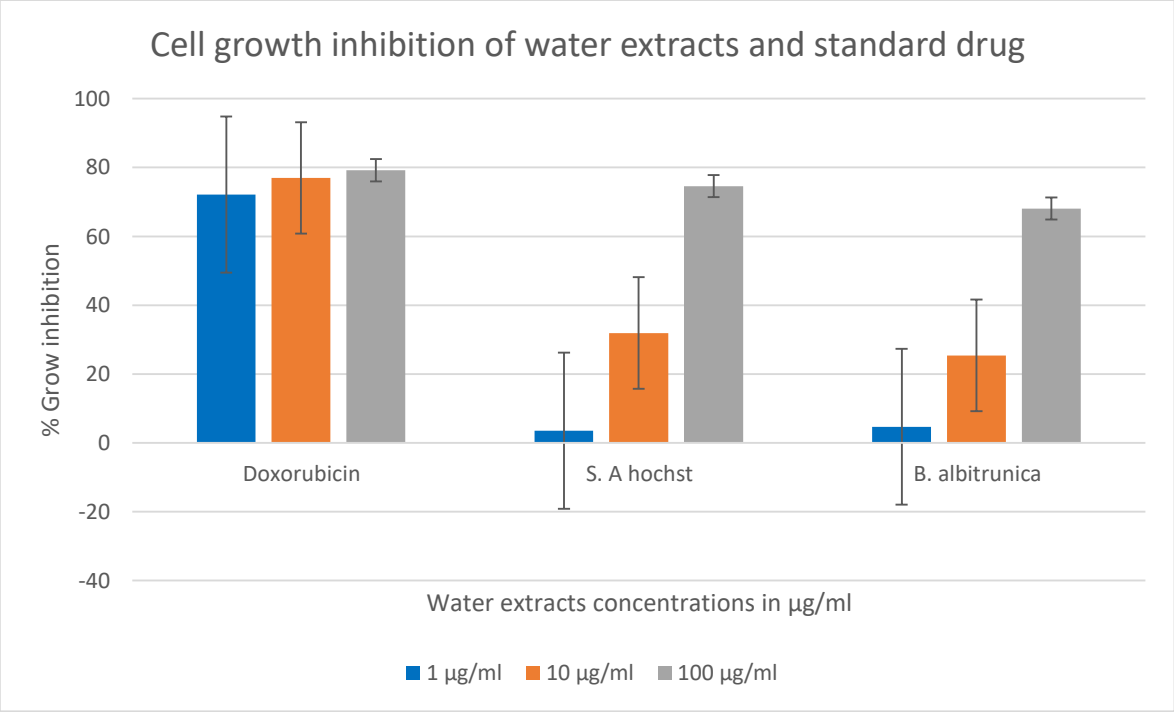

Figure 17: Cell growth inhibition of C33A cell line by the two water extracts and standard drug (Doxorubicin) at varied concentrations of 1, 10 and 100 µg/ml.

The same in **Fig 8**, the illustration in **Fig. 17** shows the relatively higher *S. araliacea* hochst water extract cell growth inhibition (30%) at the 10 µg/ml concentration. *B. albitrunica* also showed over 25% cell growth inhibition on the C33A cell line.

4.4 Thin Layer Chromatography

At least nine separations were observed in the *S. araliacea* hochst methanol extract which suggests that substantial mobility has taken place on this TLC paper than in the DCM extract of the same plant (2 bands). *B. albitrunca* exhibited 3 separations in the methanol extract as opposed to the 2 separations observed in the DCM extract. Detailed results are added in the appendices.

To investigate hindrance and to identify solvents which show effective activity, a DCM: MeOH combination was undertaken in each plant, and it was established that under these circumstances *S. araliacea* hochst (2 bands) consisted of more R_f values than *B. albitrunca* (1 band).

4.5 Fractionation

The water extract of *S. araliacea* hochst showed high anti-proliferative activity against both cell lines and was therefore selected for further fractionation by Solid Phase Extraction.

Below are the cell culture results of the five (5) *S. araliacea* hochst water fraction samples. The cell culture results are illustrated between **Fig. 18** and **22** to display cell growth inhibition of the 5 fractions (fraction 22, 35, 45, 69 and 87) from 3.125 to 100 µg/ml concentrations. Cell growth inhibition was measured in percentage. Growth inhibition over 50% suggests anti-proliferation and anti-cancer activity.

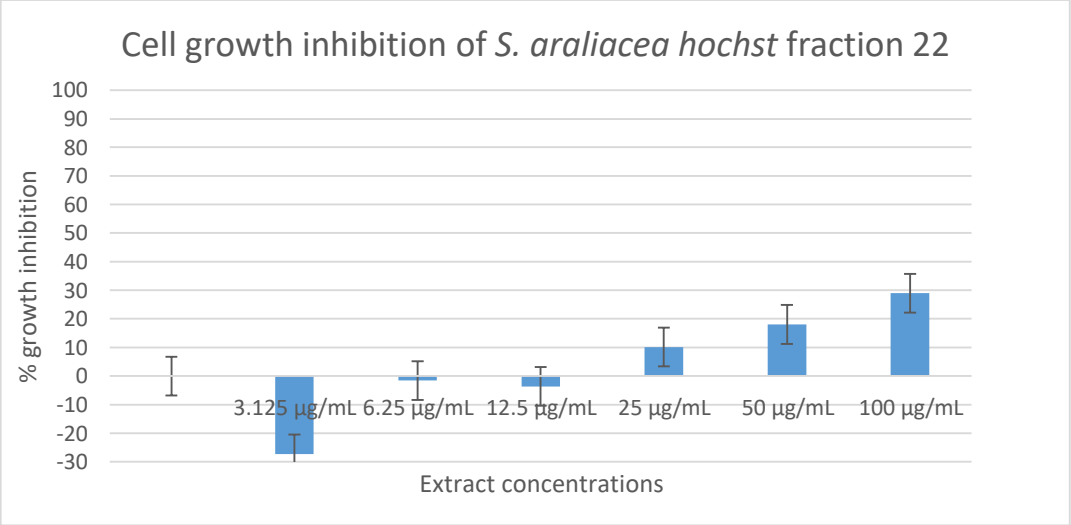

Figure 18: Cell growth inhibition of HeLa cell line for *S. araliacea* hochst fraction 22 extracts at varied concentrations between 3.125 and 100 µg/ml

Fig. 18 shows the six-extract concentrations of the fraction 22 *S. araliacea* hochst. The low extract concentrations of 12.5, 6.25, and 3.125 µg/ml showed a negative growth inhibition, suggesting that the cells grew more than the untreated control cells. The above illustration showed a slight increase in growth inhibition % of fraction 22 between the extract concentrations 25 and 100 µg/ml. The highest growth inhibition percentage of 29%.

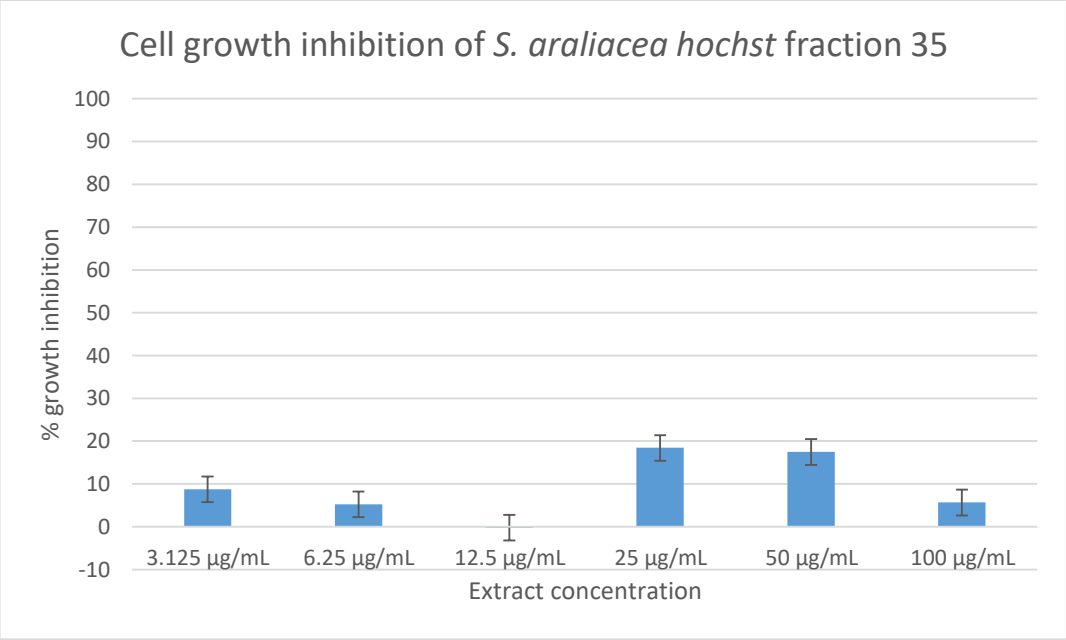

Figure 19: Cell growth inhibition of HeLa cell line for *S. araliacea* hochst fraction 35 extracts at varied concentrations between 3.125 and 100 µg/ml

Fig. 19 above shows the variable growth inhibition percentage of fraction 35 of the *S. araliacea* hochst extract. The highest growth inhibition was noted between 25 and 50 µg/ml extract concentration while the lowest can be seen at 12.5 µg/ml.

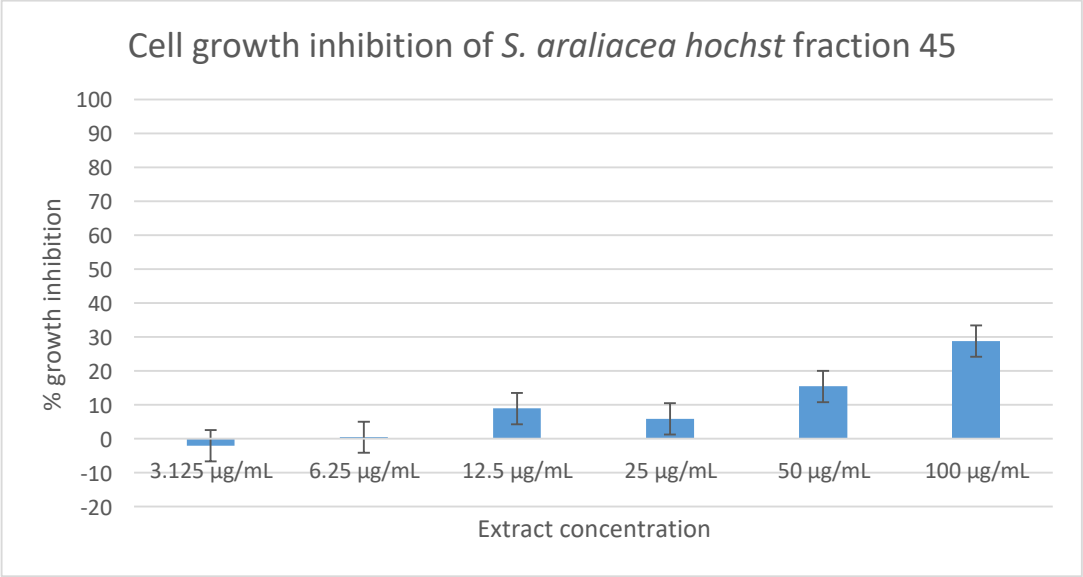

Figure 20: Cell growth inhibition of HeLa cell line for *S. araliacea* hochst fraction 45 extracts at varied concentrations between 3.125 and 100 µg/ml

Fig. 20 above shows the cell growth inhibition results between extract concentrations of 3.125 and 100 µg/ml which illustrates a gradual increase in cell growth inhibition activity. Only 3.125 µg/ml

showed cell growth. The highest cell growth inhibition of 29% is evident at 100 µg/ml concentration.

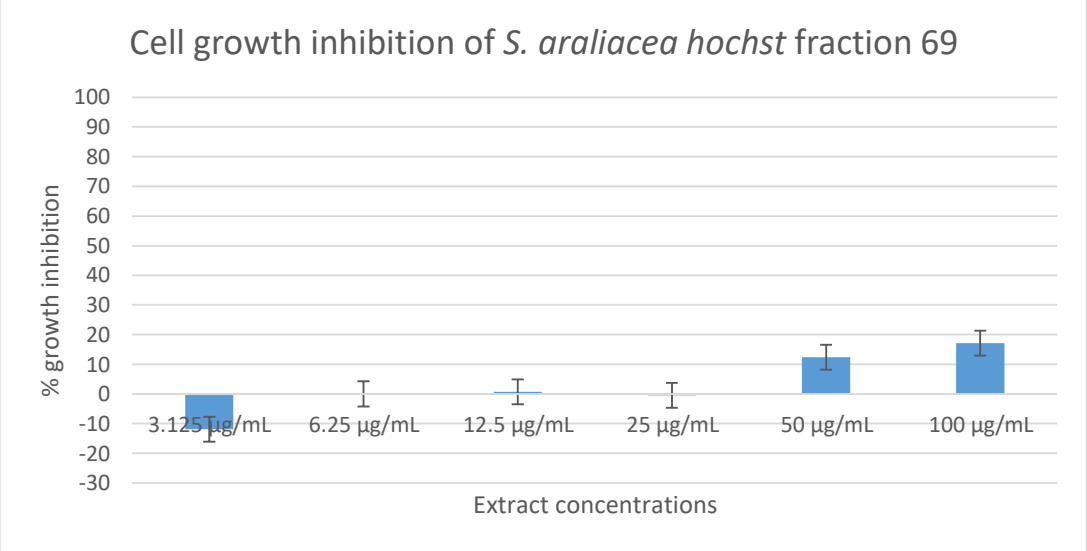

Figure 21: Cell growth inhibition of HeLa cell line for *S. araliacea* hochst fraction 69 extracts at varied concentrations between 3.125 and 100 µg/ml

Fig. 21 displays very minimal growth inhibition activity of fraction 69. The extract concentration 3.125 µg/ml showed cell growth of over 10%. The highest cell growth inhibition in the above figure was at 100 µg/ml with just under 20%.

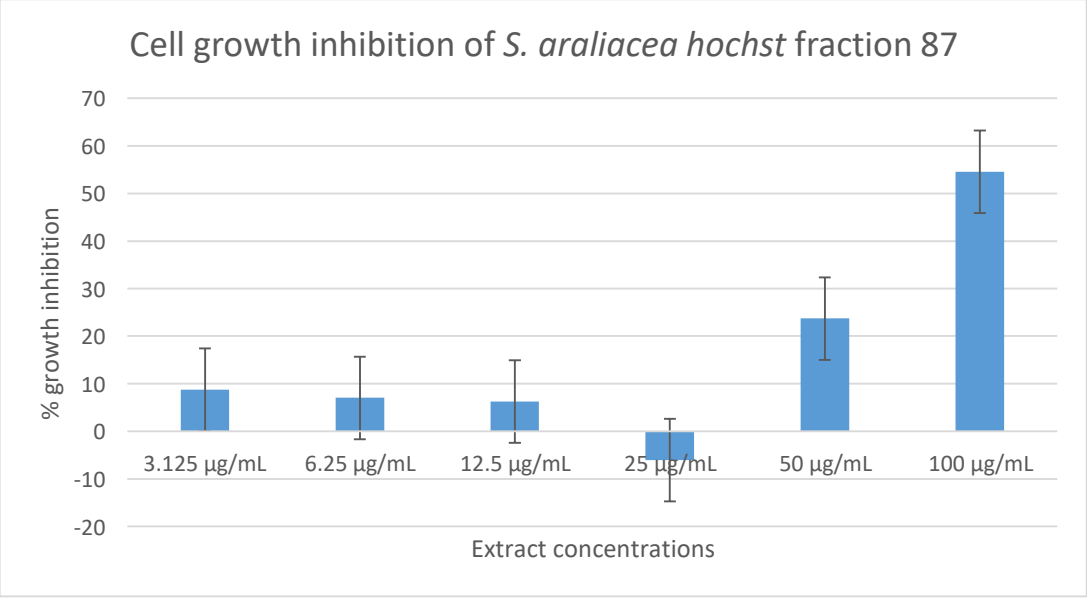

Figure 22: Cell growth inhibition of HeLa cell line for *S. araliacea* hochst fraction 87 extracts at varied concentrations between 3.125 and 100 µg/ml.

Fig. 22 illustrates the most anti-proliferative activity amongst the fractions. Proliferation was seen at 25 µg/ml concentration with around 5% cell growth. The most activity (highest growth inhibition) is noted at the highest extract concentration of 100 µg/ml. The cell growth inhibition activity was measured at over 50%. Fraction 87 was selected for further research in 2-D Matrigel sandwich cell culture model.

4.6 Two-dimensional sandwich cell culture model

4.6.1 Control cultures

The HeLa cells were grown in Matrigel sandwich model as described in section 3.7. The images in **Figure 23** shows the images of different wells captured between days 1 to 7 to illustrate the occurrence or lack of tubularization phenomena. The light white circular shaped structures in below images are live cells. The dark and light circular shape structures that have come together depict a colony of cells at their various life stages. The dark irregular shapeless patches are dead cell debris. Results of cells are treated with a standard drug Vincristine (negative control) showing the mechanism of action of anti-tabularization from day 1-7.

4.6.1.1 Positive control

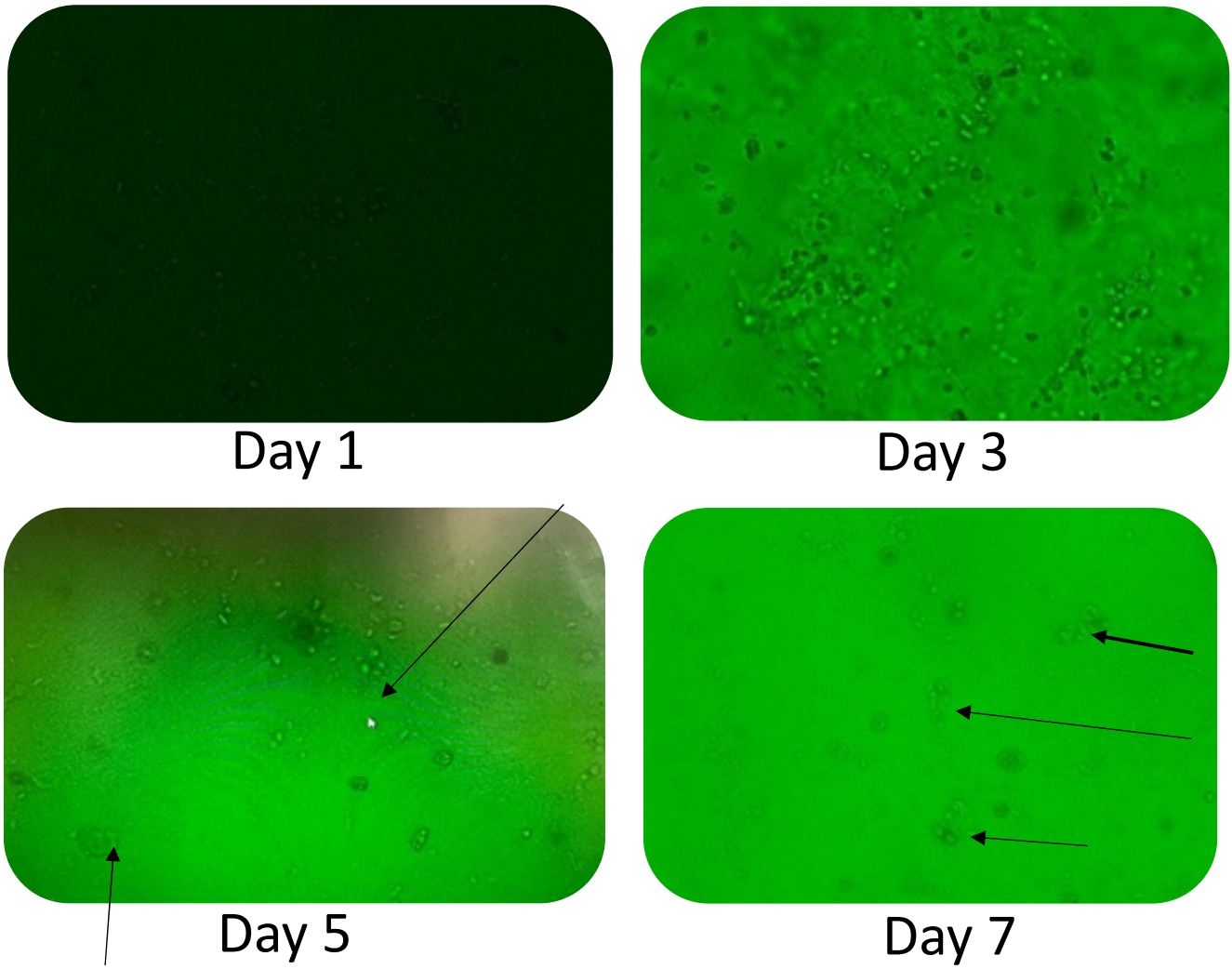

Figure 23: Images depicting the 2-D Matrigel cell culture of untreated cell well from day 1-7

Days 1 image was not clear as the rest (technical issue) but multiple scattered calls across the well could be observed with several dark patches of black. Day 3 depicts cells scattered across the well with floating dead cells. Days 5 show more organization in cells coming together to form colonies, while at day 7 colonies coming together to form super colonies.

4.6.1.2 Negative control

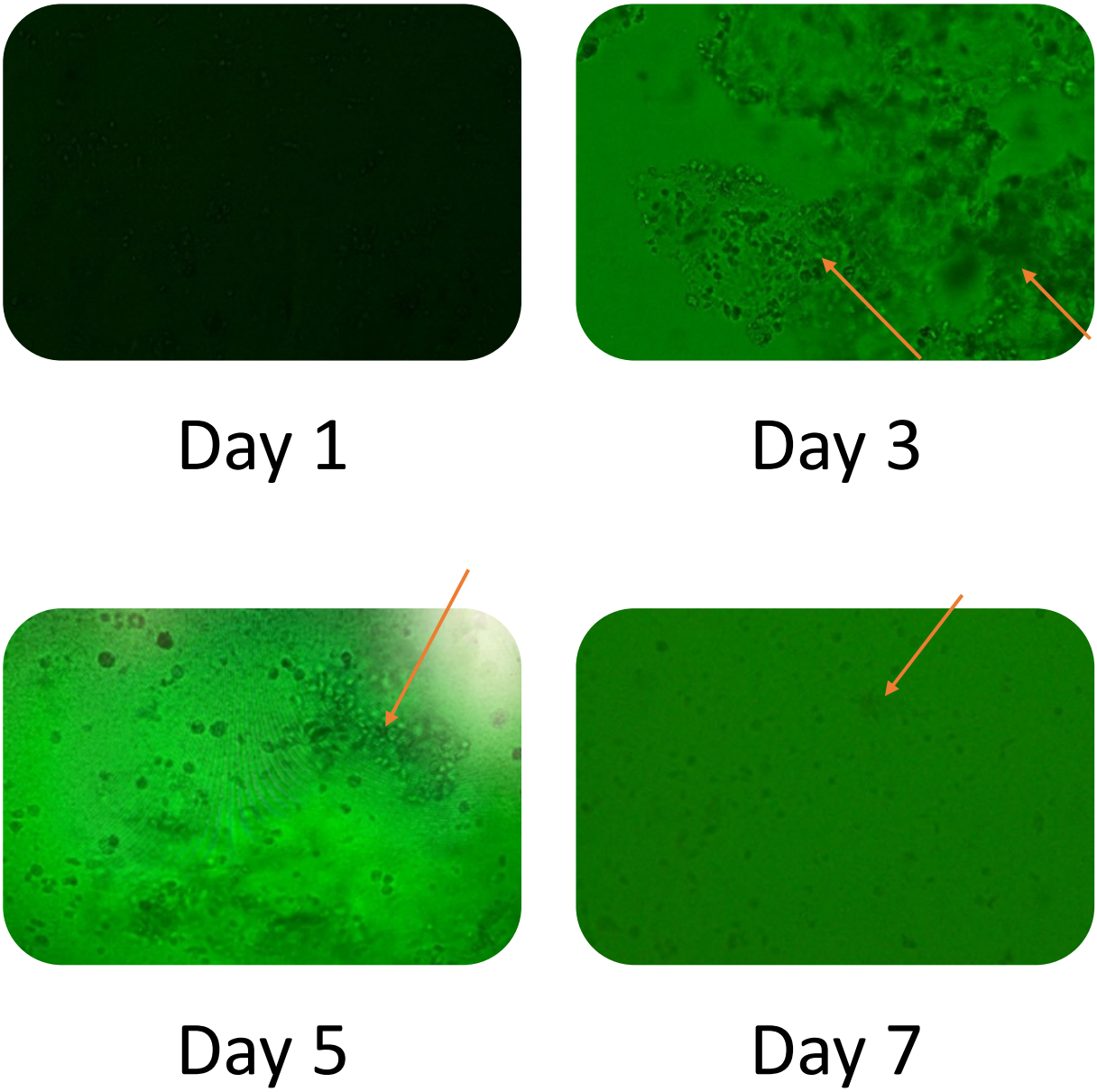

Figure 24: Above set of images (day 1-7) depict the sandwich 2-D Matrigel cell culture of a well plate treated with standard drug, Vincristine.

The results of cells shown above are treated with a standard drug Vincristine (negative control), which is expected to display its mechanism of action of anti-tabularization. Day 1 shows many more live cells than dark debris. The dark debris can be observed sporadically on day 3. Day 5 shows increased cell colonies and colonies that have started disintegrating. Day 7 images depicts a well with more debris and less colonies than all previous days.

4.6.1.3 Active extract treatment

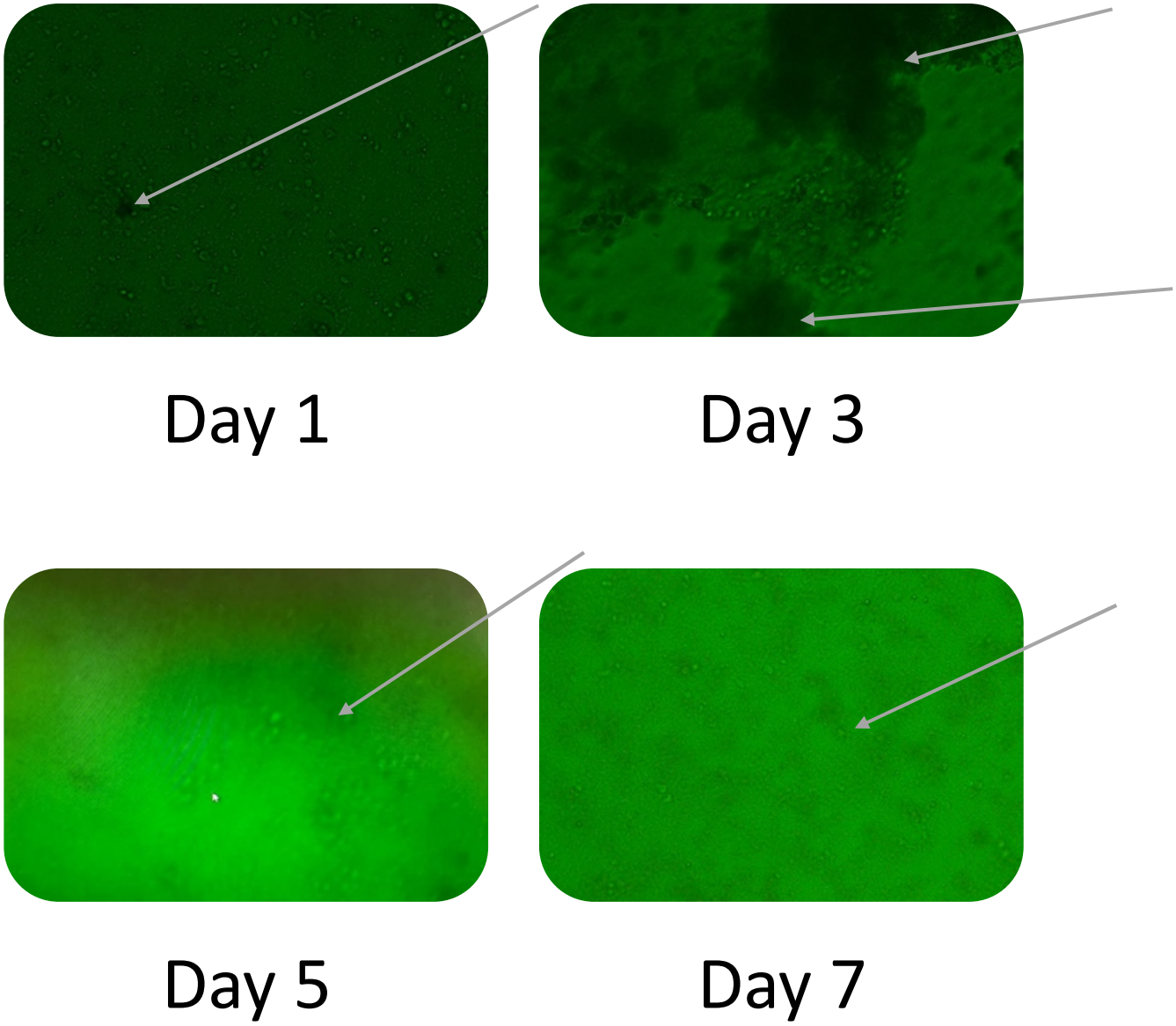

Figure 25: Above set of images (day 1-7) depict the sandwich 2-D Matrigel cell culture of a well plate treated with active plant extract (*S. araliacea* hochst).

Fig 25 shows results of cells treated with the active plant extract (*S. araliacea* hochst) showing the anti-tabularization mechanisms of the plant. Day 1 results was already showing isolated patches of dark debris. Day 3 increasing number of dark patches on the well. Day 5 shows several live cells successfully formed cell colonies and upper part of well with disintegrating cells. Day 7 shows more dark patches in majority of well, several live cell colonies can be noted on the image.

Chapter 5

Discussion

5. Discussion

This chapter discusses the results obtained from the study in relation to the aim and the 4 objectives laid out in chapter 1. Section 5.1 discusses the percentage yields obtained and the reported history of anti-inflammatory capabilities of phytochemicals identified in the two plants studied in this research. Section 5.2 discusses the cytotoxic screening of both plants against HeLa and C33A cell lines to establish their cell growth inhibition tendencies (particularly at 10 µg/ml concentrations). The chapter also discusses the phytochemical profile of extracts on the TLC plate (Section 5.3-5.4), while section 5.5 explores the validation of the use of 2-D Matrigel cell culture to assess tubularization and growth of HeLa cell line in this study.

5.1 Collection, extraction, and phytochemical analysis

High percentage yields were obtained for methanol and water extracts of both plants. These results indicate that extraction efficiency favors partially and highly polar solvents which is consistent with the extraction yields of other medicinal plants (Kuppusamy *et al*., 2015). The extraction process is an important step of this study to recover and isolate secondary metabolites from the two selected plant extracts, according to their polarity (Truong *et al*., 2019). This extraction process is crucial as it impacts the accuracy and reliability of the analysis over and above that it has been used in previous research. The selected solvents were used in this study due to their varying polarities and solubility properties which affect accuracy of results. The anti-proliferation results of the resultant extracts give an idea on whether the active constituents are polar or non-polar.

Phytochemical analysis conducted on selected plant extracts (*S. araliacea* hochst and *B. albitrunca)* showed presence of different classes of phytochemicals known to exhibit anti-cancer activities; alkaloids, phenols, flavonoids, glycosides, phytosterols and triterpenoids. (Sofowora, (1993); Yadav & Agarwala, (2011); Hamambulu *et al*., (2021)). All these were detected in *S. araliacea* hochst extract. Surprisingly saponins were not determined in this extract as previously reported in Alemika *et al*., (2006); Hamambulu *et al*., (2021). Alkaloids, triterpenoids, and glycosides among other phytochemicals are reported as powerful cytotoxins and commonly derived from medicinal plants, exhibit biological activities such as anti-inflammatory, anti-microbial, anti-malarial, cytotoxicity, anti-spasmodic and pharmacological effects (Iqbal *et al*., 2015).

The absence of triterpenoids, tannins and saponins could be one of the reasons behind the low IC_{50} value of *B. albitrunica* on HeLa cell line. These constituents possess biological properties such as inhibition of angiogenesis and cell proliferation activities (Yadav and Agarwala, 2011), the two of four pillars which are important processes for metastasis.

5.2 Cytotoxicity screening

The results **(Fig. 6-7)** showed that moderately polar and polar extracts generally displayed higher cytotoxicity than less polar extracts, particularly at 10 µg/ml and 100 µg/ml concentration. The DCM and methanolic extracts demonstrated a higher cell growth inhibition (at 10 µg/ml) than hexane extract for the *S. araliacea* hochst extract in **Fig.6**. The *B. albitrunca* plant showed some growth inhibition only above 40% for the methanolic and ethyl acetate extracts at 10 ug/ml. *Steganotaenia araliacea* hochst water extract exhibited the highest cell growth inhibition above 60% at 10 µg/ml and potential of activity even at the lowest concentration of 1 µg/ml. At this point it was feasible to observe that this extract will be a front runner as showing more potent cytotoxic properties. The concentration of 10 µg/ml was used to measure the cytotoxicity as it falls within a range where it is likely to exhibit significant effects on cell viability without being excessively high (leading to nonspecific toxicity or apoptosis). This concentration allowed the researcher to assess cytotoxic potential while minimizing confounding factors.

More dilutions of the extracts were screened to obtain more accurate IC_{50} values, as presented in **Fig 10-14**. Evidently, the methanolic extracts of both plants showed higher activity with higher IC_{50} (see **Table** 4) compared to DCM extracts. The cytotoxic screenings results showed that the *S. araliacea* hochst water extract was the better candidate for bioactivity-guided fractionation to concentrate anti-proliferative active constituents in **Fig 17**. This conclusion was based on the levels of cytotoxicity potential amongst all tested extracts.

The standard drug, Doxorubicin, achieved 100% cell growth inhibition at concentrations of 10-100 µg/ml **(Fig. 9),** reaching an IC_{50} of 2.155 (**Table 5).** This IC_{50} has been noted before in previous literature. (Lovitt *et al*., 2018).

S. araliacea hochst water extract illustrated in **Fig. 14** demonstrates a scattered and non-linear trend which may be caused by the chemical interactions between the water-based extract and MTT indicator as mentioned by Chan *et al*., 2015.

Fig. 15 and **16** depicts cell growth inhibition results of *B. albitrunca* and *S. araliacea* hochst on the C33A cell line respectively. It is noted that the *B. albitrunca* extracts exhibited low anti-proliferative activity on C33A cell line compared to HeLa cells, at drug concentrations of 1 and 10 µg/ml. Oddly **Fig. 16** results of *S. araliacea* hochst on the C33A cell line demonstrated more cytotoxic activity (1 and 10 µg/ml) of non-polar extracts (hexane) than on more polar extracts as opposed to HeLa results in **Fig. 6.** This result goes to show that a different cell line (C33A) exhibits varying sensitivities towards a non-polar extract possibly because of the lipid compositions than the HeLa cell line. The benefit of testing the two cell lines (HeLa and C33A) is because they exhibit distinct behaviors due to their unique molecular compositions (stable karyotypes which generally help them maintain a stable chromosome structure for consistent and reproducible experiments) and respond differently to treatment despite both being derived from cervical cells (Wang *et al*., 2022). HeLa cell line involvement in this study was crucial due to its genetic composition (aneuploidy), a genetic instability which is a hallmark of cancer cells (Hu *et al*., 2019). HeLa cells are also highly proliferative, which is one of the pillars of metastasis (Jiang *et al*., 2022). These qualities qualify the use of this cell line to this study (Huang *et al*., 2019; Jiang *et al*., 2022).

Just as HeLa cells, C33A cells are aneuploid but have a much slower proliferative rate (Yoshida *et al*., 2022). The use of this cell line was important for this study as the cells are known for their resistance to HPV infection – common factor in cervical cancer development (Boon *et al*., 2023). This resistance makes the cell line useful for a study such as this (Yoshida *et al*., 2022).

5.3 Thin Layer Chromatography

TLC was conducted using different solvent systems; Toulene: acetone(v/v) (8:2) for (*S. araliacea* hochst (methanol extract). The chromatogram illustrated the highest number of bands (9 bands) for *S. araliacea* hochst and 3 bands for *B. albitrunca* both which displayed several bands indicating the presence of potential active phytochemicals (see Plate 1). Plate 2 showed moderate low number of bands and thus indicating a lower presence of potential active phytochemicals in DCM extracts on both plants. The visualization of separated bands enables the estimation of constituents present, some of which may contribute to the activity of the extract. (Masoko & Eloff, 2007; Chen *et al*., 2020). The limitation with TLC is that it only provides a rough estimate, as structurally related constituents can elute at the same rate and appear as one band on the TLC chromatogram. Several recent research disputes hexane extracts as the best measure via TLC of showing active

constituents due to lingering impurities (Truong *et al*., 2019). It can be gathered from the TLC on Plates 1-3 and IC_{50} values in **Tables 5** & **6** that *S. araliacea* hochst displayed the most bands and cytotoxic activity. This provided the basis the continued investigation of the plant extract to the next phase of the study, which is fractionation.

5.4 Fractionation

The 94 eluents were examined on TLC plates, and those having similar separation patterns were combined, and eventually a total of 5 different fractions was reached. The similar separation patterns can be credited to the sameness in phytochemical composition within each fraction (See appendices). The sameness in phytochemical composition may be due to close molecular structures, isomeric compounds with same molecular formula but varying structural arrangements or same complexity (Ponce and Stortz, 2020). The five *S. araliacea* hochst fractions were screened against the HeLa cell line, and fraction 87 (see **Fig. 22**) showed the most potential cytotoxic activity at relatively low concentrations.

The more active fraction 87 has presence of previously reported class of phytochemicals with cytotoxic and anti-cancer properties such as alkaloids, flavonoids, and glycosides. The fraction pharmacological activities, phytochemical profile, and cell growth inhibition capabilities is in line with reported research by Hamambulu *et al*., 2021 that illustrated the cytotoxic benefits of this plant extract. The results supported fraction 87 further use in the 2-D Matrigel cell culture model.

5.5 Two-dimensional sandwich cell culture model

This study demonstrated how 2-D sandwich Matrigel cell culture technology was used to observe cell proliferation, colony formation, and formation of tubular like structures in the different wells plated with HeLa cells from day 1 to day 7. The Matrigel model supported colony formations, which implies that cells responded with production of extracellular molecules that promotes affinity and cell migration.

The untreated cells (positive control) demonstrated how cells grow to form cell colonies when undisturbed or undisrupted by a test sample. Cells achieved colony formation from day 5. Images (**Fig 23**) show live cells (white bright circular structures) moving toward each other. The results suggest that the environment allowed these colonies to further merge with other colonies to form super spheroids (super cell colonies) at day 7 (see pointed arrows in **Fig. 23**). This can only happen

through intuitive cell to cell communication and sharing of nutrients via tubulation between cells (Gonzalez-Molina *et al*., 2022).

Fig 24 depicts results of cells treated with the standard drug (Vincristine), which served as negative control. Day 1 results (**Fig 23 – 25**) were similar in depicting large number of live cells mostly attached, few still floating in the well. Day 3 results showed more dead cells where they were concentrated in specific areas of the well as opposite to sporadic spreading across the well.

Light colored, shape defined live cells can be seen in the other regions of the well. By day 5 the more equal appearance of dead cells on the well was observed. Cell proliferation inhibition actions are noted (**Fig. 24-25**), several cell colonies are however still apparent. By day 7, the standard drug inhibition capabilities were well observed with well presenting only dead debris. It is reasonable to state that the most inhibitory action took place between day 5-7. Vincristine exerts its activity primarily, by its anti-tubularization mode of action and secondly by imparting restraining effects on topoisomerase enzymes to subsequently stalling DNA replication and cell death (Dhyani *et al*., 2022). This alkaloid rich plant have proven efficacy for oncogenesis suppression as noted from day 3 in **Fig. 24-25** (Dhyani *et al*., 2022). The resultant cell death observed on day 5-7 (**Fig. 24**) aligns with cell proliferation inhibition and anti-tubular formation capabilities of Vincristine as opposed to cell proliferation, cell growth and initiation of tubularization process noted in **Fig. 23** (Blanco-Fernandez *et al*., 2021). These results support research by Liu *et al*., 2019 that Vincristine affect mechanisms that increase proliferation and cell migration by degrading fibronectin, inhibiting microtubule-related proteins and other ECM components.

Results from **Fig. 25** illustrate the effect of active fraction on the Hela cell line and interestingly, we observed distinctive activity in both **Fig. 24** day 3 and **Fig. 25** day 3. Day 3 (**Fig. 25**) image is similar to day 3 (**Fig. 24**) there is a predominant number of dead debris in the well than was observed on the same day in untreated cells well. The number of dark patches that is interpreted as dead cells started increasing from day 3 of the experiment, suggesting inhibition of cell growth. According to the results, the active fraction inhibited cell growth and colony formation and migration earlier than the standard drug as seen by the more dead cells in day 3 of **Fig. 25** compared to **Fig. 24**. This result confirmed the active fraction as a faster acting agent (Dhyani *et al*., 2022).

Day 5 image is very interesting in that it depicted colonies that were dead and floated while still attached while day 7 image shows dark colored irregular shaped colonies and significant number

of still floating cells. It is fair to note that the active fraction well illustrated more floating (dead) cells than 2 other controls which may be attributed to active fraction effect to discourage further attachment. It is also a fair interpretation in stating that by day 7 almost all attached cells were inhibited to metastasize (see number of dead cells in **Fig. 25** day 7) while the floating cells unable to continue forming colonies.

Day 5 images of **Fig. 24** contrary to untreated cells **Fig. 23** well on the same day showed more floating cells than attached. This observation supports the recorded effects of Vincristine, which include to minimize cell attachment by prolonging cell floatation and ultimately promote cell death. It is hypothesized that vincristine may have halted colony formations by impeding tubular formation. The result of unformed tubules meant that cells could not communicate, interact, and colonize effectively, characteristics involved in metastasis (Ojha *et al*., 2023).

Fig. 25 showed the effectiveness of the active plant extract (*S. araliacea* hochst) in halting tubularization between day 3 and day 7. Day 1 images are uniform between the positive, negative, and active drug wells, which are characterized by mostly attached live cells, patches of floating well-defined cells and numerous dead cells attributable to strained efforts to acclimatize to microenvironment.

The similarities of day 3 to day 7 of active drug and standard drug are very important in agreeing with the hypothesis that *S. araliacea* hochst is just as effective in halting metastasis as Vincristine however at a slower pace. It is rational to believe that both agents disrupted the tubular structure formations in the cells to avoid cluster integration and caused cells to die.

It was noted that *S. araliacea* hochst was fast acting in disrupting cell proliferation already attached cells by day 3. The results depicted on day 7 images showed that the *S. araliacea* hochst is just as effective in reducing cell proliferation. While an *in vitro* study is not able to conclude active process of treatment halting metastasis using anti tubularization mechanisms, the results in Fig 24 and 25 depicted a reduction of proliferation and colony formation (Hamambulu *et al*., 2021). The magnitude in cell death between day 5-7 in **Fig. 24** and **Fig. 25** was different which led us to believe that the active fraction is more fast acting than standard drug. In conclusion, the 2-D Matrigel sandwich cell culture proved a valid tool to use to investigate tubularization patterns and the active extract demonstrated positive anti-metastatic properties.

Chapter 6

Conclusions

6. Conclusion

6.1 Conclusionary comments and implications

This study demonstrated that *S. araliacea* hochst and *B. albitrunica* are both rich in phytochemicals especially alkaloids, flavonoids, phytosterols and anthraquinones, while the extract of *S. araliacea* hochst further contains glycosides, tannins, and triterpenoids. These classes of phytochemicals are well known for their pharmacological activities (anti-proliferative, and anti-tubularization effects). This continues to position Ethnopharmacology as a sensible option for drug discovery.

Band separation TLC plate for the *S. araliacea* hochst suggested the presence of several active constituents, which could be attributed to the biological activity observed in this study. The isolation of active compounds in the quest of drug discovery and development for cervical cancer could be pursued. More accurate techniques including High-Performance Liquid Chromatography (HPLC) and Mass Spectrometry (LC-MS) will have to be employed for purification and molecular identity of the constituents.

This Matrigel model supported colony formations, which implies that cells responded with production of extracellular molecules that promotes affinity and cell migration. The 2-D Matrigel cell culture model results of the selected active plant extract showed that *S. araliacea* hochst was a faster acting cell growth inhibitory agent than the standard drug and effective in a longer timeframe as well. From as early as day 3 was a steady decline in live cells noted as well as anti-tubularization activity which resulted in dark black irregular patches of debris. While the mono layer cell-based cell viability tests are still being used widely for screening of drug candidates, other techniques based of different possible modes of action should be explored. It is also clear based on the results obtained form our study that there is a need for more advanced equipment for more clear and accurate assessments. While affordability of this equipment might be a challenge, collaborations and networks across borders could provide a solution.

This study provided new knowledge on the ability of the selected plant extract in disrupting cell proliferation, and tubularization, which implies its ability to halt the initial processes involved in metastasis. Based on the cytotoxicity screening, and 2-D cell culture results, it is reasonable to suggest the use of *S. araliacea* hochst plant in traditional medicine for cancer patients could have

a complimentary effect to anti-cancer drugs. The study also suggests the use of 2-D sandwich Matrigel cell culture model as an alternative method for observing proliferative and tubularization processes. But the need for more sensitive microscopy is evidence.

6.2 Further study and recommendations

For a PhD study the use of a more biomimetic cell culture model such as the 3-D Matrigel models grown in bioreactors will illustrate a better picture of the physiological processes of metastatic activity than a 2-D model does. The development and use of 3-D spheroids and a comprehensive evaluation of the effect of active extract on more physiological parameters will advance the research.

6.3 Limitations of the study

The two other pillars (differentiation and angiogenesis) are important for the process of metastasis were not investigated in this study. These processes require conducting specific assays different from the ones used in the current study, and they could not be pursued due to limited timeline available for a master's degree programme. These pillars together with differentiation and vascularization are best studied *in vivo* or *ex-vivo* research. The collective investigation of the other 3 pillars may be more suitable for a Ph.D. study due to the extensive timeframe granted and expected applicability to real live environment. A complete understanding of the effects of active plant extracts on metastasis can therefore only be possible when all processes involved in metastasis initiation and progression have been investigated. Although the primary focus of this study was to study *in vitro* cell proliferation and anti-tubularization, more can be done in future studies to assess the effects of differentiation and angiogenesis using specific assays on the above selected plant extracts.

Conflict of interest

Authors declared that there is no conflict of interest.

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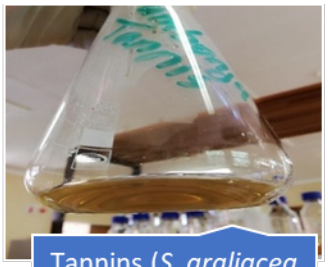
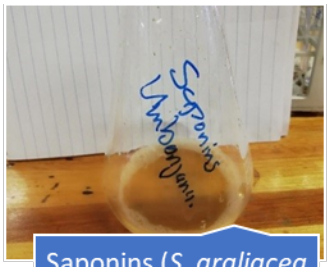
Annexures

Appendices

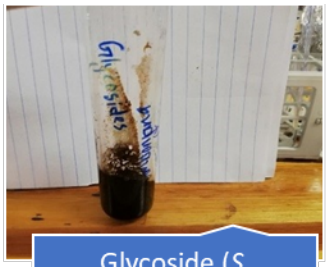
Annexure A: Phytochemical analysis *S. araliacea* hochst (Umbonjana)



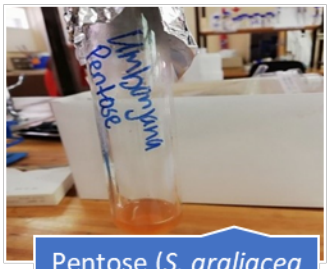
Tannins (*S. araliacea hochst*)



Saponins (*S. araliacea hochst*)



Glycoside (*S. araliacea hochst*)

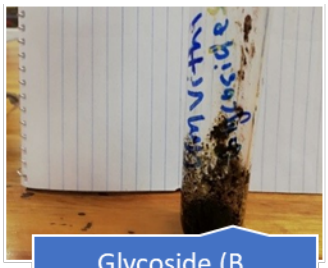


Pentose (*S. araliacea hochst*)

Annexure B: Phytochemical analysis *B. albitrunca* (Umvithi)

Pentose (B. albitrunica)

Phytosterols (B. albitrunica)



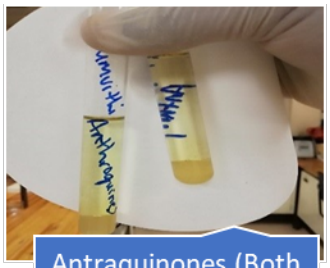
Glycoside (B. albitrunica)

Saponins (B. albitrunica)

Tannins (B. albitrunica)

Annexure C: Phytochemical analysis (Both extracts)

Flavanoids (Both extracts)

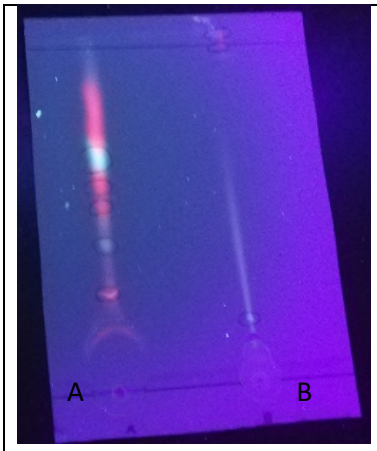


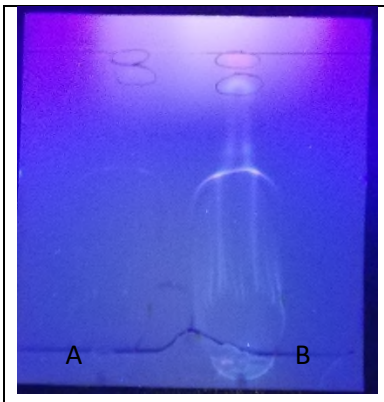
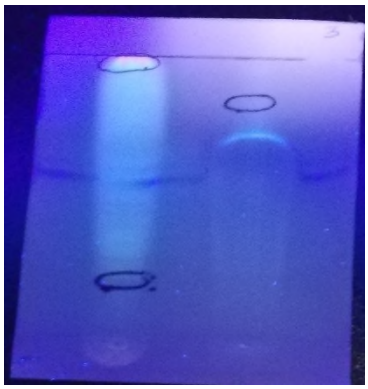
Antraquinones (Both extracts)

Alkaloids (Both extracts)

Triterpenoids (Both extracts)

Annexure D: TLC plates

	<u>Plate 1</u> **A:** *S. araliacea* hochst (MeOH) TLC plate showed the greatest number of bands (9 bands). **B:** *B. albitrunica* (MeOH) showed only 3 bands. **Solvents:** Toluene: acetone (v/v) (8:2)

	Plate 2 **A:** *S. araliacea hochst* (DCM) plate displayed separation of 2 band. **B:** *B. albitrunica* (DCM) also showed the separation of 2 bands. **Solvents:** Toluene-chloroform-acetone (40:25:35)
	Plate 3 A: S. *araliacea hochst* (DCM:MeOH) shows separation of 2 bands. B: B. *albitrunica* (DCM:MeOH) showed only 1 band separation. Solvents used: n-butanol-glacial acetic acid-water (50:10:40).

Annexure E: Sandwich 2-D Matrigel cell culture Day 7 images viewed with Nikon Eclipse TS2-S-SM Microscope (x20)