IN VITRO ANTI-CANCER ACTIVITY OF ISOLATED COMPOUND FROM *ASPARAGUS LARICINUS* BURCH. LEAVES ON BREAST CANCER CELL LINES (MCF-7)

by

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DECLARATION

I, Mmannini Francina Rangwaga, identity number [redacted] and student number _____, do hereby declare that this research project submitted to the Central University of Technology, Free State, for the Master of Health Science in Biomedical Technology degree, is my own independent work; 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 (or partial fulfilment) of the requirements for the attainment of any qualification.

_____ **30/08/2025**

SIGNATURE OF STUDENT **DATE**

DEDICATION

This dissertation is dedicated to my parents, William Lebogo Rangwaga and Botshenyane Esther Mogolo Rangwaga, for their spiritual, emotional, and financial support throughout my academic journey.

To my sisters, Seipopi Opelia Rangwaga and Sisinyane Betty Rangwaga, for always being by my side.

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LIST OF ABBREVIATIONS

ACS	American Cancer Society
ADC	Automatic Developing Chamber
ATS	Automatic TLC Sampler
BCT	Breast-conserving therapy
CCND1	Cyclin D1
DCIS	Ductal carcinoma *in situ*
DCM	Dichloromethane
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2/HER2
EGFR	Epidermal growth factor receptor
ESI	Electrospray ionisation
ESI-MS	Electrospray ionisation mass spectrometry
EtOAc	Ethyl acetate
FBS	Foetal bovine serum
FGFR1	Fibroblast Growth Factor Receptor 1
GATA3	GATA Binding Protein 3
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
HDI	Human Development Index
HER2	Human epidermal growth factor receptor 2
HIV/AIDS	Human immunodeficiency virus / acquired immunodeficiency syndrome
HPTLC	High-performance thin-layer chromatography
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
LC-MS	Liquid chromatography-mass spectrometry
m/z	Mass-to-charge
MAPK	Mitogen-activated protein kinase
MYC	MYC Proto-Oncogene
NCE	New chemical entity
NCI	National Cancer Institute
neu	Neuroblastoma expressed uponcogene
PBS	Phosphate-buffered saline

PI	Propidium iodide
PI3K	Phosphatidylinositol 4,5-bisphosphate 3-kinase
PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase Catalytic Subunit
PTEN	Phosphate and Tensin Homolog
PTLC	Preparative thin-layer chromatography
RPM	Revolutions per minute
TLC	Thin-layer chromatography
TMP	Traditional medicinal plant
Tp53	Tumor Protein p53
USA	United States of America
UV	Ultraviolet
v/v	Volume per volume
VLC	Vacuum liquid chromatography
WHO	World Health Organization

LIST OF UNITS

°C	Degree(s) Celsius
µg/mL	Microgram(s) per millilitre
µM	Micrometre(s)
cm	Centimetre(s)
g	Gram(s)
g/mol	Grams per mole
L	Litre(s)
mL	Millilitre(s)
nm	Nanometre(s)
µg/mL	Microgram(s) per litre
µL	Microlitre(s)

LIST OF CHEMICAL SYMBOLS

MeOH	Methanol
$FeCl_3$	Iron (III) chloride
Ca^{2+}	Calcium ions
Mg^{2+}	Magnesium ions

ABSTRACT

Breast cancer is a global health issue and the second leading cause of death in women. Global Cancer Incidence, Mortality and Prevalence 2020 reported 186 598 breast cancer cases, with 85 878 related deaths in Africa. While the morbidity rates appeared low, the mortality rates were relatively high in sub-Saharan Africa. Current breast cancer therapies are known to cause severe long-term adverse effects in patients. An alternative approach to combat this issue is to conduct research aimed at discovering treatments using traditional medicinal plants (TMPs) that have reduced severe adverse effects. TMPs contain secondary metabolites known to possess anti-cancer potential, as reported in previous studies.

This study aimed to evaluate the anti-cancer activity of compound N-A, isolated from the *Asparagus laricinus* Burch. plant, against breast cancer cell lines (MCF-7) *in vitro*. Various chromatographic techniques, such as vacuum liquid chromatography (VLC), column chromatography, high-performance thin-layer chromatography (HPTLC), and preparative thin-layer chromatography (PTLC), were employed to fractionate, isolate, and purify the compound. The chemical composition of the plant extract was identified using liquid chromatography-mass spectrometry (LC-MS).

A total of 18 fractions were collected by VLC and further combined based on similarities in content to formulate nine sub-fractions. Among these nine sub-fractions, sub-fraction number two was selected for compound isolation due to its higher content and better separation observed on a thin-layer chromatography (TLC) plate. Following isolation, the anti-cancer activity of compound N-A was assessed using the Hoechst 33342/propidium iodide assay on MCF-7 cell lines at different concentrations that ranged from 15.63 to 250 µg/mL.

After *in vitro* bio-assay screening, compound N-A did not exhibit a reduction of cell population by 50%. The inactivity of compound N-A was, however, unexpected, as the crude extract of this plant has previously been shown to possess an anti-cancer effect on the MCF-7 cell line. It is possible that this molecule is more effective in the presence of other compounds found in *A. laricinus* Burch. extract due to synergism. The findings of this study suggest that compound N-A isolated from *A. laricinus* Burch. may not function as an anti-cancer agent; rather, the crude extract should be considered in future studies.

Keywords: Breast cancer, secondary metabolites, compound, *Asparagus laricinus*, chromatography

CHAPTER 1:

INTRODUCTION

1.1 BACKGROUND

Cancer is one of the leading causes of death worldwide. It accounted for approximately 10 million deaths in 2020 (World Health Organization [WHO], 2022). Cancer encompasses a group of diseases characterised by neoplasms (Mathur *et al.,* 2015). With over 100 types of malignancies classified according to the tissue from which the cancer arises, breast, lung, rectum, prostate, and colon are among the most common cancers (Drebert, 2017; WHO, 2022). The risk of developing cancer is associated with, but not limited to, tobacco use, alcohol abuse, poor diet, obesity, reproductive factors, viruses, and infections (Arem & Loftfield, 2018). These factors may lead to cell mutations, which eventually result in cancerous cells. Conventional therapeutic approaches, such as chemotherapy, radiation therapy, and surgery, alongside non-conventional therapies, including immunotherapy, hormone therapy, and nano-therapy, aim to treat and manage cancer by specifically targeting cancerous cells to obstruct and reduce their growth rate (Chandraprasad *et al*., 2022). These interventions are primarily implemented to cure the disease, palliate symptoms when a cure is impossible, and enhance the patient's quality of life (Khan *et al*., 2005). Nevertheless, cancer therapeutic systems can cause severe adverse effects in patients (Debela *et al*., 2021).

Researchers focus on developing new drugs with fewer adverse effects that are derived from natural products, particularly medicinal plants (Rafieian-Kopaei, 2012). Previous studies have revealed a variety of isolated compounds from these plants that are effective in treating life-threatening ailments such as cancer, diabetes, and HIV/AIDS (Street & Prinsloo, 2013). Medicinal plants may therefore be considered a form of alternative medicine for therapeutic purposes (Mohammed, 2019). A medicinal plant is characterised by the use of any part of the plant to cure, relieve, and prevent disease or to alter physiological and pathological processes (Beyene *et al.,* 2016).

Empirical evidence on the therapeutic effects of traditional medicinal plants (TMPs) has been passed down from one generation to the next in rural communities (Marrelli, 2021). Rural residents primarily prefer TMPs because traditional healers understand their patients,

culture, and environment, and these remedies are easily accessible (Mbuni *et al.*, 2020). TMPs are typically readily available to the community and can be easily administered orally alongside the dietary intake of a patient (Amin *et al.*, 2009; Cornblatt *et al.*, 2007). Furthermore, they are less expensive than conventional medicine and are perceived as safe by long-time users (Fennell *et al.*, 2004). In sub-Saharan African countries, the conventional healthcare system is often centred in urban areas, which limits access for villagers in rural regions (Maroyi & Cheikhyoussef, 2015). In South Africa, TMPs are utilised by different tribal groups to treat various diseases in both humans and domestic animals (Kgatle, 2007). Some registered traditional healers have reported successfully using TMPs to treat a variety of cancers, including breast cancer (Matowa *et al.*, 2020).

Previous researchers have reported on plant species with anti-cancer properties that are successfully used as herbal remedies in developing countries (Greenwell & Rahman, 2015). Cancer chemoprevention is among the most vital projects in public health (Yasukawa, 2012). It is characterised by the use of specific natural and synthetic chemical agents to repress carcinogenesis and impede the progression of invasive cancer (Sofowora *et al.*, 2013). *Asparagus racemosus* Willd is one of the most studied medicinal plants in the *Asparagus* genus that has demonstrated anti-cancer properties (Shankar *et al.*, 2012). An isolated compound, Shatavarin IV, from the roots of *A. racemosus* Willd, has shown an anti-cancer effect in both *in vitro* and *in vivo* experimental studies (Shankar *et al.*, 2012).

A. laricinus Burch. has been used successfully to treat numerous illnesses in farm animals in rural areas (Van der Merwe *et al.*, 2001). In addition, previous studies, including phytochemical screening and investigations into anti-cancer, anti-oxidant, and anti-bacterial activities, have demonstrated that the plant is pharmacologically effective (Mashele & Kolesnikova, 2010; Mfengwana *et al.*, 2019; Ntsoelinyane & Mashele, 2014). However, the aforementioned analyses were conducted using crude extracts of *A. laricinus* Burch., and the compounds were not isolated. It is important to isolate compounds from crude extracts of this plant to understand the active constituents that contribute to its anti-cancer activity. The active compound could be targeted as a potential lead for cancer drug development. This research project therefore screened an isolated compound from an *A. laricinus* Burch. leaves extract for its anti-cancer activity against breast cancer cell lines (MCF-7). Multiple

chromatographic techniques were employed to fractionate and isolate a compound from this plant of interest.

1.2 PROBLEM STATEMENT

In Africa, a large proportion of the population relies on plants for their primary healthcare. Studies have reported numerous biologically active molecules with anti-cancer effects that are derived from TMPs. Natural products play a significant role in drug discovery and are continually used to meet the pressing need for novel and effective anti-cancer drugs. The current anti-cancer treatment poses challenges, such as unpleasant side effects, chemoresistance, and dose limitations. Thus, there is a need to develop novel anti-cancer agents with minimal or no side effects. According to the literature, *A. laricinus* Burch. crude extract has been reported to exhibit *in vitro* anti-cancer effects against breast and prostate cancer cells.

1.3 AIM

This research aimed to the anti-cancer activity of compound N-A, isolated from the *Asparagus laricinus* Burch. plant, against breast cancer cell lines (MCF-7) *in vitro*.

1.4 OBJECTIVES

The study's objectives were as follows:

1) To fractionate and isolate *A. laricinus* Burch. leaves extract using vacuum liquid chromatography (VLC), column chromatography, high-performance thin-layer chromatography (HPTLC), and preparative thin-layer chromatography (PTLC).
2) To identify the isolated compound from *A. laricinus* Burch. extract using liquid chromatography-mass spectrometry (LC-MS).
3) To determine the anti-cancer effect of the *A. laricinus* Burch. compound using MCF-7 cancer cell lines *in vitro*.

1.5 DISSERTATION OUTLINE

The dissertation is organised as follows:

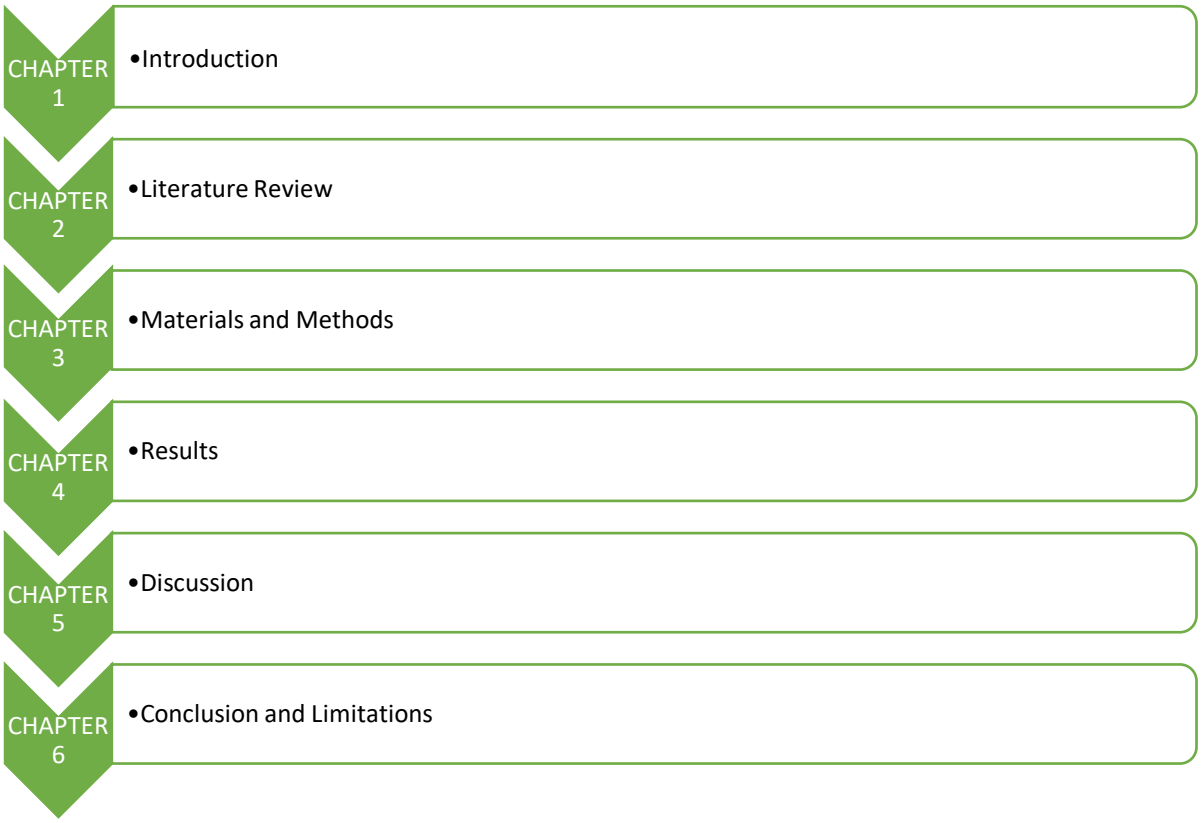


Figure 1.1: Layout of the dissertation

CHAPTER 2:

LITERATURE REVIEW

2.1 CANCER

2.1.1 History of cancer

Cancer existed long before humans on Earth, with clear evidence of tumours in animals found by palaeontologists (Hajdu, 2011). The term “cancer” originates from the Greek word “carcinos”, which means “crab” (American Cancer Society [ACS], 2018). The Greek physician Hippocrates (460 to 375 BCE) (see Figure 2.1A) described malignancy as having the appearance of a finger-like projection similar to the structure of a crab (Hajdu, 2012). Subsequently, the Roman physician Aulus Cornelius Celsus (25 BCE to 50 CE) transformed the word “carcinos” into “cancer”, which also means “crab” in Latin (ACS, 2018). The earliest recorded description of cancer is found in the Edwin Smith Papyrus (see Figure 2.1B), an ancient Egyptian document written by the physician-architect Imhotep (Faguet, 2015). The Smith Papyrus referred to the first recorded case of breast cancer (Case 45), which consisted of protruding advanced tumours that were incurable (Faguet, 2015). The oldest reported case of disseminated cancer dates back approximately 2 700 years to the Scythian king, aged 40 to 50, from Southern Siberia, who suffered from prostate carcinoma (Schultz *et al.*, 2007) (see Figure 2.2). One of the oldest theories proposed by Hippocrates stated that the body is composed of four bodily fluids (blood, black bile, yellow bile, and phlegm), and imbalances among them, particularly with black bile dominance, can result in cancer (Sudhakar, 2009).

In the 17th century, Italian anatomist Gaspare Aselli (1581 to 1625) identified the vessels of the lymphatic system and proposed that cancer arises from a malformation of lymph (Natale *et al.*, 2021). Claude Gendron (1663 to 1750), a French physician, rejected this theory and concluded that cancer emerges from a growing mass that is untreatable with drugs (Mandal, 2019). In the 18th century, French scientists Bernard Peyrilhe and Jean Astruc conducted experiments to confirm previous cancer theories (Shaikh *et al.*, 2021). As a result of their efforts, the diagnosis and treatment of cancer became better understood, which led to the establishment of medical institutions that focused primarily on cancer (Shaikh *et al.*, 2021).

In the late 19th century, improvements in microscopes enhanced awareness of cell biology (Hadju, 2012). During the early 20th century, researchers aimed to understand cell structure, function, and chemistry (Shaikh *et al*., 2021). They proposed theories on various cancer treatments, and in 1911, the first reported data in literature concerning the causes of cancer in chickens were published, which analysed both physical and chemical carcinogens (Sudhakar, 2009). In 1913, two key publications revealed various signs associated with cancer and aimed to educate the public about the disease (Shaikh *et al*., 2021).

The National Cancer Institute (NCI) Act of 1937, enacted by the United States Congress, established a research institute to develop new theories through conducting research, promoting cancer research in other institutions, and assembling cancer-related programmes (NCI, 2022). In 1971, the National Cancer Act was signed by President Richard Nixon, which launched the National Cancer Program regulated by the NCI (Brawley & Goldberg, 2021). Since the establishment of this institute, cancer research has significantly advanced.

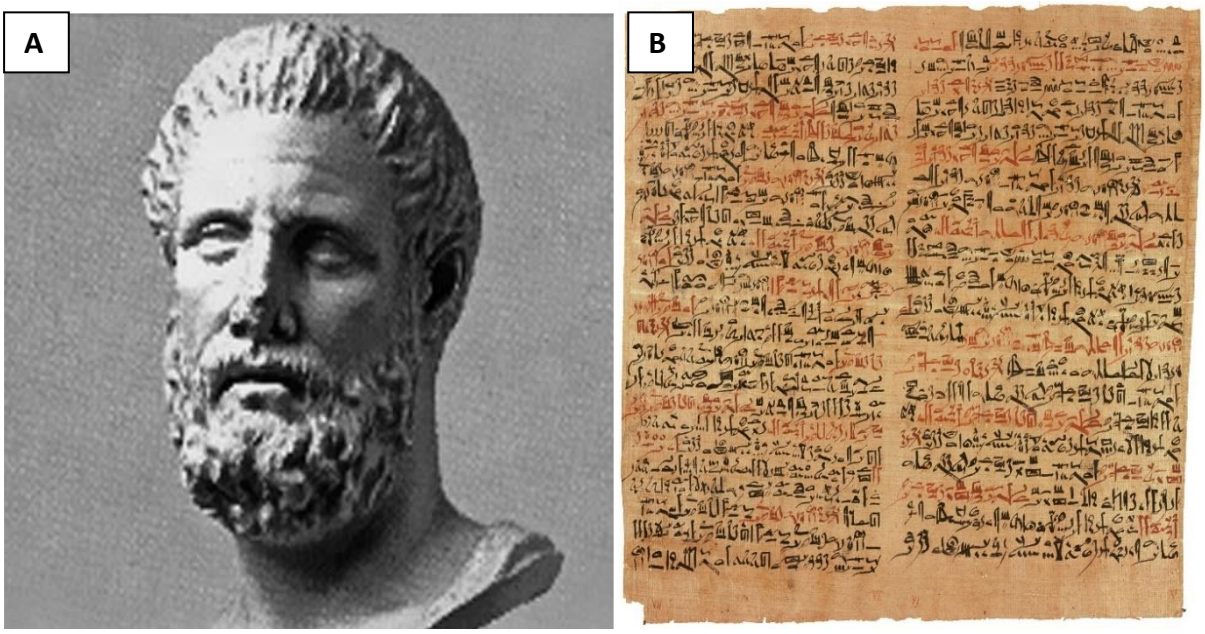


Figure 2.1: (A) Bust of Hippocrates (150 BCE) at the Archeological Museum of Kos and (B) Edwin Smith Papyrus

Source: Faguet (2015) (A); Martin *et al*. (2005) (B)

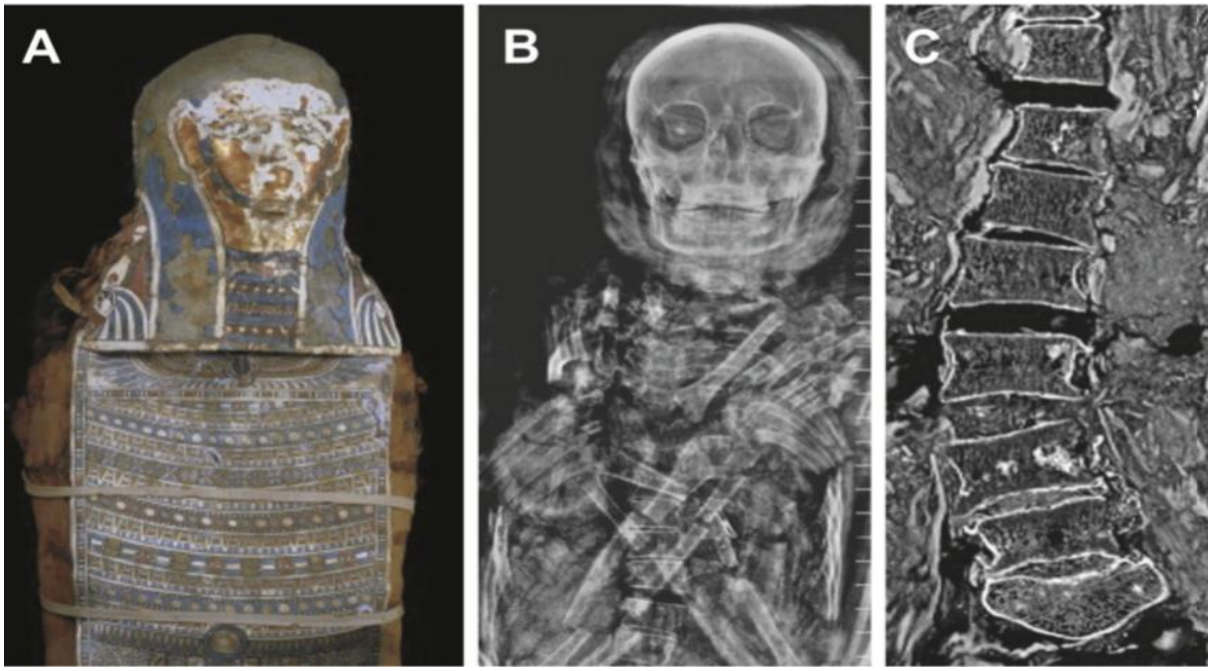


Figure 2.2: (A) The Egyptian human mummy from *Museu Nacional de Arqueologia* collection in Lisbon; (B) a digital X-ray image of the upper body; and (C) a high-resolution computed tomography scan of lumbar spine with bone lesions due to metastatic prostate cancer

Source: Prates *et al*. (2011)

2.1.2 Cancer burden

Cancer is a global burden that dreadfully affects individuals physically and emotionally (Greenwell & Rahman, 2015). In 2019, cancer ranked as the first or second leading cause of death in 112 countries and third or fourth in 23 other countries, of 183 in total, before the age of 70 years (see Figure 2.3) (WHO, 2020). The increasing global cancer statistics are due to population growth and ageing, and the economic, social, and lifestyle changes associated with human development are expected to increase the magnitude of cancer in the coming years (Fidler *et al*., 2018). Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) 2020 data, compiled by the International Agency for Research on Cancer from 185 countries, estimated 19.3 million new cases of cancer and 10 million deaths by 2020 (see Figure 2.4) (Sung *et al*., 2021). Countries with a high / very high Human Development Index (HDI) had higher incidence rates of cancer, ranging between 335.3 and 267.6 per 100 000, whereas countries with a low/medium HDI had 104.3 to 128.0 per 100 000 for both genders (Sung *et al*., 2021). The mortality rates of men in high HDI countries were higher (122.9 to 141.1 per

100 000) than in low HDI countries (76.7 to 78.0 per 100 000); however, there were small variations in HDI levels for women (67.0 to 88.4 per 100 000) (Sung *et al*., 2021).

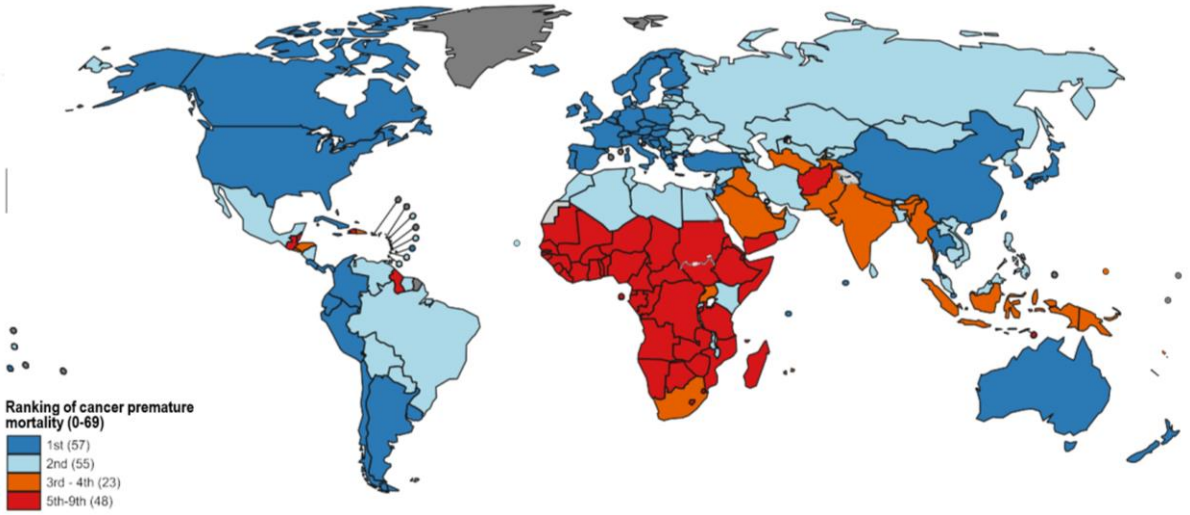


Figure 2.3: National cancer ranked as a cause of mortality at <70 years of age in 2019

Source: Sung *et al.* (2021)

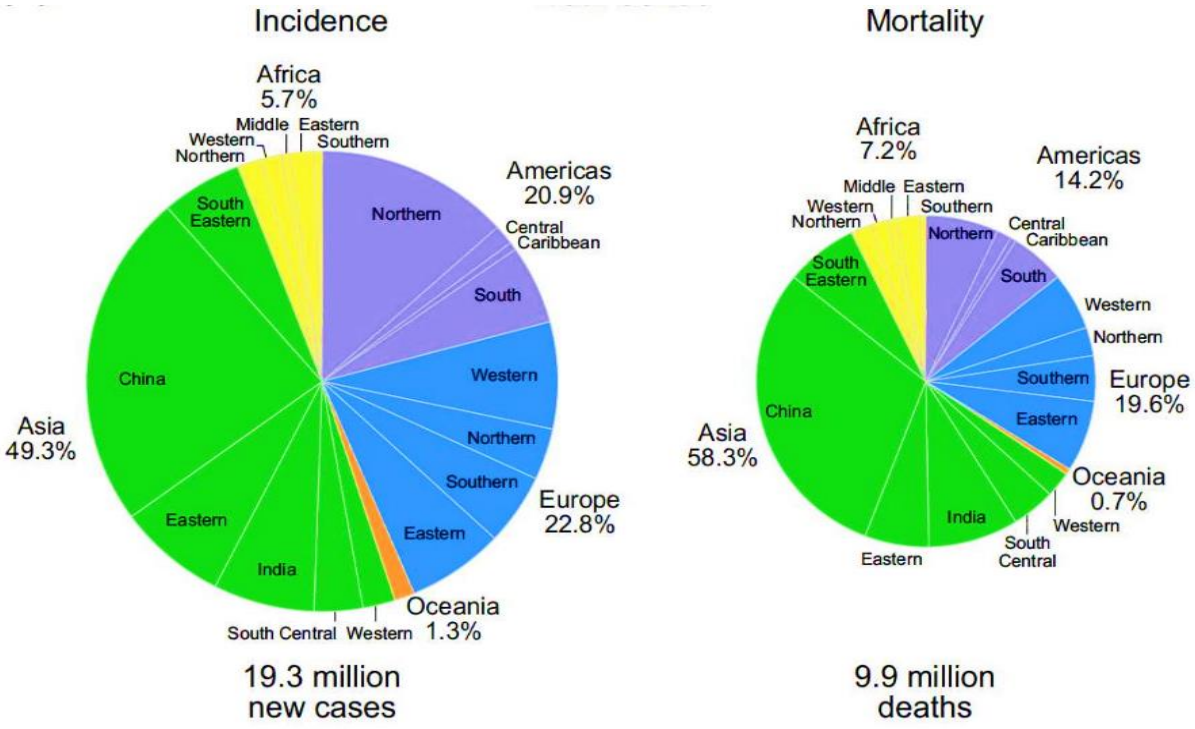


Figure 2.4: Global distribution of cancer prevalence and deaths for both sexes in 2020

Source: Sung *et al.* (2021)

In men, the most prevalent cancers are lung, prostate, colorectal, and liver cancer, while women are primarily diagnosed with breast, cervical, lung, and colorectal cancer (Hassanpour & Dehghani, 2017). Although higher cancer prevalence is linked to socio-economic development, developing countries face a significant risk of increased cancer rates as their lifestyles and economic conditions improve (Kanavos, 2006). Nevertheless, approximately one-third to one-half of cancers can be prevented by adopting a healthy lifestyle (Arem & Loftfield, 2018). Furthermore, early detection and advancements in treatment strategies have led to increased cancer survival rates (Arem & Loftfield, 2018). Despite these advances in cancer prognosis and treatment, cancer prevalence is predicted to rise by 47% by 2040, with an estimated 28.4 million new cases (Sung *et al*., 2021).

2.1.3 Cancer overview

The abnormal proliferation of cells in the human body is characterised as a tumour, which can either be benign or malignant (Sinha, 2018). Benign tumours consist of non-cancerous cells that do not invade neighbouring tissues, whereas malignant tumours grow uncontrollably and metastasise (Patel, 2020). The classification of cancer is based on the affected tissue or organ prior to metastasis (Mathur *et al*., 2015). Cancer can affect any part of the body, including the colon, breast, prostate, lungs, stomach, colorectal region, cervix, or brain (Drebert, 2017; Mathur *et al*., 2015). Breast cancer is the focus of this study.

2.1.4 Breast cancer

Cancer nomenclature is based on the body part from which it originally develops; breast cancer therefore refers to the irregular growth and multiplication of cells arising from breast tissue (Sharma *et al*., 2010). Breast cancer remains the most common type of cancer in women. In 2020, there were approximately 2.3 million new breast cancer cases globally, which accounted for 11.7% of all cases, with a mortality rate of 6.9% (Arnold *et al*., 2022). The prevalence of breast cancer is generally higher in transitioned countries with low death rates, compared to transitioning countries with low incidence and higher mortality rates (see Figure 2.5) (Kashyap *et al*., 2022; Sung *et al*., 2021).

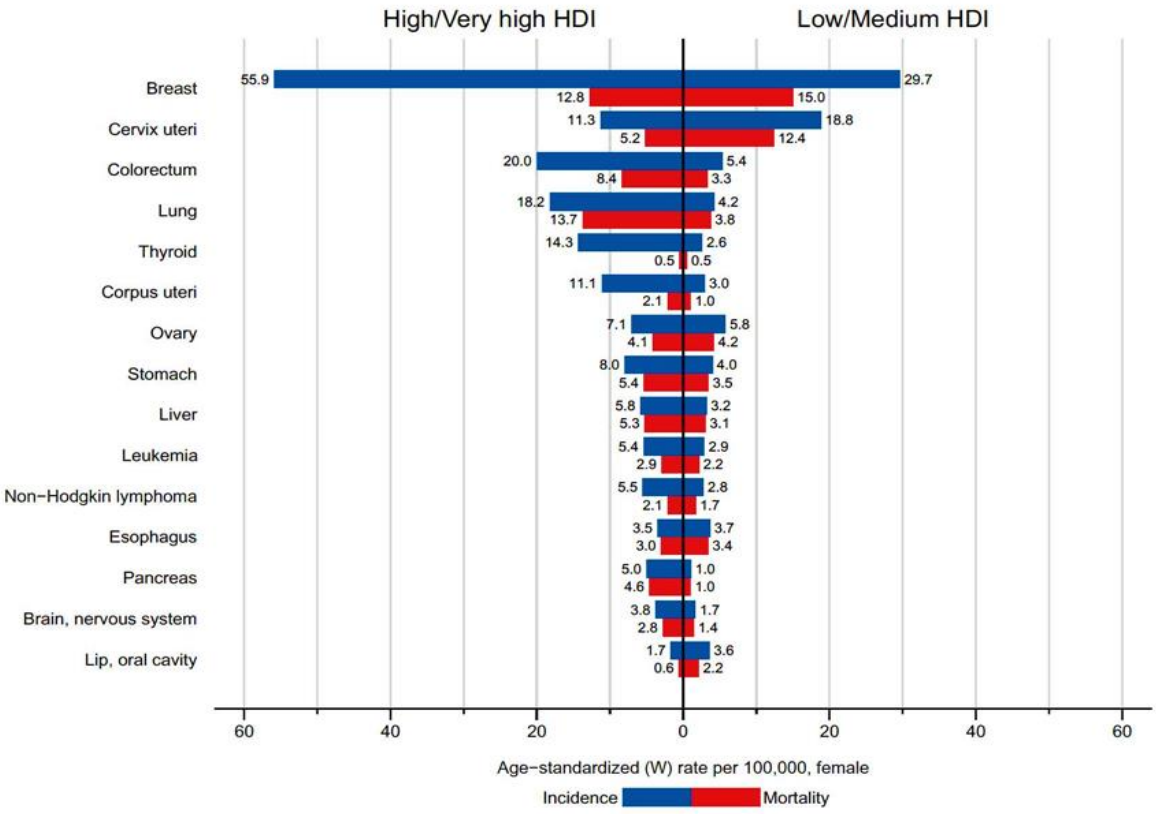


Figure 2.5: Age-standardised incidence and mortality rates of cancer in high / very high Human Development Index (HDI) countries versus low/medium HDI countries among women in 2020

Source: Sung *et al.* (2021)

Reports indicate that approximately 80% of women diagnosed with breast cancer are over 50 years of age (Łukasiewicz *et al.*, 2021). Breast cancer is characterised according to tumour grade, histological type, and predictive markers such as human epidermal growth factor receptor 2 (HER2), oestrogen receptor, and lymph node status (Tungsukruthai *et al.*, 2018). Several risk factors, including gender, alcohol consumption, obesity, family history, reproductive factors (early puberty, early menarche, late menopause), and genetic predisposition, are associated with the development of breast cancer (Kashyap *et al.*, 2022). Breast cancer management comprises various treatment strategies, including surgery, chemotherapy, radiotherapy, and others, which are implemented to prolong patients' life expectancy (Akram *et al.*, 2017).

2.1.4.1 Molecular development of breast cancer

Breast cancer is a heterogeneous disease characterised by variations in pathological characteristics, differing responses to therapies, and significant differences in the long-term survival of patients (Rivenbark *et al*., 2013). At the molecular level, breast cancer is classified into five subtypes: Luminal A, Luminal B, HER2, basal-like, and Claudin-low (Tungsukruthai *et al*., 2018). In breast cancer cells, the most altered genes include TP53, PIK3CA, CCND1, PTEN, FGFR1, MYC, ERBB2, and GATA3 (Nik-Zainal *et al*., 2016). These genes encode cell cycle regulators that can either be activated (e.g., cyclin D1) or repressed (e.g., p53) to sustain proliferation and inhibit apoptosis (Harbeck *et al*., 2019). Epigenetic modifications also play a role in carcinogenesis through hypomethylation (gene activation, oncogene upregulation, and chromosomal instability) or hypermethylation (transcriptional silencing) (Harbeck *et al*., 2019). Other epigenetic alterations may include histone modification due to deoxyribonucleic acid (DNA) methylation, which leads to chromosomal structural changes (Harbeck *et al*., 2019). In the pathogenesis of breast cancer, the most predominant altered pathways include HER2 and oestrogen receptor pathways (Song *et al*., 2014).

2.1.4.2 Human epidermal growth factor receptor 2 (HER2) signalling pathway

HER2/neuroblastoma expressed uponcogene (neu), also known as ErbB2, is a transmembrane glycoprotein encoded by the oncogene HER2/neu located on chromosome 17q (see Figure 2.6) (Lv *et al*., 2016). HER2 is part of the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors, which have an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain (Feng *et al*., 2018). When specific ligands bind to the extracellular domain of EGFR, receptor homodimerisation or heterodimerisation occurs (Shah & Osipo, 2016). This receptor dimerisation enhances the phosphorylation of tyrosine kinase residues, which further activate downstream signalling pathways such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) (Toss & Cristofanilli, 2015). Tyrosine kinase phosphorylation, in turn, activates enzymes or adapter proteins that regulate cellular processes such as adhesion, growth, apoptosis, migration, and differentiation (Esteva & Pusztai, 2005).

HER2 plays a crucial role in different cellular processes, and its expression level is kept stable in normal cells (Lv *et al.*, 2016). In contrast, the overexpression and amplification of HER2 result in tumorigenesis and metastasis (see Figure 2.7) (Lv *et al.*, 2016). The HER2-positive type of breast cancer is hallmarked by the overexpression of HER2 protein and is reported to comprise 20% to 50% of all breast cancers (Toss & Cristofanilli, 2015).

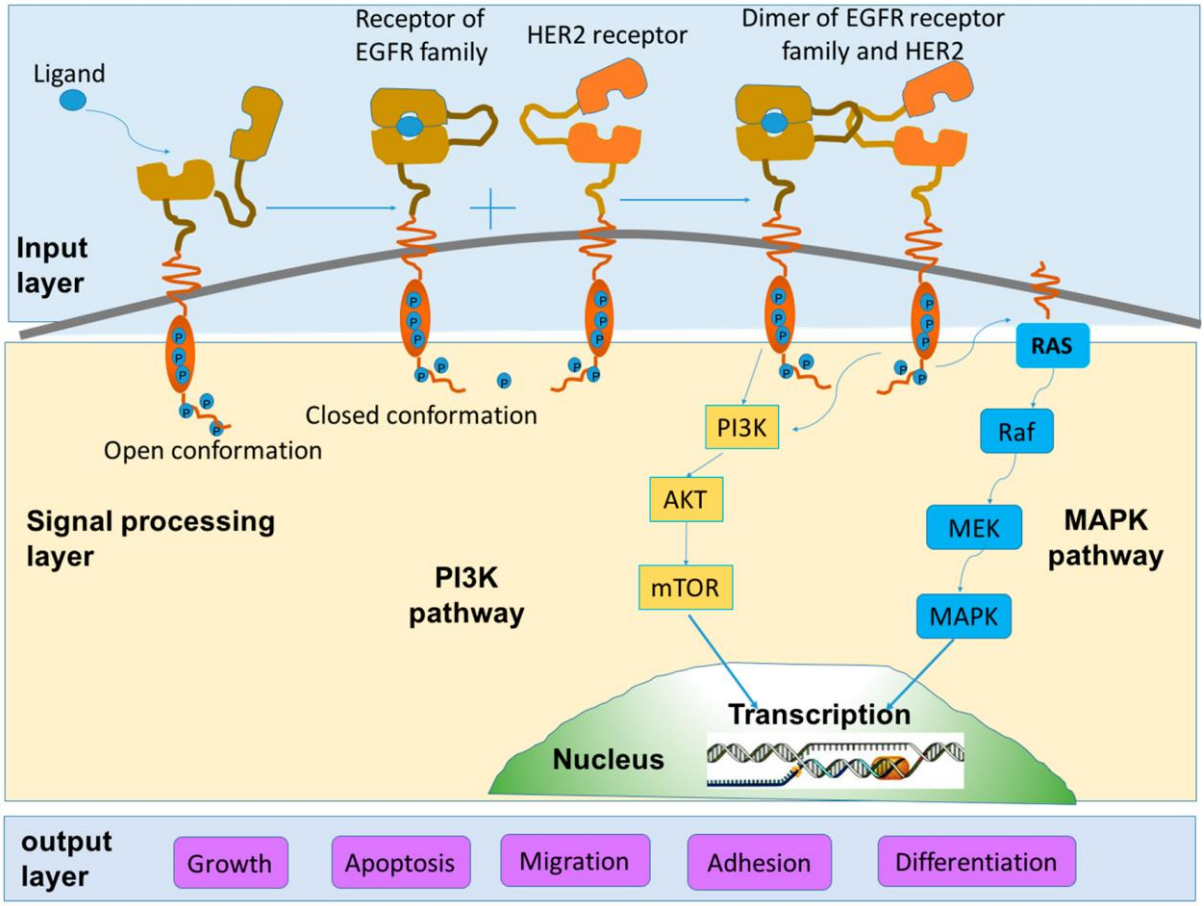


Figure 2.6: A detailed illustrative diagram of the HER2 signalling pathway

Source: Lv *et al.* (2016)

As shown in Figure 2.6, following ligand binding, dimerisation between the HER2 receptor and EGFR family is activated. The homodimers or heterodimers will then trigger the cascade signalling pathways. The two main signalling pathways, PI3K and MAPK, play a vital role in cancer proliferation apoptosis inhibition.

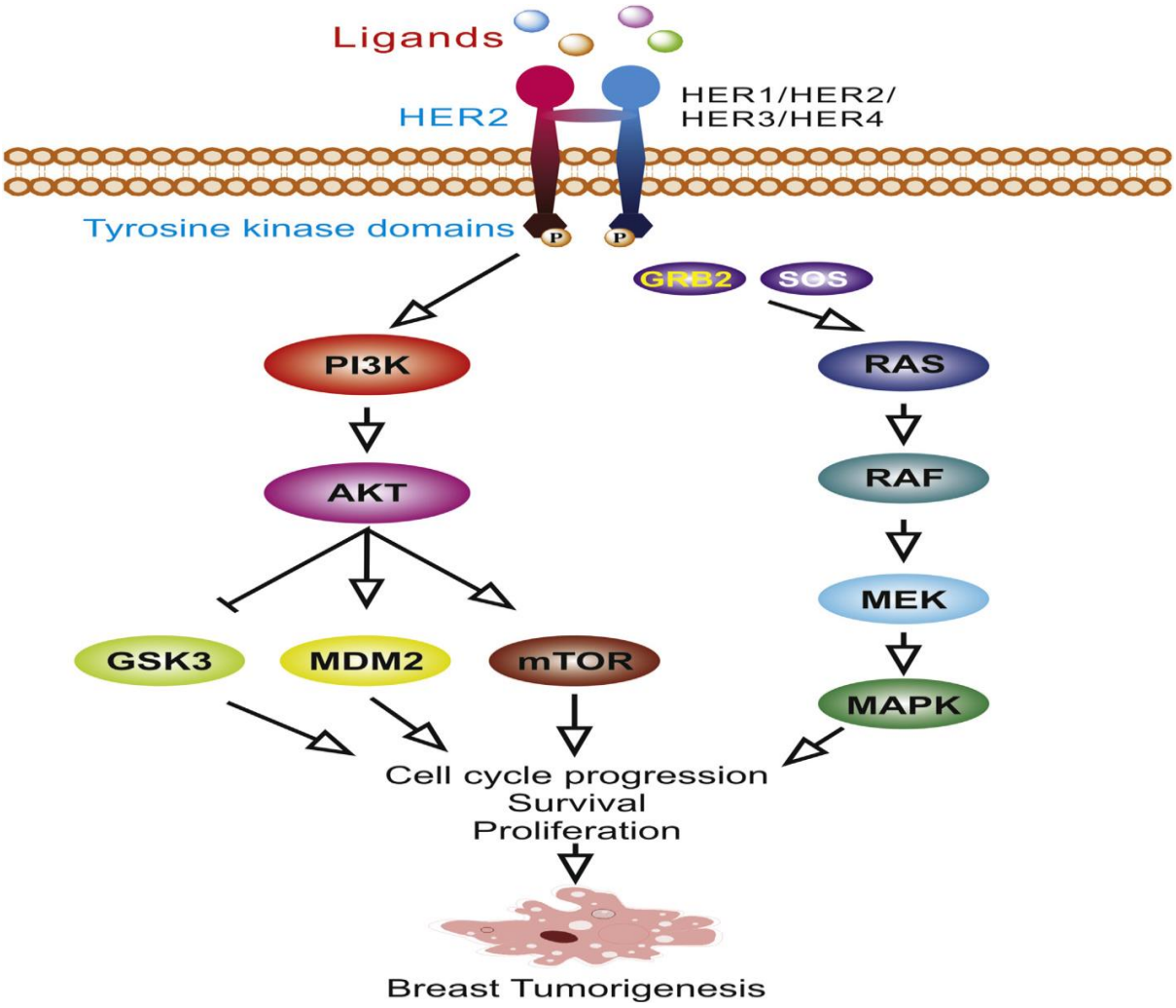


Figure 2.7: The two oncogenic signalling pathways PI3K and MAPK that are associated with breast tumorigenesis

Source: Feng *et al.* (2018)

2.1.4.3 Oestrogen receptor signalling pathway

The oestrogen receptor is a ligand-dependent transcription factor that regulates downstream gene expression (see Figure 2.8) (Chi *et al.*, 2019). It is responsible for activating or repressing the expression of ligand-binding targeted genes (Feng *et al.*, 2018). In the oestrogen receptor signalling pathway, ligand binding leads to the formation of homodimers and heterodimers, which regulate transcription in the cell nucleus (Feng *et al.*, 2018). The luminal molecular type of breast cancer accounts for approximately 60% of all breast cancers and is characterised by the expression of the oestrogen receptor, with higher expression in type A than in type B (Zhang, 2023).

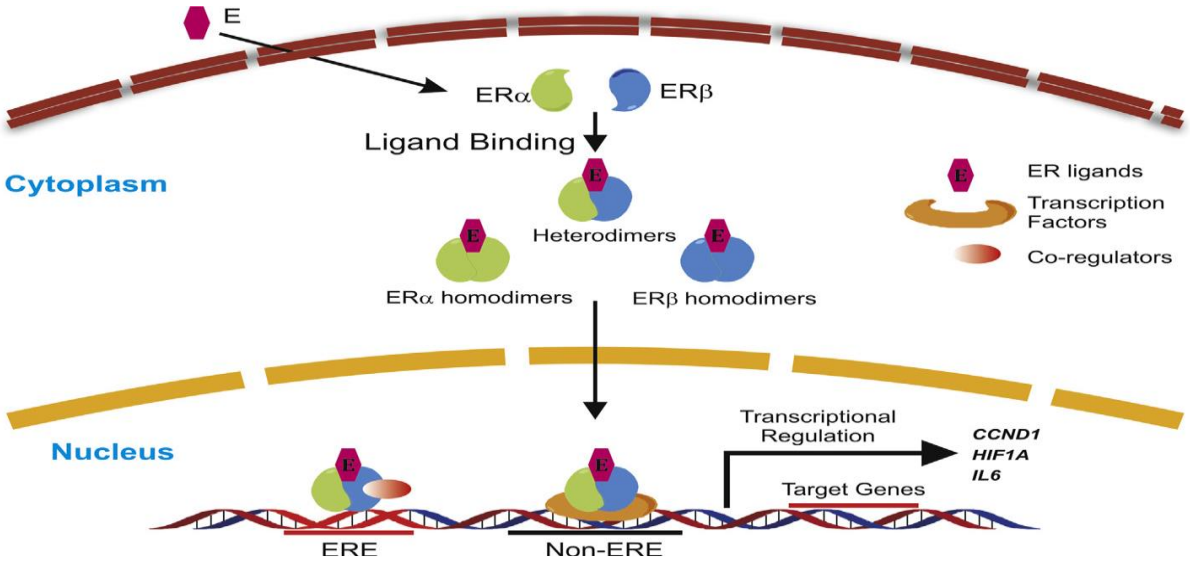


Figure 2.8: Schematic analysis of oestrogen receptor signalling pathway

Source: Feng *et al.* (2018)

Figure 2.8 illustrates that in breast cancer cells, the expression of oestrogen receptor alpha is relatively high while that of oestrogen receptor beta is low. Upon ligand binding, the two nuclear hormone receptors form homodimers or heterodimers to regulate transcription in the cell.

2.1.4.4 Pathology of breast cancer

Breast cancer initially emerges from *in situ* before it spreads by metastasis, with 85% developing from the epithelium of the ducts and 15% from the lobules of the breast tissue (WHO, 2021). Carcinomas are the most common type of breast cancer. They arise from the epithelial unit of the breast, which consists of cells that line the ducts and lobules (see Figure 2.9) (Feng *et al.*, 2018). Sarcomas are extremely rare and constitute less than 1% of all breast cancers. They originate from the connective tissues of the breast (Feng *et al.*, 2018). Ductal carcinoma *in situ* (DCIS; also known as intraductal carcinoma) is the most common non-invasive type of breast cancer. It develops from the ductal lobular unit and potentially progresses to invasive cancer over time (ACS, 2021). However, early diagnosis and treatment can prevent the malignant tumour from metastasising (Salvatorelli *et al.*, 2020). DCIS is considered the earliest stage (Stage 0) of breast cancer (Van Seijen *et al.*, 2019). Invasive breast cancer is a type that has spread and invaded the surrounding breast tissue (ACS, 2021).

Approximately two-thirds of women diagnosed with invasive breast cancer are 55 years of age or older (Feng *et al.*, 2018).

Invasive breast cancer is further divided into two types: invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). IDC initially develops from the milk duct and invades the wall of the duct, subsequently spreading to the neighbouring tissues of the breast (ACS, 2021). This malignancy can penetrate other parts of the body through the circulatory and lymphatic systems (ACS, 2021). IDC is the most common subtype, accounting for 80% of all breast cancers, and constitutes many cases of breast cancer mortality worldwide (Feng *et al*., 2018; Zangouri *et al.,* 2018). ILC begins in the lobules and progresses to other parts of the body (ACS, 2021). It is the second most common breast cancer subtype, following IDC (Zhu *et al.,* 2015), and is responsible for 10% to 15% of all breast cancers (Luveta *et al*., 2020). Unlike other breast cancer subtypes, ILC is most likely to occur in both breasts (ACS, 2021). The metastatic type of breast cancer (also called Stage IV) is an advanced or late stage where cancer cells have invaded other parts of the body from their original site (Feng *et al*., 2018). Metastasis begins with local invasion by cells from the original tumour site, which then spread and intravasate into the blood and lymphatic systems (Scully *et al*., 2012). Metastasis accounts for approximately 90% of breast cancer mortality in women (Tungsukruthai *et al*., 2018). In breast cancer, metastasis preferentially targets the brain, liver, bones, and lungs (Méndez-García *et al*., 2019). Early detection of metastatic breast cancer is significant for predicting its progression and management (Scully *et al*., 2012).

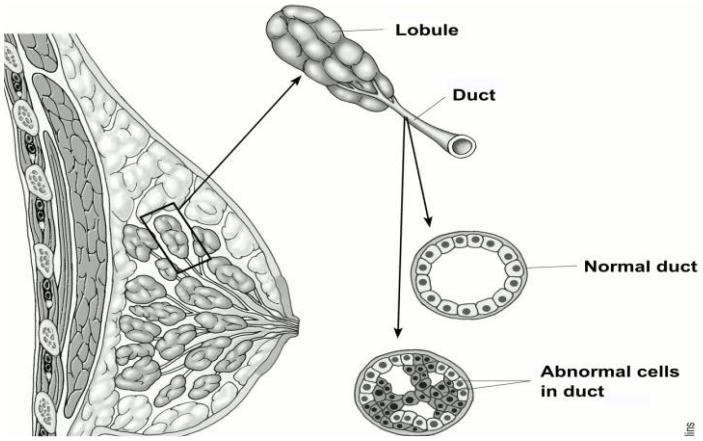


Figure 2.9: Breast cancer structure showing the difference between normal and cancerous cells in the duct

Source: ACS (2021)

2.1.4.5 Risk factors associated with the development of breast cancer

Several risk factors are associated with the development of breast cancer, including gender, alcohol consumption, family history, genetics, obesity, and reproductive factors (Feng *et al*., 2018; Mehrgou & Akouchekian, 2016; Mufudza *et al*., 2012; Protani *et al*., 2010; Richie & Swanson, 2003; Shiovitz & Korde, 2015; Sun *et al*., 2017; WHO, 2021).

- Gender: Breast cancer occurs more frequently in women than in men, with approximately 0.5% to 1% of cases occurring in men (WHO, 2021). In women, the incidence of breast cancer increases with age (Richie & Swanson, 2003).
- Alcohol consumption: Alcohol increases the risk of developing breast cancer, and the higher the consumption, the greater the risk (Feng *et al*., 2018). A woman who consumes up to three alcoholic drinks a day has about a 20% risk of developing breast cancer compared to a woman who does not consume alcohol at all (Feng *et al*., 2018). Alcohol intake can elevate oestrogen-related hormone levels in the blood and activate the oestrogen receptor pathway (Sun *et al*., 2017).
- Family history: A woman with two or more first-degree relatives diagnosed with breast cancer has a 2.5-fold increased risk of developing the disease (Sun *et al*., 2017). The risk is greater for women with a first-degree family member diagnosed before the age of 50 compared to those diagnosed after 50 years (Brewer *et al*., 2017).
- Genetics: Genetics plays a limited role in breast cancer development, with only 5% to 6% of breast cancers linked to heredity (Richie & Swanson, 2003). The high-penetrance BRCA1 and BRCA2 genes are associated with breast cancer development (WHO, 2021). A genetic alteration in either BRCA1 or BRCA2 results in an increased likelihood of breast cancer (Mehrgou & Akouchekian, 2016). Women with mutated BRCA1 or BRCA2 genes have a 50% to 85% risk of developing breast cancer (Shiovitz & Korde, 2015). There are other, albeit rare, genes such as TP53, PTEN, CHEK2, CDH1, STK11, PALB2, and ATM that increase susceptibility to breast cancer (Feng *et al*., 2018).
- Obesity: Obese women have a higher chance of developing breast cancer due to their oestrogen levels (Mufudza *et al*., 2012). The aromatase enzyme in adipose tissue produces oestrogen, which stimulates the growth of breast tumours (Bhardwaj *et al*., 2019). Obese women who are diagnosed with breast cancer have an 11% decreased chance of survival; however, this is influenced by the stage of menopause (Protani *et*

al., 2010). Additionally, obese women are more at risk of local recurrence of breast cancer compared to women of normal weight (Lee *et al.*, 2019).

- Reproductive factors: Increased exposure to oestrogen, such as early menarche, late menopause, breastfeeding, and advanced age at first pregnancy, enhances the development of breast cancer (Ataollahi *et al.*, 2015; Sun *et al.*, 2017). A one-year delay in menopause increases the chance of breast cancer occurrence by 3% (Sun *et al.*, 2017). A delay in menarche by one year and an additional childbirth reduce breast cancer risk by 5% to 10% (Sun *et al.*, 2017).

2.1.4.6 Treatment of breast cancer

Previously, breast cancer was managed solely through tissue eradication; however, it now necessitates an interdisciplinary team comprising medical, surgical, psychological, and social professionals to achieve the best possible outcomes for patients (Freeman *et al.*, 2018). Breast cancer treatments are integrative approaches that rely on prompt diagnosis. However, challenges in cancer treatment and management include a lack of resources, poor infrastructure, financial limitations in low- to middle-income countries, and insufficient healthcare personnel (Mutebi *et al.*, 2020).

Chemotherapy is a type of cancer treatment that involves using anti-cancer drugs (Shamsi & Islamian, 2017). It can be classified as either adjuvant or neoadjuvant, depending on the characteristics of the breast cancer tumour (Łukasiewicz *et al.*, 2021). Chemotherapy drugs include Paclitaxel, Docetaxel, platinum agents (carboplatin, cisplatin), liposomal doxorubicin, capecitabine, cyclophosphamide, vinorelbine, and carboplatin (Akram *et al.*, 2017). These drugs suppress cell proliferation and tumour growth and prevent invasion and metastasis. Surgical procedures, including breast-conserving therapy (BCT) and mastectomy, remain central to breast cancer treatment (see Figure 2.10) (Leclerc *et al.*, 2016). When considering surgery, the stage and type of breast cancer are taken into account (Sharma *et al.*, 2010). BCT involves excising the tumour from the breast (Moo *et al.*, 2018). This strategy has been successfully employed to enhance the cosmetic outcomes for breast cancer patients by allowing small portions of the breast to be removed (Buchholz *et al.*, 2015). To perform surgical excision, tumour position and cosmetic considerations are used as guidance (McDonald *et al.*, 2016). Examples of BCT include lumpectomy, partial mastectomy,

segmental excision, re-excision, wide excision, and quadrantectomy, which depend on the amount of tissue to be removed (Richie & Swanson, 2003). This surgical technique aims to preserve breast tissue while achieving satisfactory results (Rahman, 2011). During the procedure, the surgeon must ensure that the removed tissue has clear margins that are free of cancer; however, if this goal is not met, re-excision becomes essential (Sharma *et al.*, 2010). Mastectomy involves the surgical process of removing the entire breast tissue (Goethals & Rose, 2022).

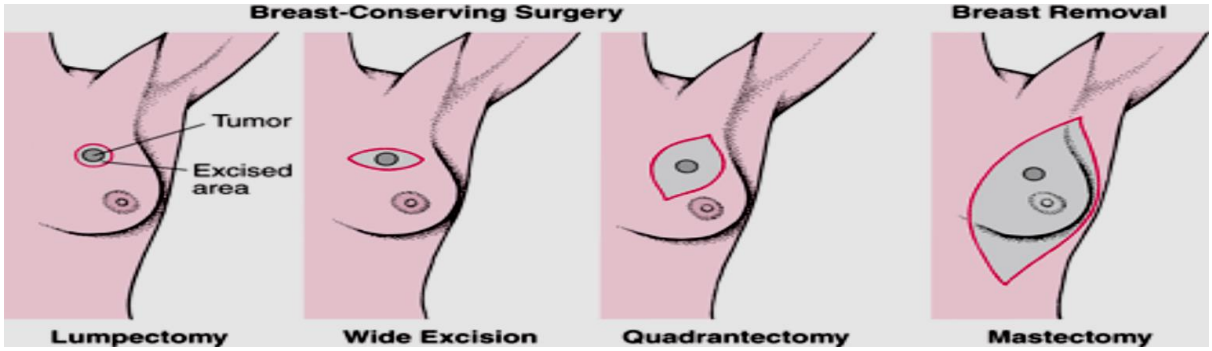


Figure 2.10: Various breast-conserving therapies (BCTs) and total mastectomy

Source: Sharma *et al*. (2010)

Radiation therapy is a fundamental part of the interdisciplinary treatment of breast cancer (Haussmann *et al*., 2020). It involves the use of high-energy rays to destroy cancerous cells and prevent their growth and division (Gianfaldoni *et al*., 2017). This type of radiation is known as ionising radiation, as it forms ion particles and releases energy to the cells (Baskar *et al*., 2012). Ionising radiation destroys the DNA of the cell, which leads to cell death and tumour reduction (Chaput & Regnier, 2021). Despite the fact that radiation causes damage to both normal and cancerous cells, the objective of this therapy is to increase the dose in abnormal cells while decreasing exposure in non-cancerous cells (Baskar *et al*., 2012). Radiotherapy can be carried out internally or externally. External beam radiotherapy is administered from outside the body and targets high radiation to the tumour area (Baskar *et al*., 2012). Internal radiotherapy (brachytherapy) occurs inside the body, using radioactive sources that directly aim at the tumour (Baskar *et al*., 2012). Radiation therapy following BCT helps to prevent recurrence and enhances survival rates for breast cancer (Maughan *et al*., 2010). Moreover, this treatment can be used after mastectomy to hinder recurrence on the thoracic wall and in the axilla (Richie & Swanson, 2003).

Treatment of breast cancer is, however, reported to cause various long-term drawbacks in patients (Leclerc *et al.,* 2016). These may include physical effects (e.g., secondary cancers, fatigue, dental problems, cardiopulmonary complications, neuropathy, lymphedema, and musculoskeletal disorders) and mental disorders (e.g., anxiety, depression, lack of concentration, dementia, and memory loss) (Kristoffersen *et al.,* 2022). There is thus a need to develop complementary or alternative medicine with fewer side effects from medicinal plants.

2.2 TRADITIONAL MEDICINAL PLANTS (TMPs)

Over the years, humans have depended on nature to meet their basic needs, such as for food, clothing, shelter, transport, medicine, fertilisers, fragrances, and flavours (Cragg & Newman, 2005). The oldest documented data on medicinal plants date back to 1500 BCE, found in the Ebers Papyrus by ancient Egyptians, which contains information about 850 medicinal plants, including aloe, cannabis, garlic, and castor bean (Petrovska, 2012). TMPs continue to play a significant role in the production of medicine in various parts of the world. Today, approximately 80% of the world's population relies heavily on medicinal herbs for treating several illnesses (WHO, 2025). The use of medicinal plants in the discovery and development of new anti-cancer agents is currently a focal point in research (Seca & Pinto, 2018).

In developing countries, many people use TMPs as their primary therapeutic strategy, as well as for alternative or complementary medicine (Sánchez *et al.*, 2020). The extensive use of TMPs by local communities is attributed to their availability, low cost compared to modern medicine, and perceived safety, as reported by users (Fennell *et al.*, 2004). Additionally, local communities often rely on alternative medicine due to their better knowledge of TMPs and limited access to healthcare facilities (Hussain *et al.*, 2018). For centuries, human and livestock ailments have been treated with TMPs in rural communities (Moges & Moges, 2019). It is estimated that 80% of the South African population, particularly in rural areas, seeks medical advice from traditional healers in addition to Western health practitioners (Boum *et al.*, 2021). Nonetheless, the use of herbal medication without consulting a medical practitioner raises concerns.

2.2.1 Uses of TMPs

The major significance of TMPs to humans lies in the medicinal benefits derived from their rich secondary metabolites (Anand *et al*., 2019). They are primarily used to treat common illnesses in communities (Mintah *et al*., 2019). TMPs are not only employed in the treatment of various ailments but are also commonly found in beauty products, such as body lotions, facial creams, and oils (Mohiuddin, 2019). Additionally, medicinal plants are used as culinary herbs due to their strong flavours in food; examples include parsley, oregano, basil, and thyme (Yudharaj *et al*., 2016). They are also utilised as aromatic herbs because of the pleasant scents found in their flowers, which are used to produce perfumes, with manufacturers often opting for mint, vanilla, and rosemary (Yudharaj *et al*., 2016).

2.2.2 Advantages of TMPs

An advantage of using TMPs is that they are composed of various compounds with multiple activities, which together produce a greater effect (Schmidt *et al*., 2008). Ethnomedicine positively contributes to the healthcare sector by isolating bioactive compounds for drug discovery (Singh *et al*., 2020). TMPs are reported to provide a source of income for some African communities, as they are commonly sold at local herbal medicine markets (Rasethe *et al*., 2019). As expected, the demand for TMPs is increasing, which results in a greater risk of extinction or loss of genetic variation (Sofowora *et al.,* 2013).

2.2.3 Limitations of TMPs

Medicinal plants play a significant role in our everyday life; however, they are reported to have some limitations. To begin with, phytomedicine is not currently fully accepted by the healthcare sector due to concerns about efficacy, safety, poor quality control, and low standardisation of different components, such as inconsistent processing methods, variability in raw materials, and unclear identification and quantification of active ingredients (Thillaivanan & Samraj, 2014; Patwekar *et al.*, 2016; Umamaheswari *et al.*, 2021). In addition, unpleasant effects may arise from the incorrect use of TMPs over long periods (Mensah *et al.*, 2019). Furthermore, the concurrent use of alternative medicine alongside conventional medicine by patients poses a threat and can lead to potentially serious effects (Meshesha *et al.*, 2020). These effects include liver disorders, kidney damage, allergic reactions,

gastrointestinal issues, and life-threatening conditions such as cancer (Ekor, 2014). Some of the negative effects of TMPs may arise from contamination with toxic metals, improper preparation methods, such as incorrect drying or extraction techniques, and adulterations (Zhang *et al.*, 2015).

2.2.4 Secondary metabolites in TMPs

The native environment continues to be an important element of therapeutic drugs (Mathur & Hoskins, 2017). Natural therapeutic agents are derived from sources such as plants, animals, microbes, and minerals (Jabeen *et al*., 2014). Plants serve as the primary natural source of medicine due to their structural and chemical diversity (Mathur & Hoskins, 2017). They produce chemical compounds that protect against herbivores through salicylic acid, a hormone involved in the plant's defence mechanisms (Hayat & Ahmad, 2007). These chemical compounds, known as active secondary metabolites, possess therapeutic properties (Al-Snafi, 2015). Secondary metabolites include flavonoids, alkaloids, terpenoids, tannins, steroids, saponins, glycosides, and others (Shakya, 2016). They are reported to be pharmacologically effective; for example, alkaloids exhibit anti-cancer, anti-malarial, analgesic, anti-spasmodic, and diuretic properties. Glycosides have anti-bacterial and anti-fungal properties, while terpenoids are known for their anti-cancer, anti-bacterial, anti-malarial, anti-viral, anthelmintic, and anti-inflammatory activities. Flavonoids and phenols possess anti-bacterial, anti-oxidant, and anti-allergic properties, and saponins demonstrate anti-viral, anti-inflammatory, and plant defence activities (Maurya *et al*., 2008; Seca & Pinto, 2018).

2.2.5 TMPs as potential anti-cancer agents

Over 3 000 TMPs worldwide are reported to possess anti-cancer properties (Seca & Pinto, 2018). According to the literature, approximately 75% of synthetic anti-cancer drugs are derived from natural sources, particularly TMPs (Shaik *et al*., 2022). Clinical trials are in progress for more than 100 drugs derived from natural products, and a minimum of 100 compounds are in the preclinical development phase (Harvey, 2008). This suggests that natural products, particularly TMPs, may be a promising source for cancer treatment and management. Nevertheless, further investigation is essential to isolate more cytotoxic bioactive compounds from TMPs (Laskar *et al*., 2020). Paclitaxel (*Taxus brevifolia* L.),

Docetaxel (*T. baccata*), vinblastine (*Catharanthus roseus*), and vincristine (*C. roseus*) are some of the approved plant-based anti-cancer drugs in clinical trials (see Figure 2.11), whereas pomiferin (*Maclura pomifera*), sulforaphane (*Cruciferous*/*Brassica*), combretastatin A-4 phosphate (*Combretum caffrum*), and many others are under investigation (Laskar *et al*., 2020). Some common herbs are effective in breast cancer treatment due to the active ingredients they contain, including garlic (*Allium sativum*), which enhances the removal of carcinogens from the body; turmeric (*Curcuma longa*), which inhibits phases of cancer growth and development; and burdock (*Arctium lappa*), which contains phenolic properties that reduce cancer propagation (Shareef *et al*., 2016). In addition, other bioactive compounds have been isolated from fruit and vegetable sources with anti-cancer activity. *Mangifera indica*, commonly known as mango, contains mangiferin (see Figure 2.12A), a phytochemical that reduces the proliferation of cancer cells (Yap *et al.,* 2021). *Solanum lycopersicum*, a red tomato, has a major compound called lycopene (see Figure 2.12B), which has been reported to hamper cell cycle progression (Holzapfel *et al*., 2013). The isolation of these bioactive compounds from medicinal plants may lead to or contribute to the discovery of anti-cancer drugs. This could be advantageous, as the active compounds in TMPs may have fewer or no side effects when taken as medication by cancer patients.

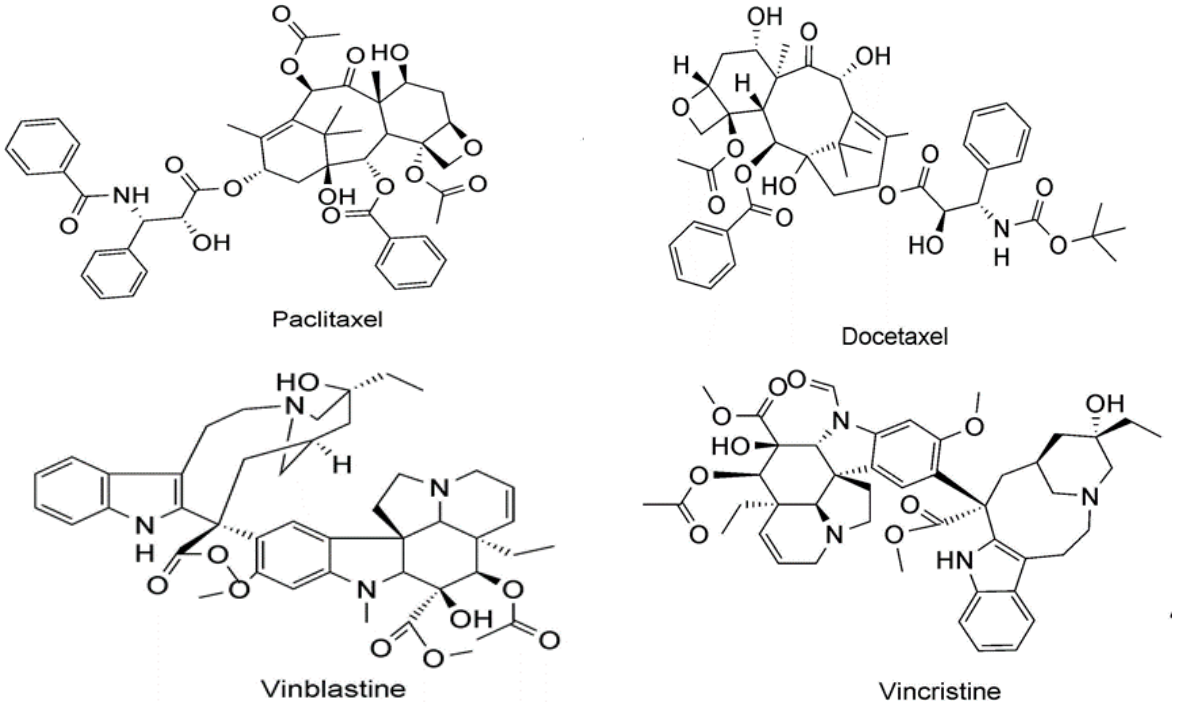
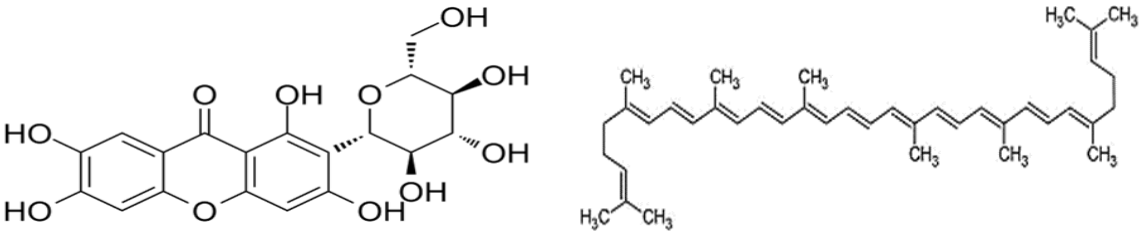


Figure 2.11: Approved plant-sourced anti-cancer drugs in clinical trial

Source: Shaik *et al.* (2022)



(A) Mangiferin (B) Lycopene

Figure 2.12: Chemical structure of compounds isolated from fruit/vegetable sources: Mangiferin and lycopene

Source: Li *et al*. (2013); Gupta *et al*. (2018)

2.2.6 Drug discovery and development from TMPs

Drug discovery is an integrated process for developing new therapeutic drugs to be used in the management and treatment of medical conditions (Deore *et al*., 2019). It aims to identify new chemical entities (NCEs) with specific druggability and medicinal chemistry qualities, which can be acquired through the isolation of bioactive compounds from natural products or through chemical synthesis (Katiyar *et al*., 2012). Active compounds can therefore serve as a guide to NCEs rather than being used directly (Salim *et al*., 2008). TMPs contain many individual bioactive constituents that vary in abundance, and the ability to isolate them for their biological effects can be a significant challenge (Enke & Nagels, 2011). In identifying compounds as potential candidates for developing NCEs, the screening method is fundamental for discovering their bioactivities (Dawurung *et al.,* 2021). This crucial step enables researchers to determine whether the isolated compounds act individually or synergistically as a mixture (Gurib-Fakim, 2006).

The overall biological activity of the crude extract is considered to result from a mixture of constituents that work synergistically, whereas the lack of activity from single compounds may have been lost during the fractionation process (Caesar & Cech, 2019). Nonetheless, individual compounds should be isolated and purified from multi-component mixtures using a combination of chromatographic techniques (Sasidharan *et al*., 2011). Several methods are utilised to acquire active compounds for drug discovery, such as extraction, isolation, chemical characterisation, synthesis, molecular modelling, and combinatorial chemistry, as shown in Figure 2.13 (Choudhury *et al*., 2020; Shakya, 2016).

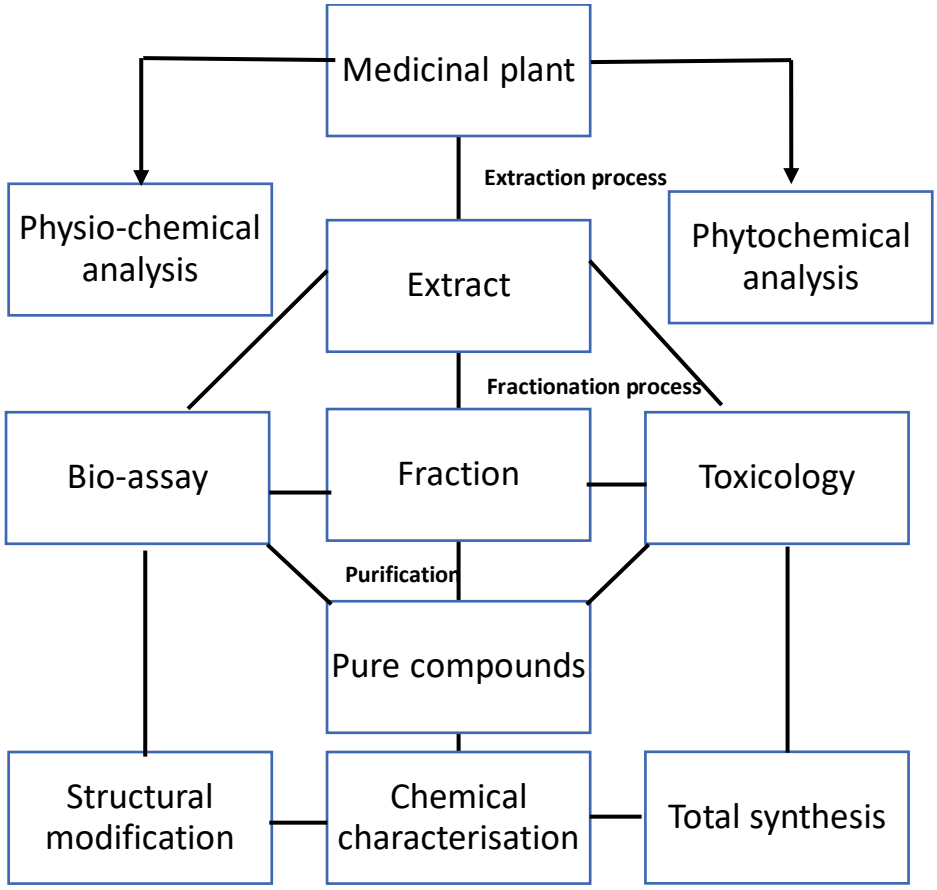


Figure 2.13: Methods for acquiring bioactive compounds from TMPs

Source: Choudhury *et al*. (2020); Shakya (2016)

The drug development process from TMPs includes four strategies: (1) isolation and synthesis of active compounds, (2) safety and efficacy evaluations using systematic pharmacological procedures, (3) safety and efficacy evaluations using conventional pharmacological techniques, and (4) regulatory approval of therapeutic drugs for market use and post-market monitoring (Pan *et al*., 2013). Cancer and infectious diseases are the principal areas in which natural products are investigated for drug discovery. However, other therapeutic areas include cardiovascular, inflammation, gastrointestinal, neuropharmacological, and metabolic conditions (Harvey, 2008). TMPs have a promising future in pharmaceutical applications, as approximately half of the plants in the world have yet to be investigated for their therapeutic potential, which could lead to definitive treatments (Yudharaj *et al*., 2016). They continue to play a significant role in drug development industries due to their minimal side effects (Dar *et al*., 2017).

2.3 *ASPARAGUS LARICINUS* BURCH.

2.3.1 Biological classification of the *Asparagaceae* family

The *Asparagaceae* family are monocotyledonous flowering plants assigned to the *Asparagales* order under the kingdom *Plantae* (see Figure 2.14) (Angiosperm Phylogeny Group, 2009; Schoch, 2020). It is estimated to consist of 300 species (iNaturalist, 2025). The *Asparagaceae* family is divided further into seven sub-families, namely *Asparagoideae*, *Agavoideae*, *Aphyllanthoideae*, *Lomandroideae*, *Brodiaeoideae*, *Scilloideae*, and *Nolinoideae* (Chase *et al.*, 2009).

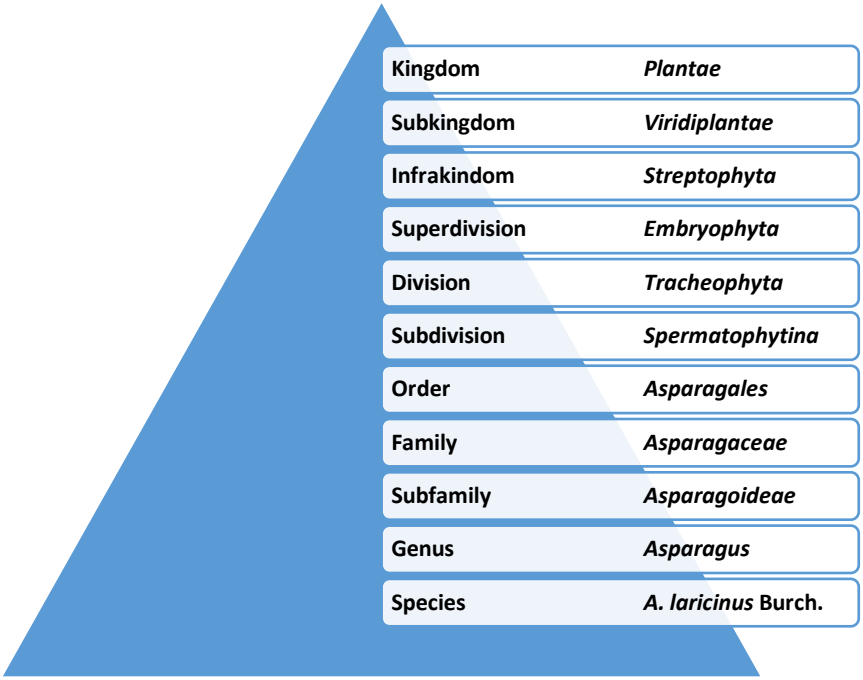


Figure 2.14: Taxonomic classification of *A. laricinus* Burch.

Source: Schoch (2020)

2.3.2 Description of *A. laricinus* Burch.

A. laricinus Burch. (see Figure 2.15) (*Protasparagus laricinus* [Burch.] Oberm.), commonly known as "wild asparagus", is a monocotyledon plant that belongs to the *Asparagoideae* subfamily in the family *Asparagaceae* (Burrows & Von Staden, 2018). It is referred to as *lesitwane* in Setswana; *katdoring*, *bergkatdoring*, *fynkatbos*, *langbeenkatdoring*, and *bergkatbos* in Afrikaans; *ibutha* in isiZulu; and *imvane* in isiXhosa (Burrows & Von Staden,

2018; Random Harvest Nursery, 2023). The shrub is evergreen, hardy, and features feathery foliage and zigzagged silvery branchlets (Wildflower Nursery, 2023). The white, fragrant flowers of the climber produce red and black berries that attract insects and birds (Wildflower Nursery, 2023).

Figure 2.15: *A. laricinus* Burch. plant with red and black berries

Source: Researcher (2025)

2.3.3 Distribution and habitat of *A. laricinus* Burch.

A. laricinus Burch. is widely distributed across the central and eastern parts of South Africa, with no danger of becoming extinct (Burrows & Von Staden, 2018). It is found in the Free State, Eastern Cape, Gauteng, North West, Northern Cape, Limpopo, Mpumalanga, and KwaZulu-Natal provinces, as shown in Figure 2.16 (Burrows & Von Staden, 2018; Random Harvest Nursery, 2023). It is not native to South Africa and is also found in Lesotho, Mozambique, Zimbabwe, Swaziland, Namibia, and Zambia (Burrows & Von Staden, 2018).

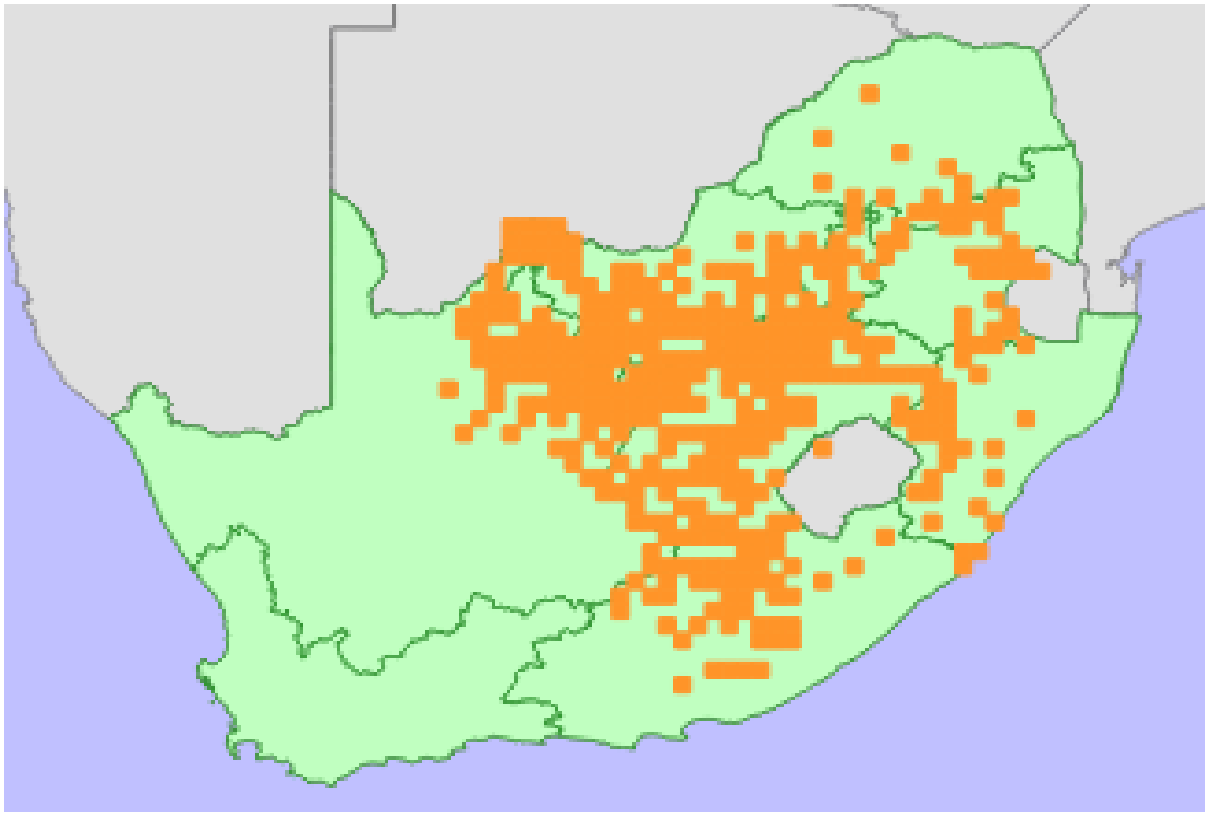


Figure 2.16: *A. laricinus* Burch. distribution in South Africa

Source: Burrows and Von Staden (2018)

2.3.4 Medicinal benefits of *A. laricinus* Burch.

A. laricinus Burch. is used to treat various ailments such as muscle pain, urinary tract infections, tuberculosis, red water, sores, umbilical cord inflammation, and general disorders (Chakale *et al*., 2022; Van der Merwe *et al*., 2001). In addition, the leaf and root extracts of this plant have been shown to possess anti-cancer properties (Mashele & Kolesnikova, 2010; Mfengwana *et al*., 2019).

2.3.5 Some of the isolated compounds from the *Asparagus* genus

Asparagus species possess active components with various biological activities, including anti-cancer, anti-inflammatory, anti-bacterial, anti-hypertensive, hypoglycaemic, anti-oxidant, anti-epileptic, anti-hepatotoxic, and anti-oxytocic properties (Guo *et al*., 2020; Negi *et al*., 2010). The most commonly isolated active compounds from the roots of *A. racemosus* Willd. are saponins known as Shatavarin I-IV (Negi *et al*., 2010). Shatavarin IV (see Figure 2.17A) is a glycoside from the sarsasapogenin class, which consists of two rhamnose molecules and one

glucose molecule, which exhibit anti-cancer activity (Pandiyan *et al*., 2022; Shankar *et al*., 2012).

A. officinalis L. has revealed various active compounds, including flavonoids (rutin, kaempferol, quercetin, and isorhamnetin), phenols (ferulic acid), anthocyanins (A1 and A2) (see Figure 2.17B), and steroidal saponin (Yamogenin II) (see Figure 2.17C) (Fan *et al*., 2015; Guo *et al*., 2020). An organic ketone, palmitone (see Figure 2.17D), isolated from *A. suaveolens*, has been reported to be effective in treating gonorrhoea and epilepsy (Olivier *et al*., 2016).

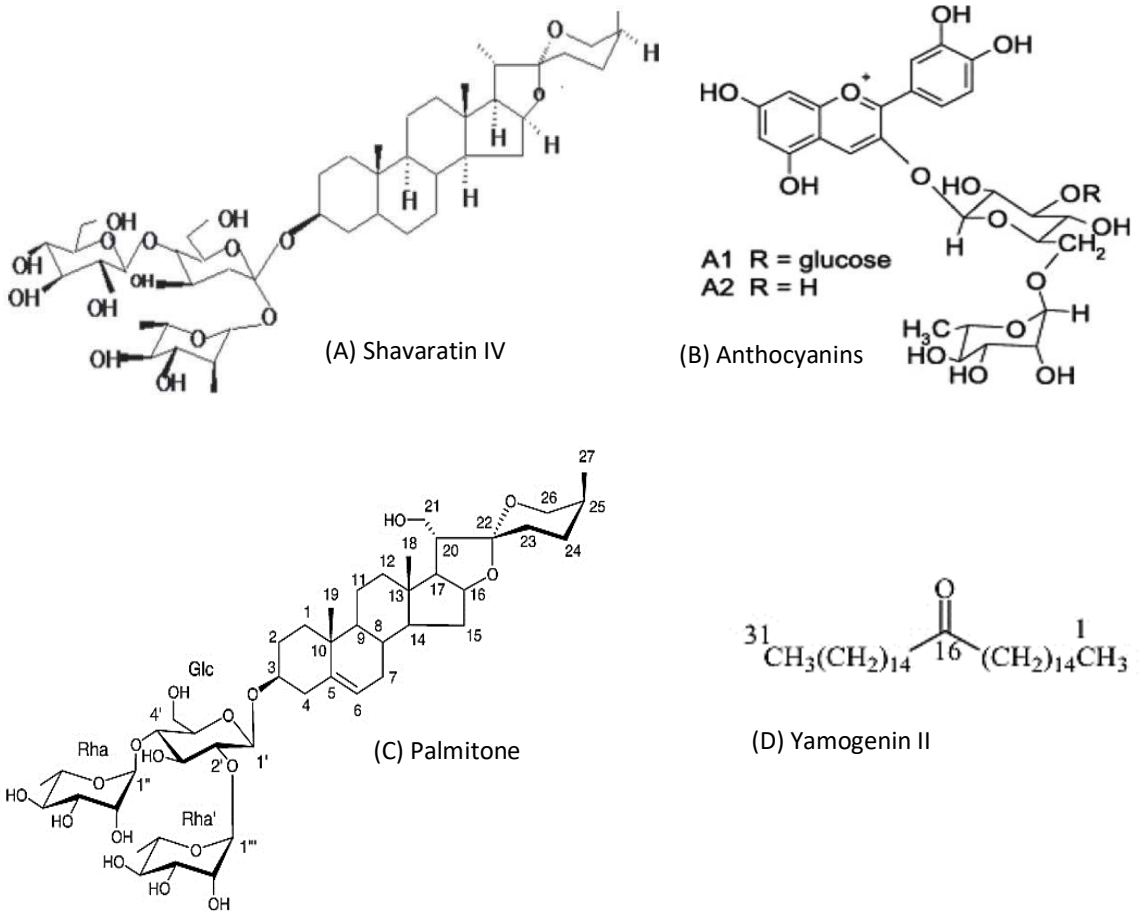


Figure 2.17: Chemical structures of Shatavarin IV (A), Anthocyanin A1 and A2 (B), Palmitone (C), and Yamogenin II (D)

Source: Shankar *et al.* (2012) (A); Guo *et al*. (2020) (B); Olivier *et al.* (2016) (C); Guo *et al.* (2020) (D)

The crude extract, fractions, and compounds from medicinal plants can be screened for biological activity (Fennell *et al*., 2004). The major steps in obtaining bioactive compounds from plant sources include extraction, pharmacological screening, compound isolation and

characterisation, toxicology analysis, and clinical evaluation (Sasidharan *et al*., 2011). Plant extracts, whether in the form of pure compounds or not, present an opportunity to develop new drugs due to their unparalleled chemical diversity (Cos *et al*., 2006). In the search for compounds with pharmaceutical value, researchers do not randomly perform screening procedures; instead, they seek knowledge from traditional users or refer to previous ethnobotanical reports to identify plants with potential medicinal benefits. It is crucial to isolate bioactive compounds from a plant of interest to identify the specific constituents that possess medicinal properties. In this study, the crude leaf extract of *A. laricinus* Burch. was subjected to a combination of chromatographic techniques to obtain a compound, which was further evaluated for its anti-cancer activity.

CHAPTER 3:

MATERIALS AND METHODS

3.1 GENERAL EXPERIMENTS

This chapter describes all the experimental procedures performed on *A. laricinus* Burch. plant material for the purposes of the study. All chemical reagents used were of analytical grade and did not require further purification. Silica gel 60 (70-330 mesh) and thin-layer chromatography (TLC) plates (60 F254 aluminium, 20 x 20 cm) were purchased from Merck KGaA, Darmstadt, Germany. Methanol (MeOH), toluene, hexane, ethyl acetate (EtOAc), formic acid, and dichloromethane (DCM) were also obtained from Merck KGaA, Darmstadt, Germany. The TLC plate staining reagents, including vanillin and iron (III) chloride ($FeCl_3$), were sourced from Merck KGaA, Darmstadt, Germany. Bis-benzamide H 33342 trihydrochloride (Hoechst 33342) and propidium iodide (PI) were purchased from Thermo Fisher Scientific (Massachusetts, United States of America [USA]). Low glucose Dulbecco's modified eagle medium (DMEM) and phosphate-buffered saline (PBS), both with and without calcium ions (Ca^{2+}) and magnesium ions (Mg^{2+}), were acquired from Cytiva (Marlborough, USA). Foetal bovine serum (FBS), non-essential amino acids, and penicillin/streptomycin were obtained from Biowest (Nuaillè, France).

3.2 PLANT MATERIAL

The plant material of *A. laricinus* Burch. was collected by Mmannini Rangwaga (the researcher) at the Free State National Botanical Garden in Bloemfontein (29°03'05.9"S 26°12'48.5"E) on 24 March 2022. Plant authentication was carried out by Prof. Z. Zietsman, a botanist at the Free State National Botanical Garden in Bloemfontein, and plant voucher number MAS001 was issued. Subsequently, the plant material was transferred to the Central University of Technology, Free State, for processing.

3.3 PLANT EXTRACTION

The leaves of *A. laricinus* Burch. were washed to remove dirt particles and allowed to dry in an oven at 40 °C for five days. The dried leaves were cut into smaller pieces using scissors and ground into a powder using a commercial coffee grinder (see Figure 3.1). A 150.0 g powdered

plant sample was dissolved in 1.5 L of 100% MeOH. The solution was placed on a shaker at a speed of 110 revolutions per minute (RPM) for three consecutive days, as shown in Figure 3.2. The solution was filtered, and the insoluble solid and solid residue were dissolved in another 1.5 L of 100% MeOH to undergo the shaking process again, which was followed by filtration. The collected filtrate was allowed to evaporate and was concentrated using a rotary evaporator (Buchi Rotavapor® R-300). A total of 46.34 g of green crude extract was collected and kept in a fume hood for several days to allow the remaining MeOH to evaporate before being stored in a fridge at 4 °C.

Figure 3.1: The grinding process of *A. laricinus* Burch. leaves before extraction

Figure 3.2: The shaking process of *A. laricinus* Burch. methanol (MeOH) samples

3.4 SOLVENT SYSTEMS

Prior to the fractionation and isolation of the crude extract, as shown in Figure 3.5, five solvent systems were developed using TLC to identify the most suitable one for separation. A small quantity of the crude extract was dissolved in EtOAc inside a small vial. Five solvent systems were prepared, and the sample was spotted at three different concentrations (1-3mg/mL) on the TLC plates. The TLC plates were developed using the five solvents listed in Table 3.1. The spots were stained with vanillin and viewed under ultraviolet (UV) light, with both short and long wavelengths observed. The best solvent system, EtOAc/toluene (3:7 v/v), was chosen based on the visibility of the spots and the degree of separation achieved.

Table 3.1: Gradient solvent systems prepared on thin-layer chromatography (TLC) plates before the fractionation and isolation process

Solvent system	Ratio
EtOAc: toluene	3:7
EtOAc: toluene	1:1
EtOAc: toluene	1:4
DCM: MeOH	9:1
DCM: MeOH	95:05

3.5 VACUUM LIQUID CHROMATOGRAPHY (VLC) METHOD FOR FRACTIONATION OF THE CRUDE EXTRACT

A 20.0 g sample of silica gel 60 (70-230 mesh) was combined with 20.0 g of crude MeOH extract of *A. laricinus* Burch. and allowed to dry for 10 minutes in a fume hood. The silica gel was filled uniformly to a height of 5 cm inside the Buchner funnel, and the prepared sample was placed on top, with cotton wool distributed evenly over the loaded sample. The packed VLC, as shown in Figure 3.3, was initially washed with 100% hexane, followed by a solvent composition of EtOAc/toluene in a 1:9 ratio, and gradually increasing the volume of EtOAc by 10% until reaching a 5:5 ratio. After this, 100% EtOAc was added, and the system was subsequently washed with 100% formic acid. A total of 18 fractions were collected using 250-mL beakers, allowed to dry for two days in the fume hood, and then spotted on a TLC plate. Similar fractions were identified and combined to formulate nine sub-fractions (see Figure 3.4).

Figure 3.3: VLC method during fractionation of *A. laricinus* Burch. crude leaves extract

Figure 3.4: VLC sub-fractions (one to nine) obtained from a combination of 18 fractions

3.6 COLUMN CHROMATOGRAPHY METHOD FOR COMPOUND ISOLATION

Column chromatography technique was performed using sub-fraction number two from nine sub-fractions. The sub-fraction was combined separately with a small portion of silica gel 60 (70-230 mesh) , stirred inside a vile and allowed to dry in the fume hood for a few minutes. The dry powdered sample was then loaded onto the pre-packed silica gel column (Merck Synthware™ chromatography column). A solvent gradient of EtOAc/toluene (3:7 v/v) was used for elution. The collected compound of interest was further purified with TLC.

3.7 PREPARATORY THIN-LAYER CHROMATOGRAPHY (PTLC) FOR COMPOUND PURIFICATION

PTLC was performed by dissolving the collected compound in EtOAc and loading it onto 20 x 10 cm TLC plates. The sample was applied three times in a straight line, 1 cm apart from the lowest point of the plate, using a spotting tube. An EtOAc/toluene (3:7 v/v) solvent system was prepared, and the TLC plates were developed five to six times in separate developing chambers. The dried plates were examined under both short and long wavelengths, and the separated bands were marked with a pencil. The bands were then cut into smaller pieces with scissors and extracted from the silica using EtOAc. The solutions were filtered using syringe filters, and the collected filtrate was allowed to evaporate under vacuum.

3.8 HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY (HPTLC) METHOD FOR COMPOUND ANALYSIS AND IDENTIFICATION

To perform HPTLC, the Camag TLC Visualizer 2, Automatic TLC Sampler (ATS) 4, Automatic Developing Chamber (ADC) 2, and TLC Plate Heater 3 were used. A total of nine sub-fractions from VLC were dissolved in EtOAc, filtered, and added to HPTLC vials. The technique was conducted on 20 x 10 cm (silica gel 60 F254) aluminium-coated TLC plates. The samples were applied from the centre as bands on the TLC plate using ATS 4, employing a spray-on technique. Chromatography development was carried out at a humidity of 33%. A total of 10 mL of the prepared solvent system (EtOAc/toluene, 18:85 v/v) was added for development, while 25 mL was used for saturation in ADC 2. The TLC plate heater was set to 100 °C, and the TLC plate was stained with 5% $FeCl_3$. The plate was placed on the heater, allowed to dry, and then viewed again under UV light at 254 and 366 nm.

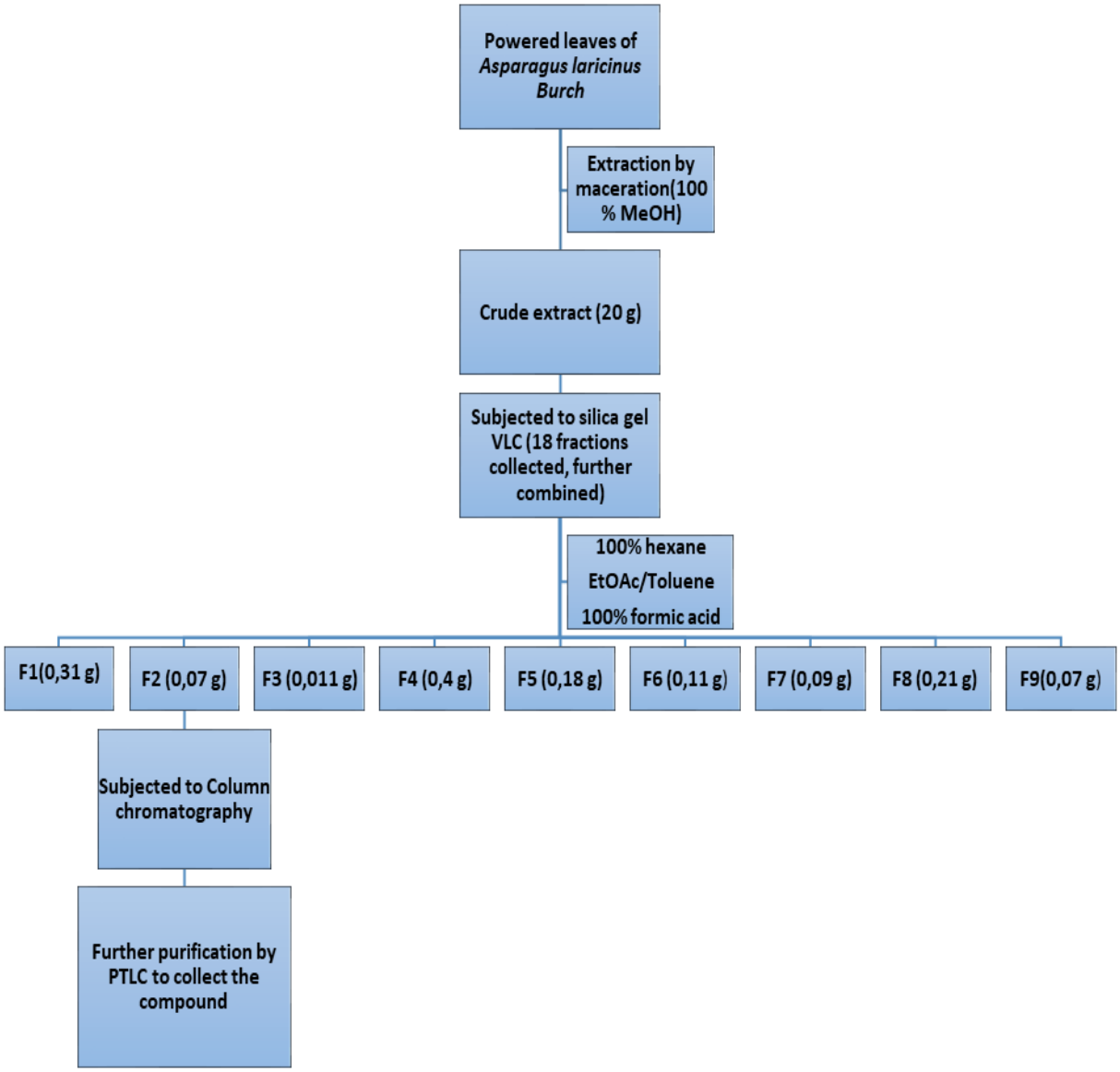


Figure 3.5: Isolation flow chart

3.9 COMPOUND IDENTIFICATION

3.9.1 Liquid chromatography-mass spectrometry (LC-MS)

LC-MS analysis was conducted at Stellenbosch University by the Central Analytical Facilities to determine the molecular ion mass of *A. laricinus* Burch. crude extract and isolated compounds. A Water Synapt G2 instrument was used to perform this experiment. The samples were introduced to the electrospray ionisation (ESI) probe, and ions were detected by the ESI positive source at a cone voltage of 15 volt.

3.10 CELL CULTURE

3.10.1 Cell lines

Human breast carcinoma cell lines (MCF-7) and African green monkey cell lines (Vero) were used in this study. The cell lines were obtained from Cellonex, South Africa. Cells were cultured in DMEM with 10% FBS, followed by incubation at 37 °C in a humidified atmosphere containing 5% carbon dioxide.

3.10.2 *In vitro* cytotoxicity assay

Cells were seeded in 96-well plates in 100 µL aliquots at a density of 4 x 10^3 and left overnight to attach. The prepared treatments were added to the cells and incubated at 37 °C in a 5% humidified incubator for 48 hours. Melphalan was used as a positive control. The culture medium was removed from each well and replaced with 100 µL of Hoechst 33342 staining solution (5 µg/mL in PBS with Ca^{2+} and Mg^{2+}), and the cells were incubated for 30 minutes at room temperature. The cells were further stained with 10 µL of PI (100 µg/mL in PBS with Ca^{2+} and Mg^{2+}) to assess cell viability. Fluorescent photographs were captured immediately using an ImageXpress Micro XLS Widefield Microscope (Molecular Devices) with a 10x Plan Fluor objective and DAPI and Texas Red filter cubes. The obtained images were analysed using the MetaXpress software and the Multi-Wavelength Cell Scoring Application Module.

CHAPTER 4:

RESULTS

4.1 INTRODUCTION

This chapter reports on a compound isolated from *A. laricinus* Burch. and its anti-cancer activity on MCF-7 cell lines. Subsequent fractionation and isolation procedures were designed to comprehensively explore the plant's composition in order to identify the bioactive compound(s). The methodological approach utilised in this research integrated multiple analytical strategies, including chromatographic separation techniques, tentative identification, and bioactivity screening. A multi-step chromatographic approach was employed to fractionate and isolate the compound, which integrated complementary techniques to ensure comprehensive extraction and purification. The approach included VLC, column chromatography, TLC, HPTLC, and PTLC. Tentative identification of the compound was performed using LC-MS, which enabled precise molecular identification. The compound underwent *in vitro* cytotoxicity screening against MCF-7 and Vero cell lines to evaluate its potential biological significance, in order to provide initial insight into its pharmacological properties.

4.2 ISOLATION AND IDENTIFICATION OF THE COMPOUND

4.2.1 TLC profiling of *A. laricinus* Burch. extract

TLC was employed to profile and characterise the phytochemical composition of *A. laricinus* Burch. leaf extract, which enabled systematic evaluation of optimal solvent systems for comprehensive chemical separation and analysis. The preliminary screening technique facilitated the assessment of mobile phase compositions to achieve maximum resolution and separation of complex plant metabolites. A comprehensive solvent system screening was conducted, encompassing five different mobile phase compositions: EtOAc/toluene (3:7 v/v), EtOAc/toluene (1:1 v/v), EtOAc/toluene (1:4 v/v), DCM/MeOH (9:1 v/v), and DCM/MeOH (95:5 v/v). The solvent system optimisation protocol involved a comparative analysis of each mobile phase's chromatographic performance, specifically emphasising spot resolution, band separation, and chemical component migration characteristics. The EtOAc/toluene (3:7 v/v)

solvent system was selected as the optimal mobile phase due to its superior spot resolution and enhanced chromatographic separation capabilities.

Assisted by the process described above, the methanolic extract of *A. laricinus* Burch. was sequentially fractionated by VLC using hexane, EtOAc/toluene, and formic acid to wash off the silica gel. Eighteen fractions were collected and spotted on a TLC plate, employing EtOAc/toluene (3:7 v/v) as the solvent system (see Figure 4.1).

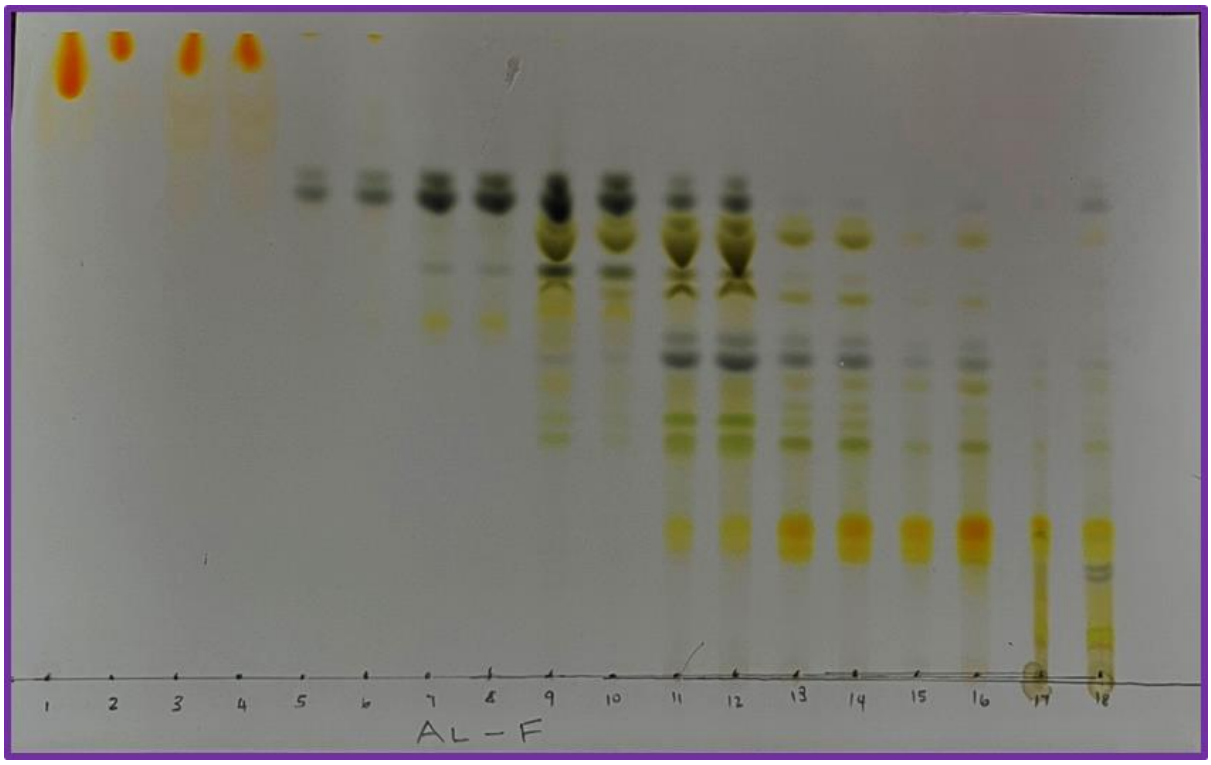


Figure 4.1: TLC profile of the 18 fractions under visible light (EtOAc/toluene, 3:7 v/v, was used as a solvent system to develop the plate)

4.2.2 HPTLC profiling of *A. laricinus* Burch. fraction

HPTLC has emerged as a sophisticated advancement of conventional TLC. It offers enhanced resolution, sensitivity, and reproducibility in analytical chemistry. This technique has revolutionised the fields of pharmaceutical analysis, natural product research, and quality-control processes through its robust separation capabilities and versatile detection methods (Reich & Schibli, 2007).

Following the analysis detailed in Section 4.2.1, similar fractions were combined from the 18 fractions to formulate nine sub-fractions. Phytochemical profiling was then conducted using

the Camag HPTLC system, described in Chapter 3, to identify constituents in the nine sub-fractions. The TLC plate, pre-coated with silica gel 60 F254, was developed using an EtOAc/toluene (18:85 v/v) solvent system with 10 mL as the volume. The plate was then dried on a heater and viewed under UV short wavelength, long wavelength, and white light (see Figure 4.2). Phytochemicals were visualised using 5% $FeCl_3$ as the staining agent (see Figure 4.3). This procedure was performed to identify the sub-fraction of interest from which the compound would be isolated.

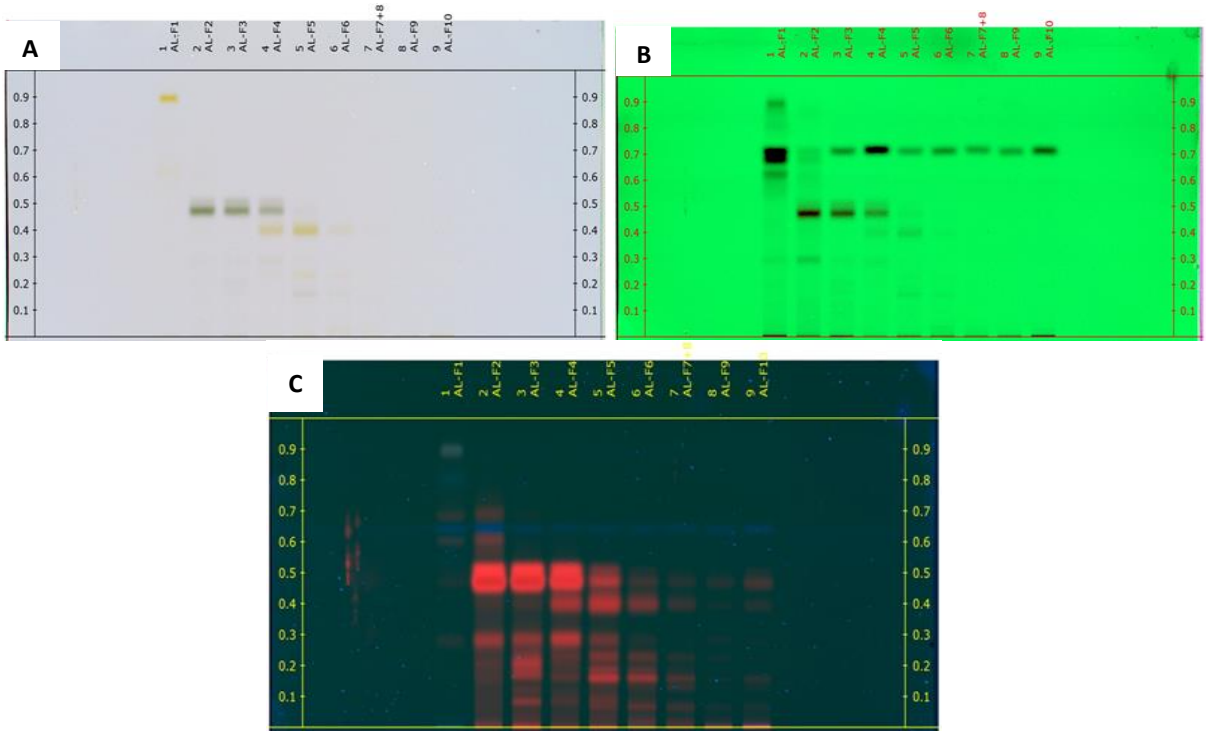


Figure 4.2: HPTLC chromatograms of nine sub-fractions of *A. laricinus* Burch. viewed under (A) white light, (B) short wavelength, and (C) long wavelength

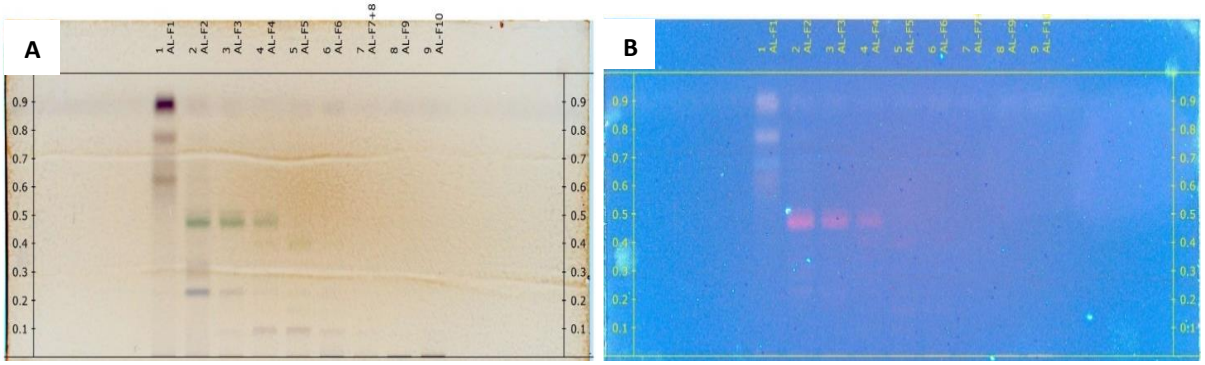


Figure 4.3: Profiling of derivatised plate with 5% $FeCl_3$ viewed under (A) white light and (B) UV at 366 nm

4.2.3 LC-MS analysis of the crude extract

LC-MS was employed to tentatively identify the chemical composition of the crude extract from *A. laricinus* Burch. The molecular ion peaks obtained from the LC-MS chromatograms of the MeOH extract were comparatively analysed against standard mass spectra from referenced databases, including the National Institute of Standards and Technology mass spectral library and MassBank, to determine the preliminary chemical profile of the extract. The strategic application of ESI in positive and negative modes facilitated tentative molecular species identification through accurate mass determination and adduct formation analysis. This methodological framework enabled the detection and preliminary characterisation of potential anthraquinone derivatives, which provided insight into the chemical complexity of *A. laricinus* Burch.

Figure 4.4 presents the LC-MS chromatogram of the *A. laricinus* Burch. MeOH extract. Positive-mode electrospray ionisation mass spectrometry (ESI-MS) detected multiple molecular ion peaks, as detailed in Table 4.1. The complex chemical profile illustrates why this plant species has remained poorly characterised from a phytochemical perspective. Analytical modifications, including adjusted instrumental parameters and solvent systems, were crucial in resolving the extract's molecular components. The retention time variations in Table 4.1 indicate potential structural isomers or closely related molecular compounds previously identified in the *Asparagus* genus. Multiple molecular ion signals with distinct mass-to-charge (m/z) ratios and chromatographic peaks suggest diverse chemical constituents and potential structural similarities to known *Asparagus* metabolites. These findings highlight the intricate chemical composition of *A. laricinus* Burch., demonstrate the challenges of comprehensive plant metabolite analysis, and provide insight into the plant's complex chemical landscape.

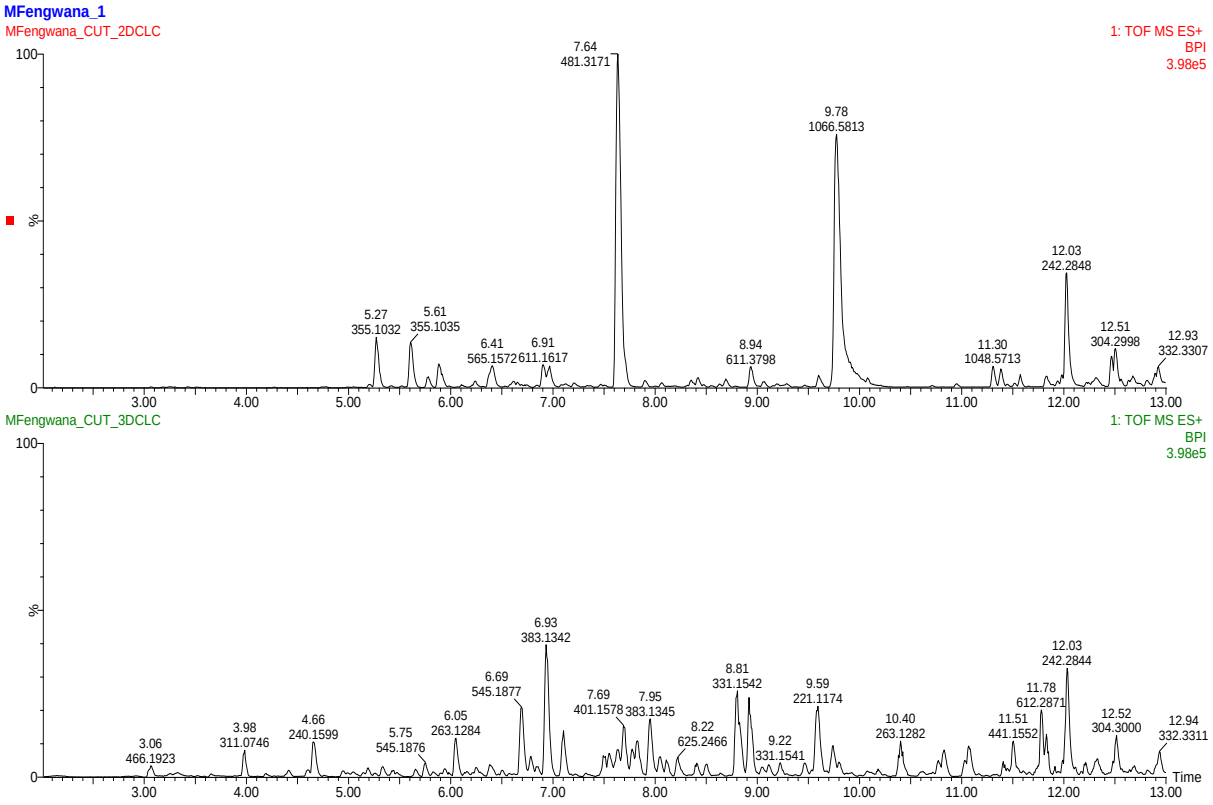


Figure 4.4: LC-MS chromatogram of the *A. laricinus* Burch. MeOH leaves extract

Table 4.1: LC-MS analysis of some tentative identified compounds from the *Asparagus* genus

Retention time (minutes)	Molecular weight (m/z)	Name	Elemental composition	Compound class
5.887	595.16705	Isovitexin 2''-O-glucoside	$C_{27}H_{30}O_{15}$	Flavonoid C-glycosides
5.887	595.16705	Apigenin 7-O-beta-D-glucopyranosyl (1->6)-beta-D-glucopyranoside	$C_{27}H_{30}O_{15}$	Flavonoid-7-O-glycosides
6.905	611.1617	Rutin	$C_{27}H_{30}O_{16}$	Flavonoid-3-O-glycosides
6.905	611.1617	Quercetin 3-galactoside 7-rhamnoside	$C_{27}H_{30}O_{16}$	Flavonoid-7-O-glycosides
6.905	611.16174	Panasenoside	$C_{27}H_{30}O_{16}$	Flavonoid-3-O-glycosides
6.051	263.12836	Pentalenolactone E	$C_{15}H_{18}O_4$	Terpene lactones
7.636	481.31711	Inokosterone	$C_{27}H_{44}O_7$	Hydroxy bile acids, alcohols, and derivatives
7.636	481.31711	26-Hydroxyecdysone	$C_{27}H_{44}O_7$	Hydroxy bile acids, alcohols, and derivatives
8.926	329.13882	4'beta-Hydroxyeriobrucinol	$C_{19}H_{20}O_5$	Linear pyranocoumarins
9.776	1031.5448	Solasodoside A;(-)-Solasodoside A	$C_{51}H_{82}O_{21}$	Steroidal saponins
9.776	1031.5448	Diosgenin 3-[glucosyl-(1->4)-rhamnosyl-(1->4)-[rhamnosyl-(1->2)]-glucoside]	$C_{51}H_{82}O_{21}$	Steroidal saponins

LC-MS analysis is useful as it provides initial insight into the phytochemical composition of *A. laricinus* Burch. The detected mass signals serve as preliminary evidence of potential molecular structures, which necessitate further investigative steps for definitive structural confirmation. Isolation and comprehensive structural characterisation techniques, including nuclear magnetic resonance spectroscopy and additional mass spectrometric analyses, are critical for verifying the identity of the observed compounds. The data suggest the presence of molecular species potentially related to known phytochemicals, but the possibility of isomeric structures cannot be excluded without rigorous subsequent analytical validation. These findings underscore the complexity of metabolite profiling and the importance of multi-stage analytical approaches in elucidating plant secondary metabolite compositions.

4.2.4 Isolation of a compound

Subsequent to the tentative identification through LC-MS, a targeted isolation strategy was imperative for obtaining a single isolated compound. Among the nine generated sub-fractions outlined in Section 4.1.2, sub-fraction two was strategically selected based on two critical analytical criteria: distinctive visible chromatic characteristics and significant band intensity observed in the HPTLC profile. The selected sub-fraction underwent systematic column chromatography to facilitate the separation of potential compounds. PTLC was applied for further purification, and EtOAc/toluene (3:7 v/v) was used to develop the plate and isolate a compound coded N-A (see Figure 4.5). This approach aimed to generate a sufficient sample amount for further analysis.

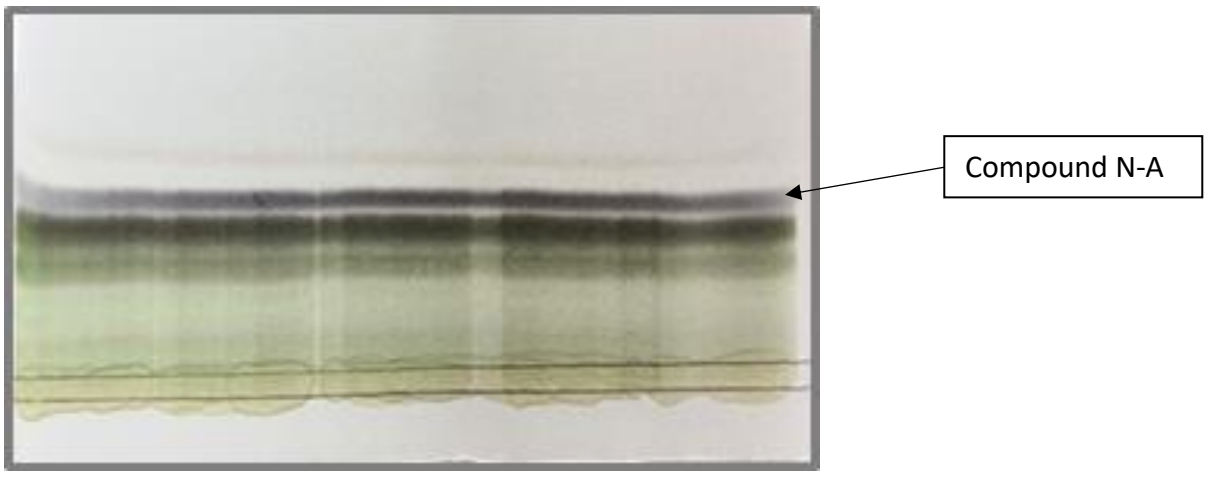


Figure 4.5: TLC profiling of isolated compound

The isolated compound N-A was evaluated using single mass analysis via LC-MS (see Figure 4.6). Mass spectrometric analysis revealed a molecular ion at m/z 267.135 in positive ESI mode, which corresponded to the molecular formula $C_{16}H_9O_4$ with an $[M + 2H]^+$ adduct. This observation was consistent with the calculated exact mass of 265.0494 g/mol, with the molecular structure confirmed through mass spectrometry. This isolated structure aligns with the tentative identification of the compound.

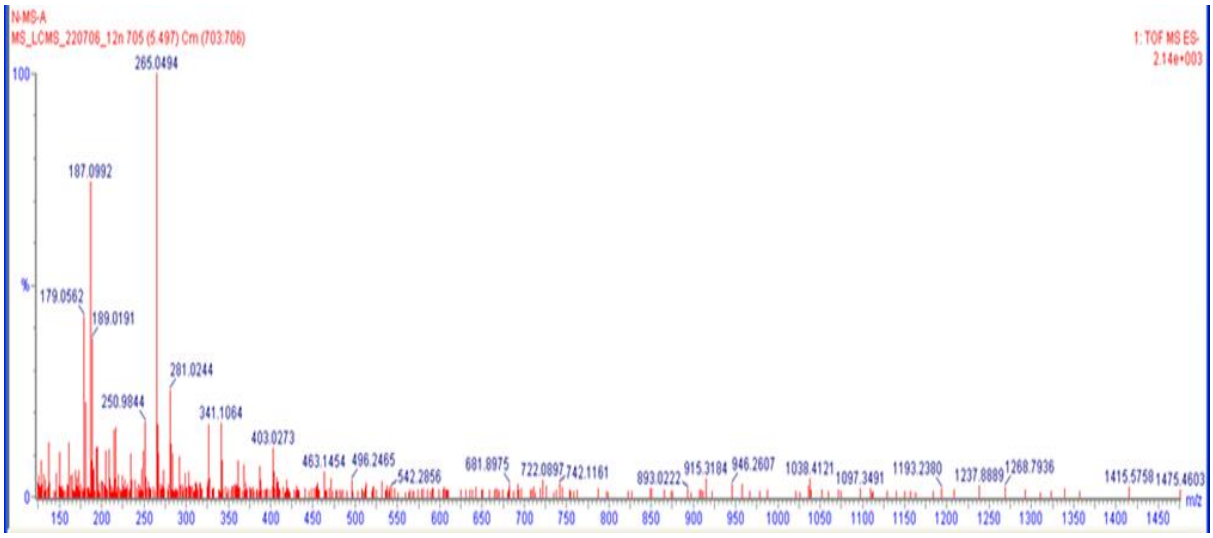


Figure 4.6: Compound N-A single mass analysis

4.3 *IN VITRO* CYTOTOXICITY ACTIVITY

In this study, compound N-A extracted from *A. laricinus* Burch. was used to evaluate cytotoxic activity in both normal and cancerous cells. This is notable as no compounds have previously been isolated from this plant; however, the crude extracts are reported to contain phyto-constituents with anti-cancer properties. The cytotoxicity of compound N-A was assessed using the Hoechst 33342/PI dual staining assay. Compound N-A was subjected to *in vitro* cytotoxicity screening against MCF-7 and Vero cell lines. Both cell types were treated with varying concentrations that ranged from 15.63 to 250 µg/mL of the compound to determine the percentage of cell viability. Melphalan (50 µM) served as a positive control, while untreated cells were used as a negative control in the experiments. Experiments were carried out in quadruplicate. There was no reduction in cell viability by 50% after 48 hours of treatment with compound N-A (see Figure 4.7). It was thus concluded that compound N-A was not cytotoxic to the MCF-7 cell lines. The compound also proved to be non-toxic to

normal cells (see Figure 4.8). The inactivity of compound N-A was unexpected, as previous studies showed that the crude extract of this plant possesses anti-cancer activity.

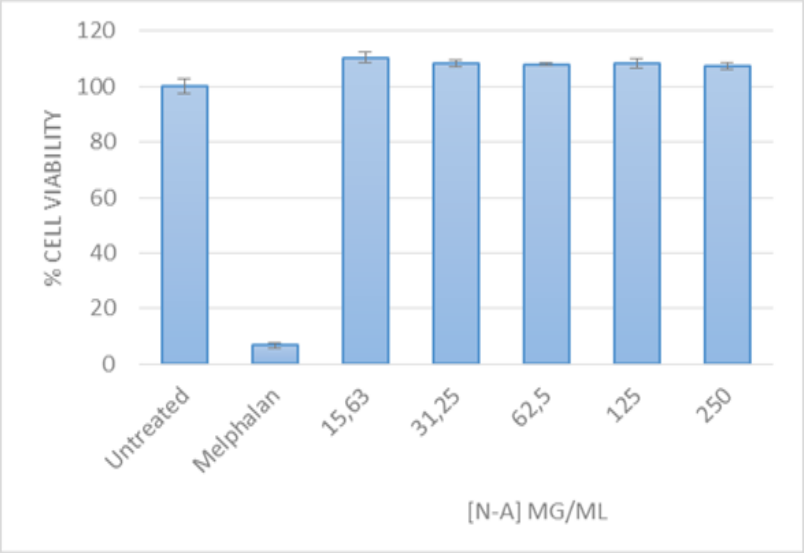


Figure 4.7: Cytotoxicity of compound N-A in MCF-7 cells after 48 hours of treatment

Melphalan (50 µM) was used as a positive control. The error bars indicate the standard deviation of the mean for a single experiment performed in quadruplicate.

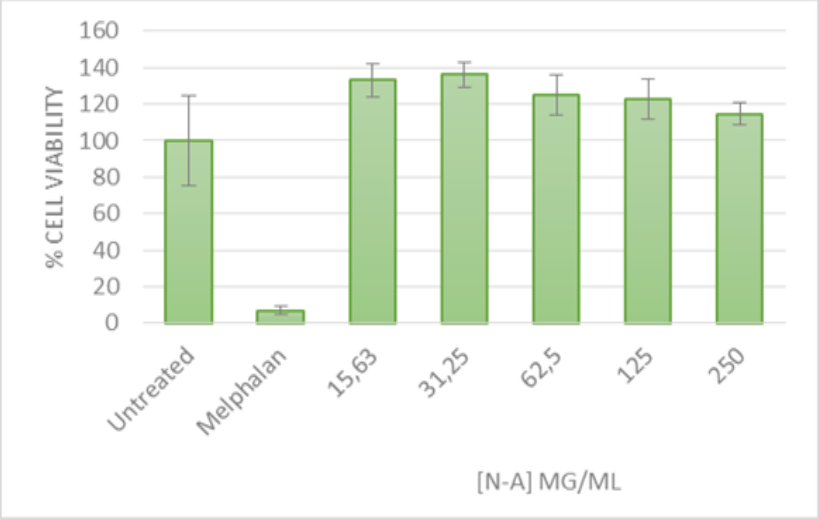


Figure 4.8: Cytotoxicity of compound N-A in Vero cells after 48 hours of treatment

Melphalan (50 µM) was used as a positive control. The error bars indicate the standard deviation of the mean for a single experiment performed in quadruplicate.

CHAPTER 5:

DISCUSSION

5.1 INTRODUCTION

TMPs continue to be of major interest in pharmaceutical, nutritional, and cosmetic applications (Rasul, 2018). They are considered an alternative medicine in African communities, as they are easy to access and have fewer side effects than modern treatments (Pandiyan *et al*., 2022). For many years, traditional healers have used TMPs to treat various ailments in their patients (Matowa *et al*., 2020). Medicinal plants are a rich source of constituents that may be employed in drug synthesis and development (Yudharaj *et al*., 2016). These plants contain phytochemical components such as phenols, flavonoids, glycosides, alkaloids, steroids, tannins, fixed oils, volatile oils, and resins, which can be found in the roots, leaves, bark, or stems (Idris *et al.,* 2015). These secondary metabolites possess pharmacological activities and protect plants against predators, pathogens, competitors, and UV light (Rabizadeh *et al*., 2022).

Phytochemical screening is a molecular technique that identifies various classes of phytoconstituents present in different parts of the plant and evaluates their biological properties (Sharma *et al*., 2020). In phytochemistry research, it is crucial to not only screen the existing phytochemicals in a plant but also to separate and purify the constituents into pure compounds for further analysis. Traditional methods of obtaining bioactive compounds include extraction methods, phytochemical screening assays, and chromatographic techniques. Following these procedures, the compounds may be further evaluated for their pharmacological effects.

Asparagus species, which belong to the *Asparagaceae* family, are endemic shrubs known for their biological properties. The genus *Asparagus* comprises approximately 300 species worldwide (iNaturalist, 2025). The main bioactive constituents found in the *Asparagus* genus are a group of steroidal saponins with various medicinal properties, including anti-oxidant, anti-inflammatory, anti-oxytocic, anti-cancer, anti-bacterial, anti-hepatotoxic, immunostimulant, and reproductive agents. Other chemical compounds that have been isolated from *Asparagus* and reported to be cytotoxic include asparacoside, asparacosin A,

asparacosin B (*A. cochinchinensis*), steroids (*A. officinalis*), and norlignans (*A. gobicus*) (Negi *et al*., 2010). *A. racemosus* Willd is one of the widely studied *Asparagus* species and has been reported to contain steroidal saponins (Shatavarin I-IV). In a study conducted by Shankar *et al*., (2012), Shatavarin IV isolated from the root extract of *A. racemosus* Willd exhibited potent anti-cancer activity against MCF-7 *in vitro*.

A. laricinus Burch., a perennial plant endemic to Southern African regions, represents a valuable source of bioactive compounds with potential pharmaceutical and medicinal applications. Despite its widespread distribution, comprehensive phytochemical investigations of this species remain limited, which creates a significant knowledge gap in understanding its chemical composition and potential therapeutic properties. No compounds have previously been isolated from *A. laricinus* Burch.; however, qualitative phytochemical screening of the aqueous leaf extract displayed the presence of carbohydrates, flavonoids, glycosides, phlobatannins, saponins, steroids, tannins, and terpenoids, while the aqueous stem extract contains saponins, tannins, flavonoids, and phlobatannins (Ntsoelinyane & Mashele, 2014). Phytochemical analysis from a study by Fuku *et al*. (2013) showed the presence of saponins, tannins, alkaloids, and terpenoids in the aqueous root extract. In the same study, gas chromatography-mass spectrometry revealed the presence of three phytoconstituents: ferulic acid, α-sitosterol, and indole-3-carbinol (Fuku *et al*., 2013).

Moreover, the aqueous and ethanol extracts of *A. laricinus* Burch. have shown anti-cancer activity against the MCF-7, melanoma (UACC62), and renal cancer (TK10) cell lines (Mashele & Kolesnikova, 2010). The MeOH leaf extract of *A. laricinus* Burch. has demonstrated cytotoxic and apoptotic potential against MCF-7 (Mfengwana *et al*., 2019). LC-MS analysis has revealed the presence of 12 known compounds in the cladodes extract of *A. laricinus* Burch., including luteolin 7-O-rutinoside, apigenin arabinoside-glucoside, apigenin galactoside-arabinoside, 3-methoxynobiletin, apigenin 7-O-apiosyl-glucoside, quercetin 3-O-rhamnosyl-galactoside, rutin (quercetin 3-O-rhamnosyl-glucoside), kaempferol 3-O-galactoside 7-O-rhamnoside, ecdysterone, isorhamnetin 3-O-glucoside 7-O-rhamnoside, syringaresinol, and 20-hydroxyecdysone (Mfengwana *et al*., 2023). Approximately 75% of these known compounds are flavonoids, which are reported to have anti-cancer properties (Mfengwana *et al*., 2023). The secondary metabolites found in this plant are known to exhibit various therapeutic properties, including anti-cancer effects (Dar *et al*., 2017). As a result, it is evident that

A. laricinus Burch. has the potential to be an anti-cancer agent. It is therefore crucial to investigate the possible bioactive compounds responsible for the anti-cancer activity of this plant.

5.2 DISCUSSION OF THE FINDINGS

In this research, the crude extract of *A. laricinus* Burch. was fractionated to isolate and purify a compound using a combination of chromatographic techniques. The results obtained from the LC-MS provided valuable insight into the chemical profile of the *A. laricinus* Burch. extract. The chromatographic separation (see Figure 4.4) indicated the presence of multiple compounds, with notable peaks at retention times of 7.64 minutes (m/z 481.3171) and 9.78 minutes (m/z 1086.5813), which suggested the presence of relatively high-mass and possibly structurally complex constituents. In addition, Table 4.1 highlights a diverse chemical profile of *Asparagus*, dominated by flavonoid glycosides, steroidal saponins, and terpenoids. The repeated m/z values (e.g., 595.16705 and 611.1617 corresponding to multiple compounds) suggest isomers or structural analogues with identical molecular weights but different glycosidic linkages. The retention time differences help to distinguish structurally similar compounds. These LC-MS results aligned with the TLC profile (see Figure 4.1), which indicated that the extract contained predominantly mid- to non-polar compounds. Most bands were concentrated in the middle of the plate (Rf ~0.3–0.7), which suggests the dominance of flavonoid glycosides and phenolic derivatives, which are moderately polar metabolites. The TLC profile also showed the presence of polar steroidal saponins, which are known to have anti-cancer activity. Further inspection of the mass spectrum (see Figure 4.6) showed dominant ions at m/z 265.0494, 187.0992, and 281.0244, which pointed to a range of mid- to high-mass compounds. The peak at m/z 265.0494 was particularly abundant, which potentially indicated a major component of interest. The presence of different molecular ions and their intensities points towards a variety of chemical structures, potentially including bioactive constituents responsible for the cytotoxic and apoptotic activities reported in previous studies. Overall, the LC-MS results demonstrated the chemical diversity of the MeOH extract and highlighted key compounds that may contribute to the biological activity reported in earlier studies.

Cytotoxicity testing revealed that the investigated compound (N-A) did not exhibit any effect against the MCF-7 breast cancer cell lines. This inactivity of N-A was not expected due to evidence of cytotoxicity from the crude extracts of the roots and leaves of the *A. laricinus* Burch. plant (Mashele & Kolesnikova, 2010; Mfengwana *et al.*, 2019). In addition, compound N-A was the major compound in the leaves' crude extract, which is the reason it was chosen for biological analysis. The negative result obtained does not necessarily mean that the plant used does not have active compounds or that compound N-A does not have biological activities. Instead, there are various factors that might influence the inactivity of a compound, which are discussed below.

To begin with, numerous steps were taken to isolate the compound using a crude extract, which was then further purified for bio-assay analysis. The combination of chromatographic techniques employed carries a greater risk of diluting the compound or completely damaging it. In other words, separating components from each other during multiple fractionation or further purification methods can account for the loss of biological activity. As a result, an isolated compound (such as N-A) might exhibit anti-cancer effects; however, its activity could have been compromised during the fractionation steps.

In addition, synergistic interaction can be observed in pharmacology, where a combination of different chemical substances produces a greater effect than that of a single compound (Rasoanaivo *et al*., 2011). This means that crude extracts or mixtures of plants typically have greater biological activity than isolated compounds at an equivalent dose. Synergistic interactions of phytochemical constituents in plant extracts have previously been observed in anti-cancer activity. The isolated compound (N-A) may act synergistically rather than individually, which explains why it might need to be combined with other constituents to exhibit activity.

Furthermore, the drying method employed prior to extraction, the polarity of the solvent used during plant extraction, and the temperature conditions during the extraction and isolation processes are additional factors that may affect the results obtained. In this study, the *A. laricinus* Burch. plant material was oven-dried at 40 °C. Although this method of heating was conducted within the recommended temperature range, oven heating can sometimes lead to the breakdown of heat-sensitive constituents and result in the loss of volatile

compounds (Ramesh *et al*., 2024). During the fractionation of the crude extract, it was observed on a TLC plate that this plant consists mostly of mid- to non-polar compounds; however, it was extracted using MeOH, which is known to be a more polar solvent. Polar solvents are highly effective at dissolving polar or ionic substances because their molecules can form strong intermolecular interactions, such as hydrogen bonds and dipole-dipole forces. This facilitates the efficient extraction and isolation of polar and hydrophilic compounds (Subhash *et al*., 2017). The polarity of a solvent typically influences the ability of compounds to dissolve; polar solvents perform best with polar compounds, while non-polar solvents work better with non-polar components (Altemimi *et al*., 2017). Mfengwana *et al*. (2019) reported that the MeOH leaf extract of *A. laricinus* Burch. exhibited cytotoxic and apoptotic activity against breast cancer cells. Based on these promising findings, MeOH was selected as the extraction solvent in this study to facilitate the isolation of bioactive compounds responsible for the observed activity. If mid-polar solvents, such as EtOAc, chloroform, or DCM, had been used during the plant extraction, a more efficient crude extract and better separation of compounds might have been achieved. Nevertheless, the use of MeOH was scientifically justified, particularly for compounds already associated with previously reported biological activity. Exposure to excessive light or higher temperatures during extraction and isolation processes might also lead to the degradation of bioactive compounds. However, in this study, the temperature in the laboratory was well monitored; this could therefore not be one of the contributing factors.

Lastly, *A. laricinus* Burch. is a very complex plant with many phytochemical constituents observed on a TLC plate. This complexity makes it a challenging subject for analysis in phytochemistry research. Additionally, the limited number of previous studies conducted on this plant made it difficult to obtain sufficient information. Despite earlier reports of anti-cancer activity in *A. laricinus* Burch., the isolated compound (N-A) from *A. laricinus* Burch. showed no cytotoxic effect against MCF-7 cells, which suggests limited promise as an anti-cancer agent in this model. system. Additional studies are therefore warranted to assess the potential of its crude extract in the development of nanoparticles.

CHAPTER 6:

CONCLUSION AND LIMITATIONS

6.1 CONCLUSION

Breast cancer is reported to be the second leading cause of mortality in women. In the health sector, the adverse effects associated with standard breast cancer therapies continue to be a major concern. It is crucial to develop new strategies for implementing therapeutic agents derived from TMPs. *In vitro* and *in vivo* studies have shown that bioactive compounds from TMPs may contribute to drug discovery and development and may provide an alternative and safer treatment for cancer. Previous studies have reported the isolation of phytoconstituents from extracts prepared using the leaves, roots, bark, stems, and fruits of plants. However, there is insufficient research on African medicinal plants for anti-cancer activity, which limits the potential to isolate compounds and formulate NCEs for breast cancer management and treatment.

This research study demonstrated the isolation and identification of a compound from the crude leaf extract of *A. laricinus* Burch. A combination of chromatographic techniques, such as VLC, TLC, column chromatography, HPTLC, PTLC, and LC-MS, was employed. The results confirmed the diverse phytochemical profile of *Asparagus*, dominated by flavonoid glycosides, steroidal saponins, and terpenoids. Overall, the separation pattern supports the LC-MS results, which highlighted that *Asparagus* contains a mixture of compounds falling mostly into the mid-polar category, but extending into both polar and non-polar classes. Compound N-A was identified using the MCF-7 and Vero cell lines. The isolated compound exhibited no cytotoxicity against the MCF-7 cell lines.

The inactivity of compound N-A could have been influenced by multiple fractionation, the synergy of chemical constituents, higher laboratory temperatures, the plant-drying process, and the solvent used during the extraction process. While compound N-A should not serve as an anti-cancer agent, the crude extract should be considered. This research project represents the first attempt to isolate compounds from *A. laricinus* Burch. as a follow-up to studies conducted on this plant. More compounds should be isolated from this plant and further screened for anti-cancer activity and other biological activities.

6.2 LIMITATIONS

The researcher acknowledges the following limitations of the study: Firstly, this study aimed to isolate compounds from *A. laricinus* Burch. To the best of the researcher's knowledge, no compounds have been isolated from this plant before. While the crude extract has been scientifically studied, there is no documented information on the specific compounds isolated from it. Secondly, although the screening of the isolated compounds was completed, the data indicated that the compounds were not cytotoxic to MCF-7 cells. The sub-fractions obtained during the fractionation process could not be tested due to limited funding.

6.3 RECOMMENDATIONS

The current data demonstrate the anti-cancer potential of compounds isolated from the leaves of *A. laricinus* Burch. These findings can be further explored through various approaches. One approach involves combining the plant material with allopathic chemotherapeutic drugs to evaluate potential synergism. Another approach entails the isolation of specific bioactive phytochemicals, such as glycosides, flavonoids, and saponins, among others, that may be responsible for the anti-cancer activity. Finally, the MCF-7 cells were treated for only a 48-hour period; results could differ if shorter or longer treatment durations are investigated.

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APPENDIX: LC-MS SPECTRA OF COMPOUND N-A