

***In vitro* anticancer and toxicological evaluation of *Gunnera perpensa* L. isolated compounds**

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BLOEMFONTEIN
2024

DECLARATION

I, Motiki William Ramatsa, student number #####, hereby declare that this dissertation, titled “*In vitro* anticancer and toxicological evaluation of *Gunnera perpensa* L. isolated compounds”, submitted to the Central University of Technology, Free State, for the degree Master of Health in Biomedical Technology, is my own independent work. It 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 previously been submitted by me for any qualification at another institution of higher education. I declare that this dissertation does not contain other persons’ data, pictures, graphs, or other information, unless specifically cited/acknowledged as being sourced from other persons.

SIGNATURE:

DATE: October 2024

DEDICATION

I dedicate this dissertation to myself for the many years of studying, crying, sweating, and celebrating. Self, the overwhelming feeling was temporary; the satisfaction of achieving it is forever.

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LIST OF ABBREVIATIONS

~	Approximately
¹³ C	Carbon
¹³ C-NMR	Carbon nuclear magnetic resonance spectroscopy
¹ H	Proton
¹ H-NMR	Proton nuclear magnetic resonance spectroscopy
AIDS	Acquired immunodeficiency syndrome
C ₁₅ H ₁₆ O ₈	Molecular formula of Z-venusol
COSY	Correlation spectroscopy
d	Doublet
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarisation transfer
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DXR	Doxorubicin
ER	Estrogen receptor
ESI	Electrospray ionisation
EtOAc	Ethyl acetate
FDA	Food and Drug Administration
GAE	Gallic acid equivalents
GC-MS	Gas chromatography–mass spectrophotometry
H ₂ O	Water
HER2-	Human epidermal growth factor receptor 2
HMBC	Heteronuclear multiple bond coherence spectroscopy
HPTLC	High-performance thin-layer chromatography
HRMS	High-resolution mass-spectrometry

HSQC	Heteronuclear single quantum coherence
IC ₅₀	50% inhibitory concentration
IV	Intravenous
<i>J</i>	Spin-spin coupling constant
LC-MS	Liquid chromatography–mass spectrometry
MDA-MB-231	Human breast cancer cell line
MCF-7	Breast adenocarcinoma cells
MDR	Multi-drug resistant
MeOH	Methanol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
PR	Progesterone receptor
PTLC	Preparative thin-layer chromatography
QE	Quercetin equivalent
R _f	Retardation factor
SD	Standard deviation
SI	Selectivity index
TFC	Total flavonoid content
TLC	Thin-layer chromatography
TPC	Total phenolic content
UV	Ultraviolet
VLC	Vacuum liquid chromatography
WHO	World Health Organization
δ _c	Carbon chemical shift
δ _H	Proton chemical shift

LIST OF UNITS

°C	Degree(s) Celsius
µg/mL	Microgram(s) per millilitre
µL	Microlitre(s)
cm	Centimetre(s)
eV	Electronvolt
g	Gram(s)
Hz	Hertz
m	Metre(s)
M	Molarity
mg	Milligram(s)
mg GAE/g	Milligrams gallic acid equivalents per gram
mg QE/g	Milligrams quercetin equivalents per gram
mg/g	Milligram(s) per gram
mg/kg	Milligram(s) per kilogram
mg/mL	Milligram(s) per millilitre
MHz	Megahertz
mL	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar
nm	Nanometre(s)
V	Volt(s)
v/v	Volume per volume

CONFERENCE PRESENTATIONS AND WORKSHOPS

Ramatsa, M. W., Adegoke, A. M., & Mfengwana, P. H. (2022, October 19-21). *Phytochemical analysis and cytotoxic effects of Gunnera perpensa L. extracts and fractions on breast cancer cell lines*. Oral presentation at the 2nd African Traditional and Natural Product Medicine Conference, University of Limpopo, Polokwane, South Africa.

Attended the 5th Research Collaboration Symposium, Central University of Technology, Bloemfontein, Free State (2020).

Mfengwana, P. H., & Ramatsa, M. W. (2019, October 2). *In vitro investigation of cytotoxic and genotoxic properties of Gunnera perpensa roots extracts*. Presentation at the Prestige Research Day (PRD) Conference, Central University of Technology (CUT), Bloemfontein, South Africa

PUBLICATIONS

In process:

Ramatsa, M. W., Adegoke, A. M., & Mfengwana, P. H. *Gunnera perpensa*: Overview of herbal medicine used traditionally across Africa for the management of different diseases.

ABSTRACT

INTRODUCTION: The leading cause of mortality worldwide is cancer. According to estimates, 11.5 million people will die from cancer by 2030. A painful cancer fact is that this disease kills more people than malaria, tuberculosis, and AIDS combined. Breast cells are where breast cancer first develops. When it comes to cancers affecting women, research suggests that breast cancer ranks second. Unfortunately, modern therapies like targeted therapy, radiation, and chemotherapy are toxic, often barbaric, and usually ineffective. There is thus an obvious need for alternative treatments and therapies against breast cancer.

The identification of new lead compounds for cancer treatment may be accomplished by the use of natural medicines, including medicinal plants. The creation of different anticancer drugs has been influenced by plant-derived compounds, which include vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan, etoposide, and paclitaxel. It has been discovered that some medicinal plants possess phytochemicals that have biological activity. The presence of secondary metabolites can indicate the biologically active molecules that are known to be anticancer, antimicrobial, anti-oxidant, anti-inflammatory, antifungal, antiviral, and anthelmintic compounds. In addition to treating parasitic diseases, kidney problems, urinary complaints, and sexually transmitted infections, herbal remedies containing *Gunnera perpensa* have been used to induce labour and ease menstrual pain. Certain chemical compounds extracted from *G. perpensa* were reported to be from the class of phytochemical constituents known as triterpenes. Triterpenoids have been demonstrated to possess a wide range of medicinal uses, including fighting cancer, malaria, and pain.

AIM: This study aimed to separate natural compounds from *G. perpensa* and test how harmful, selective, and poisonous they were to cells. In addition, the phytochemicals, together with their total phenolic content (TPC) and total flavonoid content (TFC), were evaluated.

METHODOLOGY: Phytochemical testing was performed on crude aqueous and methanol (MeOH) extracts. The Folin-Ciocalteu colorimetric technique, using Gallic acid as the standard, was used to determine the TPC. The TFC was quantified using a colorimetric technique with aluminium chloride, using quercetin as the standard. To obtain the best results from thin-layer chromatography (TLC) for separating the compounds of the active extracts, different mobile phases were created. The preparation and fractionation of the MeOH extract was done through vacuum liquid chromatography (VLC). Purified compounds were obtained through preparative thin-layer chromatography (PTLC). Structural elucidation of the pure compound was facilitated by the use of nuclear magnetic resonance (NMR). The *in vitro* anticancer and toxicological effects of crude extracts, active fractions, and pure compounds were tested on MDA-MB-231 and MCF-7 cell lines and non-cancerous Vero cells. The cells were grown using standard methods and cytotoxicity and clonogenic tests were performed using standard methods. In each experiment, cells were exposed to dimethyl sulphoxide (vehicle control). Calculating the selectivity index was done using the Vero normal cell line. The data that was acquired was evaluated statistically. GraphPad Prism 5 was the tool employed to analyse data and figure out the IC₅₀.

RESULTS: The phytochemical testing showed that there are terpenoids, alkaloids, flavonoids, reducing sugars, tannins, and phenols which may function inhibitors. The TPC obtained were 17.11 ± 0.036 mg GAE/g and 16.02 ± 0.012 mg GAE/g for MeOH and water (H₂O) extracts respectively. Similarly, the TFC values obtained were 10.33 ± 0.01 mg QE/g and 19.69 ± 0.05 mg QE/g for MeOH and H₂O extracts respectively. Six major sub-fractions concentrated from VLC were obtained. It was possible to isolate two pure compounds, one of which crystallised. MR-A was identified as methyl lespedezate, whereas MR-B was determined to be Z-venusol. Its chemical formula is (7,8-Dihydroxy-6-(hydroxymethyl)-3-[(Z)-(4-hydroxyphenyl)methylidene]-tetrahydro-4aH-pyrano (2, 3-b) (1, 4) dioxin-2-one] and the molecular formula C₁₅H₁₆O₈. Incorporating ¹H and ¹³C NMR spectroscopy into the process improved our understanding of the chemical structure. The compounds' names were confirmed by adding

standards to some of them and matching the results from ultraviolet data, tandem mass spectrometry, and high-resolution mass spectrometry with data that had already been published. Following MTT-based *in vitro* cytotoxic screening the results of the extract, fractions, and isolated compounds of *G. perpensa* against the tested cancer cells displayed moderate to low cytotoxicity. The compound Z-venusol (MR-B) had the best selectivity, suggesting that it might be a safe chemotherapeutic option. The *in vitro* toxicity of Compound MR-B showed weak cytotoxic effects. The diluted MCF-7 cells responded differently to *G. perpensa* extracts depending on the concentration of the extracts. This means that a lower dose rate leads to more cell survival. The findings suggest that this compound may exhibit enhanced efficacy in conjunction with other compounds found in the crude extract of *G. perpensa* due to synergism.

CONCLUSION: The findings provide evidence that *G. perpensa* isolated compounds reduce the survival of MCF-7 breast cancer cells in a way that depends on the concentration, and with apparently low toxicity.

KEYWORDS: *Gunnera perpensa* L., cancer, phytochemicals, vacuum liquid chromatography, active fractions, pure compounds, cytotoxicity activity, clonogenic activity

CHAPTER 1:

GENERAL INTRODUCTION

1.1 INTRODUCTION

Since the very beginning of human existence, people have familiarised themselves with plants and used them in various ways throughout the ages. The use of plants extends beyond food production to include furniture, shelter, fuel, and clothing, as well as disease prevention, management, and treatment. Due to this special past, people have been using natural plants as medicine for many years to treat a wide range of illnesses (Buwa & Van Staden, 2006). Healing practices have typically been handed down from one family to the next through verbal communication, which may cause such information to be lost with future generations. Documentation of indigenous healing systems is insufficient and for this reason, it becomes quite a challenge to continue the use of natural plants without a thorough understanding of the efficacy and safety of herbal products.

However, scientists have done much work in Africa and the world in many ethnobotanical studies to report on the ethnobotanical uses of plants with medicinal value. The impact of journals publishing data on medicinal plants is increasing and, as a result, there is adequate data from laboratory and animal/human studies to support their traditional use. The first written records of natural products appeared in around 2 600 BCE in Mesopotamia (Dias et al., 2012).

Chemical scientists who study natural products started their path in the 19th century when they were asked to figure out the structures of difficult natural products so that the pharmaceutical industry could use them as templates to create new drugs (Howes, 2018). By the 20th century, natural products had become a hot topic in biological sciences like clinical biochemistry, drug interactions, toxic substances, microorganisms, and cell processes. Antibiotics made the study of natural products much more popular and made sure that natural products would stay an important part of growing drug companies. As early as the year 2000, 11% of the 252

medications deemed fundamental and important by the World Health Organization (WHO) came from flowering plants alone (Veeresham, 2012).

Some examples of the most famous drugs isolated from plant origin include the following (see Figure 1.1): aspirin, which is derived from the natural product salicin; morphine, a commercial drug from the opium poppy plant in 1803; taxol, obtained from *Taxus brevifolia* and vincristine and vinblastine, which are obtained from periwinkle (*Vinca rosea*) and used as an antitumor drug (Samuelsson, 1992; Carney et al., 1999; Khalid et al., 2005).

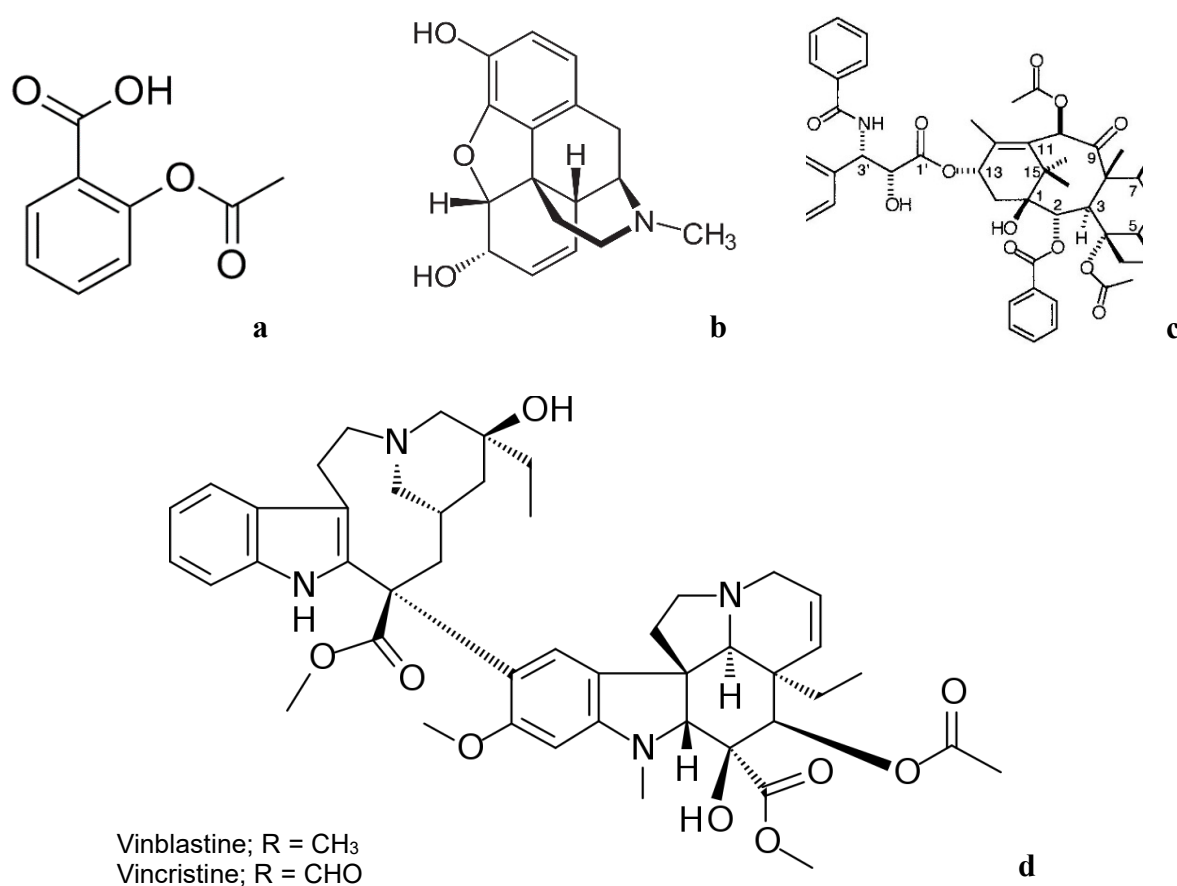


Figure 1.1: The chemical structures of (a) aspirin, (b) morphine, (c) taxol, and (d) vinblastine and vincristine

Source: Gurnani et al. (2014)

1.2 BACKGROUND AND MOTIVATION OF THE STUDY

Natural remedies have been practiced throughout Africa for over a millennium. It is common practice in developing countries to treat various diseases using plant materials (Nahar, 1993;

Ozioma & Chinwe, 2019). *Gunnera perpensa* is a remedy that is widely recognized for its medicinal properties in various traditional medicine systems. In Southern Africa, it is used to induce labor, ensure smooth childbirth, and facilitate the expulsion of the placenta and cleansing of the womb after birth in both women and animals (Maroryi, 2016). The genus *Gunnera* part of the family Gunneraceae, is among the oldest living angiosperm genera and is geographically distributed worldwide. However, *G. perpensa* L. is the only African member of the genus that is widespread in tropical Africa from sub-Saharan African countries like Zaire and Southern Africa, including Lesotho and Swaziland (Chigor, 2014).

According to Ncube et al. (2011), 60% of the South Africans were thought to be using traditional medicinal herbs as a form of treatment in 2000. With over 3 000 kinds of medicinal plants, South Africa is home to a large number of traditional healers who prescribe a wide variety of traditional medicines (Dyubeni & Buwa, 2012; Hübsch et al., 2014). Even though these numbers seem low, it should be noted that South Africa is very rich in medicinal plants and contains 10% of the world's plant diversity (Eloff, 1998).

The plant of interest in this investigation was *G. perpensa*. Based on ethnomedicinal evidence suggesting its historic use in the treatment of several illnesses, this plant was selected. Numerous phytochemicals have been discovered in *G. perpensa* rhizomes by scientific investigation. Brookes and Dutton (2007), isolated seven bioactive compounds from methanol (MeOH) extracts, all of which were identified as phenolic compounds with antiseptic and antioxidant activities. These compounds are recognized for their anticarcinogenic, antimutagenic, and antihaemorrhagic capabilities, which enhance the plant's benefits, particularly in pregnancy-related medicines. As previously stated, traditional medicinal plant use is good for human health; however, there isn't enough published evidence to support this claim.

While medicinal plants used in traditional medicine provide some advantages, they are assumed to be completely safe, even though this is not true. Many times, it has been said that some traditional medicinal plants could cause cancer, mutations, and other harmful effects if

they are not used correctly. Because of this, the safety of these plants is therefore necessary (Fennell et al., 2004). This research aimed to examine the toxicity of isolated compounds from *G. perpensa*.

1.3 PROBLEM STATEMENT

Multiple factors, including DNA changes and environmental exposure, can lead to breast cancer (Kemp, 2015). Reports suggest that breast cancer is a genetic disease (Vogelstein & Kinzler, 2004); however, inherited genetic predisposition makes very little contribution. BRCA1 and BRCA2, two tumour-suppressor genes, are responsible for just 5% of breast cancer cases, with the reason for the remaining 95% being unknown (Grover & Martin, 2002; Bishayee & Mandal, 2014; Kemp, 2015). The main health concern is that breast cancer is a group of diseases that are closely related, and the occurrence of breast cancer is influenced by lifestyle-related factors such as obesity and diabetes (Bishayee & Mandal, 2014; Minari et al., 2016). Supporting the latter idea, increased exposure to endogenous or exogenous oestrogen has also been found to cause breast cancer. The most common mutation that arises in and contributes to certain cancers, including that of the breast, is caused by exposure to environmental pollutants, including polycyclic aromatic hydrocarbons, as well as diet (Singletary et al., 1996; Grover & Martin, 2002).

Cancer of the breast is a widespread and fatal illness. It has been recognized as the second most common cause of death from cancer in women around the world (Klug & Rehli, 2006; Gunnarsson et al., 2008). Most anticancer drugs are hydrophobic and therefore lead to poor bioavailability (Naderinezhad et al., 2017). Moreover, current medical therapies such as chemotherapy, radiation therapy, and targeted therapy are toxic, often barbaric, and usually ineffective. The concerning side effects of current anticancer medications can lead to significant health risks and complicate adherence to treatment. Additionally, the high costs associated with these therapies create barriers for many patients. This highlights a clear demand for alternative treatment options (Sabiou et al., 2015). Barks, leaves, flowers, roots,

fruits, and seeds have been identified as plant products that have the potential to reduce the incidence and mortality of specific malignancies with minimal or no side effects (Rastogi et al., 2007). Even though many natural drugs have been found, the search for new cancer-fighting drugs is still going on.

Gunnera perpensa is an underutilized medicinal plant that is found in Africa, traditionally used to treat a wide range of ailments, including inflammation, infections, and reproductive health issues. Despite its conservation status, little research has been conducted, especially regarding its anticancer potential and safety profile (Maroyi, 2016). Scientific investigations have shown that compounds isolated from *G. perpensa*, such as Z-venusol, induce apoptotic cell death in breast cancer cells in vitro, highlighting its potential as a source of new anticancer agents (Mathibela et al., 2016; Mfengwana & Sone, 2023). However, much of the plant's phytochemistry and pharmacology remain unexplored, and many isolated compounds have not yet been thoroughly evaluated biologically. Additionally, toxicological assessments of these compounds are limited, though preliminary results suggest a lack of genotoxicity, supporting the plant's relative safety for medicinal use (Mfengwana, 2019).

Filling these gaps helps ensure that warnings about the toxic effects of *G. perpensa* isolated compounds can be issued, allowing people to make informed decisions about its use. Hence, the choice of *G. perpensa* as a case study plant.

1.4 AIM OF THE STUDY

G. perpensa is of considerable ethnobotanical interest in traditional medicine because of its wide usage. The rhizomes are widely used and indiscriminately collected in large quantities from the wild to meet the ever-increasing demand of the traditional medicine markets. As a result, the species is mostly affected because its underground parts are harvested.

This study effort examined the chemical variation in the rhizomes and the preliminary bioactivity of the *G. perpensa* medicinal plant. This project sought to determine the selective cytotoxicity of *G. perpensa* (crude extract, fractions, and isolated compounds).

The research project intended to achieve the following objectives:

- To determine the phytochemical profile of the *G. perpensa* plant, through qualitative and quantitative screening
- To isolate and characterise structure of *G. perpensa*; and
- To screen crude extracts and isolated fractions of *G. perpensa* for their anticancer activity and toxicity through *in vitro* biological assays.

This project's primary aim was to isolate compounds from *G. perpensa* and to investigate their cytotoxicity on breast cancer cells and toxicity *in vitro*.

1.5 STRUCTURE OF THE DISSERTATION

The dissertation's remaining work is structured as follows:

- Chapter 2 presents a literature review on *G. perpensa*.
- Chapter 3 discusses the phytochemical profile of *G. perpensa* as a medicinal plant through qualitative and quantitative study.
- Chapter 4 explains the isolation and structural characterisation of *G. perpensa*.
- Chapter 5 presents the results of the clonogenic assay to demonstrate the effectiveness of *G. perpensa* as an anticancer drug, as well as a toxicological evaluation on the active fractions.
- Chapter 6 provides the conclusion and limitations of the study.

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CHAPTER 2:

LITERATURE REVIEW

2.1 WHAT EXACTLY IS CANCER?

The Greek word *carcin*os, meaning crab, is where the term “cancer” originates. Hippocrates (460 to 370 BCE) was the first to use this phrase to describe lumps in the body that look like fingers or claws, that extend into surrounding tissues (Stephens & Aigner, 2009). Celsius (28 to 50 BCE) finally turned *carcin*os into cancer, which is the Latin word for it (American Cancer Society, 2018).

Cancer is a leading cause of death in both developing and developed nations worldwide. Globally, the GLOBOCAN 2020 estimates of cancer incidences and mortality, as reported by the International Agency for Research on Cancer report (Sung et al., 2021), indicate that there were ~ 10 million cancer deaths and 19.3 million new cancer cases in 2020. The International Agency for Research on Cancer Statistics projects, 1.28 million additional cancer cases and 970 000 cancer-related deaths in Africa by 2030, mostly attributed to aging and the population growing (Ferlay et al., 2010). By 2030, the number of cancer cases in South Africa may rise by 78% (Bray et al., 2012). Adoption of lifestyles connected with economic development, such as smoking, poor diet, and limited physical activity, may cause the development to become even worse [Cancer Alliance, 2021(Finestone et al., 2021)].

There are many things that can cause cancer. It starts with the buildup of mutant proto-oncogenes, tumour-suppressor genes, and deoxyribonucleic acid (DNA)-repair genes. This causes uncontrolled abnormal cells to develop, which can then turn into a growth or tumour or spread throughout the body, and start abnormal growth in other places (Minari, 2014). Instead of dying before cell division, cells lose their ability to stop dividing and more abnormal cells are formed, which accumulate to form a mass of cancerous tissue known as a tumour

(Karnam et al., 2017). Tumours can either be benign (non-cancerous) or malignant (cancerous).

Despite increased interest in developing anticancer drugs, cancer prevention is a complex topic because the root causes of cancer are non-specific; it thus becomes harder to avoid it (Twombly, 2005). The disease is complex because sometimes the use of cancer-treating methods such as chemotherapy may lead to further mutations by having serious side effects on healthy cells (Amjad et al., 2022).

2.2 BREAST CANCER

There are over 100 different types that can occur (National Cancer Institute, 2015), and many of them are named after the organ or the type of cells they come from. There are five main types of cancer cells, namely:

- 1) Carcinoma, a form of cancer beginning in the internal organs or the skin;
- 2) Sarcoma, a type of cancer beginning in bone, cartilage, fat, muscle, blood vessels, or other connective or supporting tissue;
- 3) Leukaemia, a form of cancer that originates in blood-forming tissue like bone marrow;
- 4) Lymphoma and myeloma, two cancers beginning in the immune system; and
- 5) Central nervous system cancers, begin in the brain or spinal cord (Cancer Research UK, 2014).

This section discusses breast cancer, which is the most common cancer in women around the world and the main reason women die (Parkin et al., 2005).

Globally, breast cancer is the second most common cause of death among women and a widespread disease (Gunnarsson et al., 2008; Sharma et al., 2010; Obeagu & Obeagu, 2024; Menon et al., 2025). Men only make up ~1% of breast cancer cases in both developed and developing countries, making it the most common cancer in women. Cancer of the breast is a worldwide public health issue (Ojeswi et al., 2010; Bishayee & Mandal, 2014; Minari et al.,

2016; Giordano, 2018). Breast cancer has recently surpassed cervical cancer as the most common cancer in women in sub-Saharan Africa (Mfengwana et al., 2019; Minari et al., 2016). Multiple factors can lead to breast cancer, such as getting your first period early, going through menopause late, getting pregnant late, being overweight, using oral contraceptives or hormone replacement therapy, gender, eating habits, drinking alcohol, body movement, family history, breastfeeding, and having had benign breast diseases in the past (Krishnamoorthy & Sankaran, 2016; Shareef et al., 2016). These factors together are responsible for the most (~80%) of all breast cancer cases.

Men and women have slightly different breast structures. The milk glands and fatty tissue in a woman's breasts make breastfeeding easier, while the nerves in a man's breast nipple enhance sexual arousal. The female breast is composed of three different tissue types: glandular, connective, and fatty tissues (Sharma et al., 2010). The glands that produce milk (lobules) and the ducts (the milk passages) are located in glandular tissue. Both the glandular and fatty tissues of the breast are held in place by connective tissue. The size of the breasts is determined by the amount of fatty tissue that fills the spaces between the glandular and connective tissue (Katulski et al., 2024).

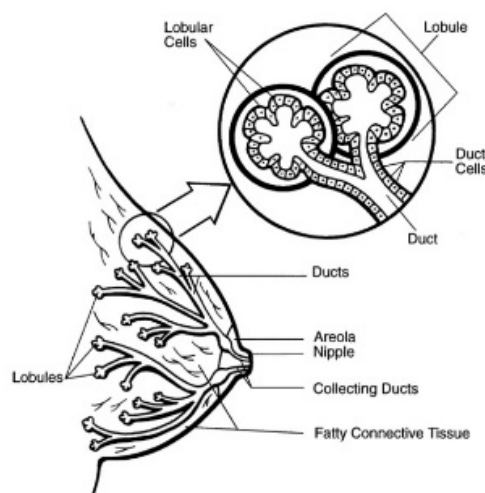


Figure 2.1: The schematic structure of the breast

Source: Haider (2023)

According to Breastcancer.org (2025), states that there are many forms of breast cancer and they are named after the tissue of origin of the cancer, and whether it has spread into the breast tissue:

- **Ductal:** These are the tubes that carry milk to the nipple. They are where cancer cells develop.
- **Lobular:** The lobes or lobules, which produce milk, are where cancer cells began
- **In situ:** All cancer cells are contained within the ducts or lobes.
- **Invasive:** Cancer has progressed from the ducts or lobes to the tissue surrounding them.

The following groupings of breast cancers may be found using these classifications of breast cancer types:

- **Ductal carcinoma in situ:** The most basic kind of breast cancer. Although cancer has begun to metastasize (spread to other parts of the body), it is still contained in the breast ducts.
- **Invasive ductal carcinoma** is when cancer cells enter the surrounding breast tissue from outside the duct or lobule lining. Two subtypes of invasive breast cancer are as follows:
 - *Invasive ductal breast cancer:* When cancer cells lining invade neighbouring breast tissue. It accounts for 80% of all cases of breast cancer.
 - *Invasive lobular breast cancer:* A type of breast cancer that develops in the milk-producing glands of the breast. Invasive lobular breast cancer accounts for ~10% of the two kinds of invasive cancers.
- **Paget's disease of the breast** is an unusual kind of breast cancer, which develops when cells from the breast's milk ducts spread to the nipple. It shows up on the nipple skin as a red, scaly rash that looks like eczema.

- **Inflammatory breast cancer** is a rare form of breast cancer that develops when cancer cells proliferate and interfere with the lymph vessels in the breast's epidermis. This disorder is named after the swelling and inflammation that occurs in the lymph vessels and the breasts due to the illness.
- **Triple-negative breast cancers** lack receptors for estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2). Consequently, the proliferation of the malignancy is neither facilitated by the hormones estrogen and progesterone nor by the presence of HER2 receptors. This kind of breast cancer is mostly treated with surgical intervention, followed by chemotherapy, since hormonal therapies like tamoxifen or aromatase inhibitors don't work on this type of breast cancer.

2.3 CURRENT TREATMENT REGIMENS

In most developed countries, conventional breast cancer treatments include the “Big 3”, known as surgery (to remove a tumour), radiation (X-rays and gamma rays of a cancerous tumour that causes cancer cell death or apoptosis, which protects the structures around the tumour but kills non-visible cancer cells), and chemotherapy (systemic administration of anticancer drugs that travel through the bloodstream and kill cancer cells) (National Cancer Institute, 2015). Hormone therapy and targeted therapy (National Cancer Institute, 2015; Harfindal & Helms, 2006) are therapies that are currently used by oncologists to control tumour growth, prolong survival time, and improve the quality of life of a patient to some extent. Most treatment plans include a combination of the abovementioned treatment options and are not limited to using a single agent. However, these therapies are toxic and have limitations in safety and efficacy (Karia et al., 2018).

2.3.1 Surgery

Surgery is the least harmful option because in some instances it can be a life-saving treatment. The majority of patients are diagnosed too late to undergo surgery due to poor diagnosis and

other factors. Once cancer has metastasised to other areas in the body, surgery has no benefit and can cause problems like bleeding, infections, bile reflux, and lymphedema. Also, patients who have undergone a resection often experience postoperative recurrence and metastasis. Surgery only removes tumours at specific, known locations, and does not remove the original cause of cancer (National Cancer Institute, 2015).

2.3.2 Chemotherapy and radiotherapy

The National Institute of Health reports that these therapies are subjected to more than 65% of patients (Cheng et al., 2000). Chemotherapy drugs are used to eradicate cancer cells present throughout the body. They may be provided in many ways such as by slow intravenous (IV) drip, oral tablet, or short IV injection, depending upon the specific medication. More often than not, chemotherapy is effective when given after surgery to prevent cancer recurrence (adjuvant therapy). Even though it may be effective, numerous publications and studies have reported on the toxicity of the drugs used in chemotherapy as they destroy not only cancer cells but other cells in the rest of the body as well. According to Amjad et al. (2022), chemotherapy drugs can be classified according to the following mechanisms of action:

- **Alkylating agents (cyclophosphamide, chlorambucil)** work by attaching chemical groups to DNA, which damages the cancer cell's genetic material and prevents it from dividing, eventually leading to cell death.
- **Antifolates (methotrexate)** block the body's ability to use folic acid to make DNA and other essential molecules, stopping cancer cells from growing and multiplying.
- **Purine and pyrimidine analogues (clofarabine, nelarabine, 5-fluorouracil)** mimic the building blocks of DNA, getting mistakenly used by cancer cells disrupting their ability to copy genetic material correctly.
- **Antitumor antibiotics (actinomycin D, bleomycin, daunomycin)** insert themselves into the DNA of cancer cells or generate free radicals that damage the DNA, making it impossible for the cells to grow and divide.

- **Antimicrotubular Agents (anthracyclines)** inhibit DNA synthesis. In addition, it inhibits topoisomerase II, causing inhibition of DNA repair, resulting in a blockade of DNA synthesis.

Moreover, chemotherapy weakens the immune system and causes multiple complications such as increased infections, nausea, fatigue, alopecia, and irreversible damage to the heart, lungs, liver, and kidneys; thus leaving the body without a defence mechanism and making a cancer patient more susceptible to disease, including new forms of cancer (Amjad et al., 2022).

Radiation has all the disadvantages of surgery and uses high doses of radiation to shrink cancer. Along with surgery or combined with systemic therapy, it may improve cure rates and allow for less extensive surgery. There are two ways to deliver radiation to the cancer site: external radiation therapy, which targets tumour from outside the body by using high-energy rays (photons, electrons, protons) and internal radiation therapy (brachytherapy) which involves placing radioactive seeds directly into the tumour site from inside the body (Baskar et al., 2012; Gale & Spivak, 2024).

2.3.3 Targeted therapy

Targeted therapy is a newly introduced treatment protocol that has the advantage of precisely detecting and attacking cancer cells. Certain medications, however, aim at substances that are more common in cancer cells but may also be present in healthy cells. This can lead to a number of problems, such as high blood pressure, renal damage, cardiac problems, and allergic reactions. It should also be noted that targeted anticancer drugs are costly and thus not affordable to all cancer patients for socio-economic reasons (National Cancer Institute, 2015; Saini et al., 2020).

2.3.4 Hormone therapy

Most tumours that test positive for either ER or PR (called ER positive or PR positive) may be effectively treated with hormone therapy, often called endocrine therapy. The goal of this kind of treatment, known as neoadjuvant hormonal therapy, is to reduce the size of a tumour before surgery so that the procedure may go smoothly and lowers the risk of recurrence. Additionally, it might also be administered as adjuvant hormonal therapy after surgery in order to reduce the likelihood of recurrence (National Cancer Institute, 2015).

2.3.5 Immunotherapy

Biological therapy is a relatively new method of treating breast cancer that aims to strengthen the immune system so that it can better fight cancer cells. Immunotherapy forms part of the therapies that are currently used and includes cytokine infusions, cancer vaccines, and T-cell therapy (American Cancer Society, 2021).

Most people who need immunotherapy can't afford it because it costs so much. So far, a lot of progress has been made in finding and making chemotherapy drugs that work and are safe. However, every day more people die from cancer. This circumstance necessitates the urgent exploration of alternative therapies to enhance patients' quality of life (American Cancer Society, 2021). Drugs from plants are readily accessible, cheaper, more efficient, and are believed to be safe. Several plant components, vitamins, and synthetic compounds have been found to reduce the occurrence and death rate of certain cancers in high-risk human populations (Sauter, 2020).

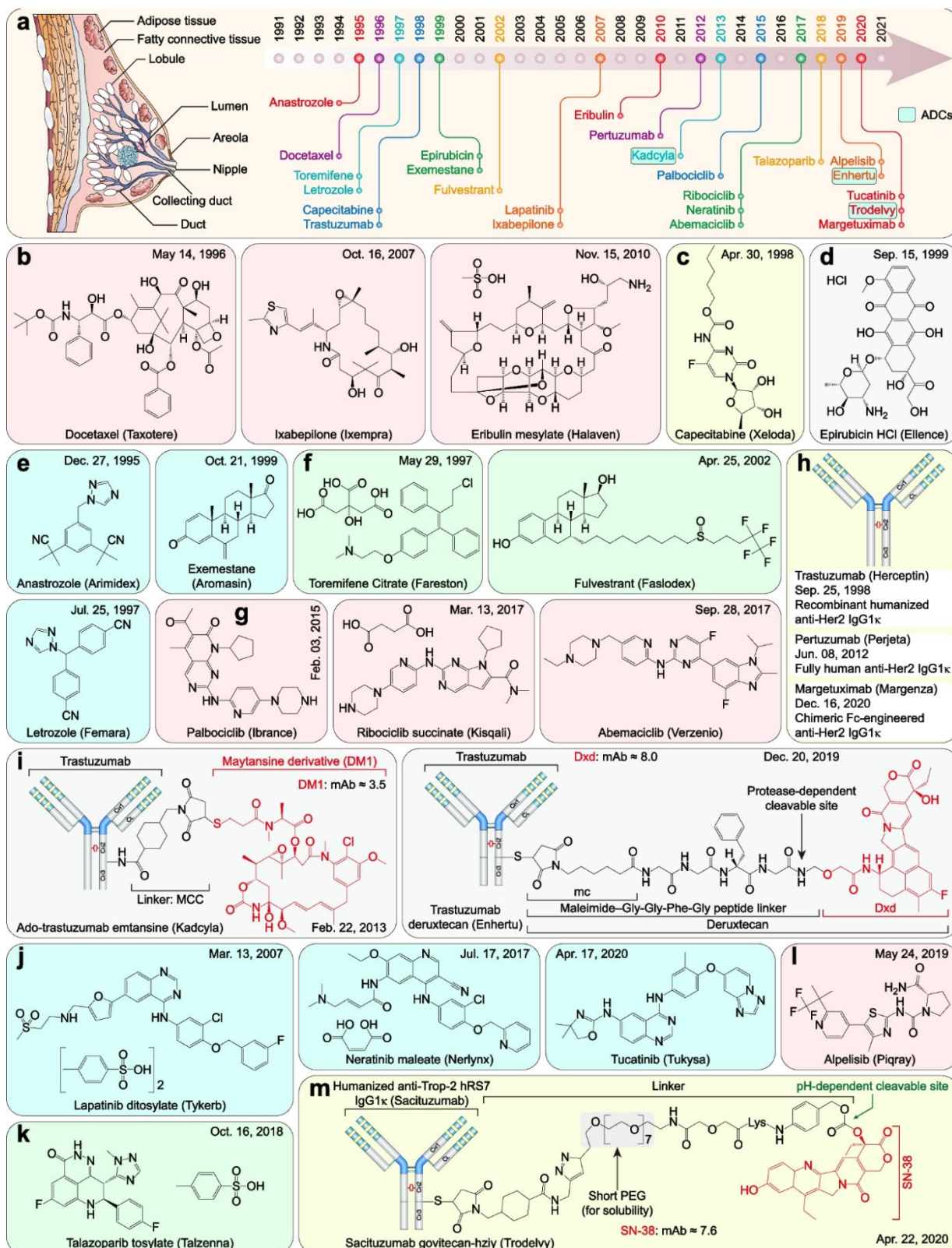
2.4 THE SEARCH FOR NEW ANTICANCER LEAD COMPOUNDS FROM NATURAL SOURCES

According to the WHO (2009), poverty and the absence of contemporary medication compel 65% to 80% of the global population to rely only on plants for basic healthcare.

During the early 1950s, scientists began to search for anticancer agents from plant sources, which led to the discovery and development of vinca alkaloids and the isolation of cytotoxic podophyllotoxins (Moraes et al., 2017). The American National Cancer Institute launched an extensive plant collection effort in 1960 as a result of these findings (Cragg et al., 2007). In the 21st century, natural products represent more than 50% of all drugs in clinical use (Thomford et al., 2018). Up to 50% of the herbal medications that have been licensed in the past three decades originate directly or indirectly from natural sources, including plants, marine organisms, and micro-organisms. Molecules derived from natural sources (so-called natural products) have played and continue to play a major factor in the identification of leads for the development of conventional drugs to treat the majority of human illnesses (Calixto, 2019).

Among the many beneficial anticancer drugs that scientists have found are the discovery of vinca alkaloids (vinblastine, vincristine, and vindesine), epipodophyllotoxins (toposide and teniposide), taxanes (paclitaxel and docetaxel), and camptothecin derivatives (camptotecin and irinotecan), which are sourced from medicinal herbs (Stopek & Covidien, 2015). Taxol is a diterpene that was isolated from the Pacific yew tree, *Taxus baccata*, and is used in the majority of traditional chemotherapeutic medicines. Nowadays, taxol is semi-chemically synthesized as paclitaxel by using needles and twigs from yew species that are cultivated under agricultural conditions (Mohanlall & Naicker, 2020). According to Kintzios & Barberaki (2019), the Food and Drug Administration (FDA) has authorized this medication for the treatment of lung, breast, ovarian, and other cancers. Among these cytotoxic drugs, docetaxel represents one of the most prominent microtubule-stabilising agents. Although docetaxel and its analogue paclitaxel shares the taxane-binding site of β -tubulin (Diaz & Andreu, 1993), docetaxel has stronger anticancer activity (Wang et al., 2011). It exerts its activity by binding to free β -tubulin and inducing microtubule polymerization, which results in cell cycle arrest and death (Morse et al., 2005; McKeage, 2012).

The discoveries of medicinal herbs as major sources of chemotherapeutic agents led to increased evaluation of the efficacy of several extracts by industries and research institutes worldwide (Mondal, 2012). The targets of these compounds tend to broadly fall into three categories, namely those targeting topoisomerases, those inducing apoptosis, and those disrupting oncogenic signalling pathways (Hsiao & Liu, 2010). Resistance to apoptosis is a common hallmark of cancer cells (Hanahan & Weinberg, 2011) and the induction of apoptosis is a common mechanistic endpoint of many effective anticancer therapies. Among the compounds that have been isolated, many conventional drugs or their precursors are derived from plants, and derived compounds elicit their antitumor effects (Parekh et al., 2009). Figure 2.2 shows other plant-derived compounds and/or therapeutic drugs for breast cancer that have been commercialised according to the year of approval. The main advantage that plant-derived compounds have over synthetic drugs in the treatment of cancer is their relative non-toxicity and better bioavailability profiles (Amin et al., 2009). By mechanisms of action, most of the plant-derived conventional anticancer drugs are methyltransferase inhibitors, DNA damaging/pro-oxidant, histone deacetylase inhibitors, and mitotic disruptors (Amin et al., 2009).



2.5 PLANTS AS A SOURCE OF MEDICINE

Herbal plants are useful in both preventing and curing with the use of human diseases. For thousands of years, people have used plants as traditional medicine. The WHO defines traditional medicine as “the totality of knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the prevention, diagnosis, and improvement of physical and mental illnesses as well as in the maintenance of health” (Akerlele, 1991).

There is mounting scientific evidence that proves that healing with medicinal plants is as old as humankind, and more than two billion people worldwide use medicinal plants to treat various ailments (Nicholson, 2016). African traditional medicine is perhaps the oldest, and most diverse of all therapeutic systems (Mahomoodally, 2013). Africa is widely regarded as the cradle of humankind, with rich biological and cultural diversity marked by regional differences in healing practices (Gurib-Fakim, 2006). Plant-based medicine has been used in Africa since 3 200 BCE (Dalziel, 1937). The use of medicinal plants was brought to light through many years of hardships and struggles against illness, which prompted humans to seek medicines in leaves, roots, barks, and other parts of plants (Bijauliya et al., 2017). The bioactive substances in these plant species have been linked to secondary metabolites such as phenolic compounds (curcumins, flavonoids, and tannins), saponins, terpenoids, and alkaloids. These plant metabolites are thought to have biological and medicinal qualities such as anti-oxidant and antimicrobial (that improve the removal of waste products and toxins), anti-inflammatory compounds (that reduce swellings and pain), and anticancer (that initiate apoptosis by turning on the expression of signal molecules) (Sabiou & Ashafa, 2016; Sabiou et al., 2016).

Traditional use of plants has continued into modern times, as the WHO (2009) estimated that 80% of people living in developing countries rely on traditional medicine (mostly herbal medicines) to meet their primary healthcare needs (Chaudhary et al., 2010). In South Africa,

many people still use plants as medicine as an alternative to visiting healthcare practitioners (Van Wyk & Gericke, 2000). This is not surprising given that South Africa is one of the top countries in terms of cultural diversity, as well as its large floral biodiversity (Hoveka et al., 2020). South Africa is home to over 30 000 species of higher plants and 3 000 of these species have been found to be used in traditional medicines across the country (Van Wyk & Gericke, 2000). Such alternatives usually cost only a fraction of the price of conventional breast cancer treatment.

2.6 PLANT SELECTION

For thousands of years, herbal remedies have been used to treat various diseases. The roots of *G. Perpersa* L. have traditionally been used to induce labour, expel the placenta after birth, for milk letdown, and to relieve menstrual pain (Nkomo et al., 2010; Simelane et al., 2010). Scientific reports indicate that *G. perpersa* contains several bioactive compounds, which makes this plant beneficial in managing many disorders. The bioactive compounds of *G.perpersa* and their uses are described in Table 2.1. There are limited studies on the chemical composition of *G. perpersa*.



Figure 2.3: *G. perpersa* showing dark bluish-green, kidney-shaped leaves, tiny flower buds, and pinkish or reddish-brown flowers

Source: Random Harvest (n.d.)

Table 2.1: Bioactive compounds of *G. perpensa* L. and their Uses

Compound	Pharmacological Uses	Target Diseases
Ellagic acid, 3,3',4'-tri-O-methyl ellagic acid	Antioxidant, anti-inflammatory, anti-mutagenic, anticarcinogenic	Potential benefits during pregnancy and birth (Drewes et al., 2005)
Ellagic acid lactone	Antimutagenic, anticarcinogenic	Cellular protection (Brookes & Dutton, 2007)
Z-venusol	Antiseptic, antioxidant, anticancer	Enhance uterine response, postpartum haemorrhage (Khan et al., 2004; Mathibe et al., 2017)
Z-methyl lespedezate	Not specified, but isolated as a bioactive compound	Not reported (Khan et al., 2004)
Punicalagin	Antioxidant, anti-inflammatory, anti-mutagenic	Cardiovascular protection, cancer prevention, COVID-19 management (Brookes & Dutton; Invernizzi et al., 2022)
2-methyl-6-(3-methyl-2-butenyl)-benzo-1,4-quinone	Antimicrobial, anticancer	Bacterial and fungal infections, cancer prevention (Drewes et al., 2005; Chavan et al., 2013)
3-hydroxy-2-methyl-5-(3-methyl-2-butenyl) benzo-1,4-quinone	Not specified, but isolated as a bioactive compound	Not reported (Drewes et al., 2005)
1,1'-Biphenyl-4,4'-diacetic acid	Not specified, but isolated	Not reported (Brookes & Dutton, 2007)
p-Hydroxybenzaldehyde	Antioxidant, anti-inflammatory	Inflammation-related conditions (abdominal pain, kidney inflammation) (Brookes & Dutton, 2007)
β -sitosterol	Not specified, but isolated as a bioactive compound	Not reported (Mammo et al., 2017)

G. perpensa L, commonly called river pumpkin, is a perennial, robust, erect herb that grows up to 1 m tall, with petioles that vary from 150 to 750 mm in length (Mendes, 1978). The plant belongs to the genus *Gunnera* (family Gunneraceae), which is a genus of herbaceous

flowering plants comprising 40 to 50 species. *G. perpensa* is the only African member of the genus that is widespread in tropical Africa in Sudan, Ethiopia, Zaire, Rwanda, Uganda, Kenya, Tanzania, Zimbabwe, Mozambique, Lesotho, and Swaziland (Hinkley, 2007). In South Africa, it is distributed in the Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, Northern Cape, North West, and the Western Cape provinces (Williams et al., 2008). The plant has large, dark bluish-green, kidney-shaped leaves that are covered with hairs on both surfaces and arise from a central tuft near the top of the apex, just above the soil level (Mendes, 1978). The margin of the leaves is irregularly toothed. The flowers are numerous, small, pinkish or reddish-brown, not very noticeable, and borne on a long slender spike that is taller than the leaves (Mendes, 1978). The plant flowers between September and February. The roots are up to 300 mm thick and creep in black, muddy soil. Like any other plant that grows under various conditions, *G. perpensa* has conditional seasons, as it is unable to endure frost and cold conditions (Mendes, 1978). According to Dold and Cocks (2002), despite the plant's widespread distributional range, its population is declining due to overexploitation of its roots, which are sold at medicinal markets throughout the country. Most medicinal plants in the country are collected from the wild, and so is *G. perpensa* L.



Figure 2.4: (A) *G. perpersa* plant showing a perennial, robust, erect herb that always grows near water, (B) fresh root stock, and (C) powdered plant material

Source: Researcher

2.7 THE THERAPEUTIC POTENTIAL OF SECONDARY METABOLITES

Phytochemistry (*phyto* in Greek means plant) is the study of naturally occurring phytochemicals, which are bioactive (or not) chemical compounds found in plants that provide health benefits to humans, which are not found in macro- and micronutrients (Hasler & Blumberg, 1999). Plants synthesise a wide variety of chemical compounds that have specific biological functions that defend against a variety of diseases (Kumar, 2019). These are called phytochemicals.

Organic compounds known as secondary metabolites provide specific physiological action in the human body (Edeoga et al., 2005; Yadav & Agarwala, 2011). Most of the bioactive compounds with a wide variety of free radical scavenging molecules and therapeutic effects include phenolic compounds (phenolic acids, flavonoids, quinones, coumarins, lignans,

stevens, and tannins), nitrogen compounds (alkaloids, amines, and betalains), vitamins, terpenoids (including carotenoids), and a few other endogenous metabolites that are rich in anti-oxidant activity (Pan et al., 2013). The synthesis of different classes of secondary metabolites from primary metabolites is presented in schematic form in Figure 2.5.

Plants typically contain mixtures of different phytochemicals (secondary metabolites) that may act individually, additively, or in synergy to improve health. Unlike pharmacological drugs, medicinal plants typically contain multiple chemicals that catalyze and synergistically to produce a combined effect that speeds up or slows down the body's absorption of the primary medicinal constituent, surpassing its total activity (Sabiu et al., 2016).

Throughout history, plants' secondary metabolites have been utilised by humans. It is not surprising that many are used as pharmaceuticals, spices, fragrances, pesticides, poisons, hallucinogens, stimulants, or colorants (Barz & Ellis, 1981; Wink, 1988). Phytochemicals are inexpensive, effective, readily applicable, and easily accessible bioactive compounds that neutralise free radicals that cause cell damage. The inherent potential of these phytochemicals in the chemoprevention of cancer cannot be overemphasised, especially considering robust cell damage (Harborne, 1998; Hossain & Nagooru, 2011). Various secondary metabolites have the ability to indicate biologically active molecules such as anti-oxidant, antimicrobial, antifungal, anticancer, antiviral, and anthelmintic compounds (Daboor & Haroon, 2012). This has extended the field of research for potential anticancer compounds, as some derived anticancer compounds are already extensively used.

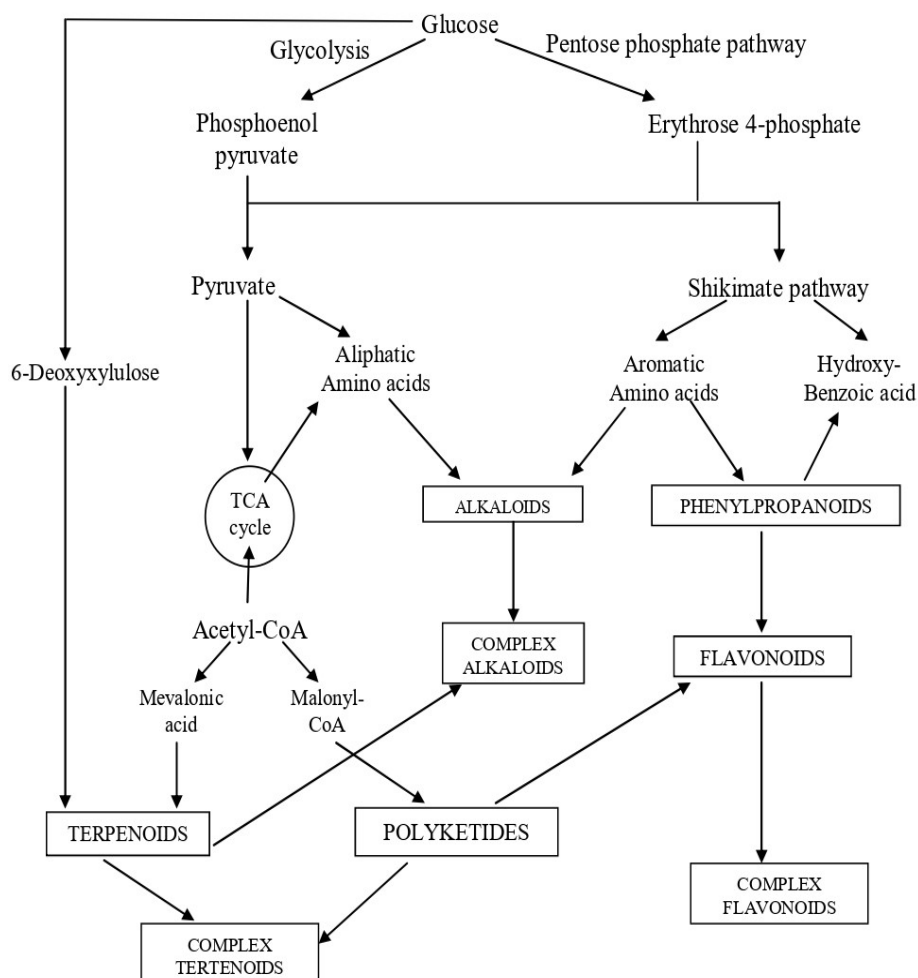


Figure 2.5: Principle biosynthetic pathways leading to the synthesis of secondary metabolites

Source: Adapted from Fordham-Skelton et al. (1999, p. 195)

2.8 CHEMICAL SCREENING

The isolation of pure, pharmacologically active constituents remains a long and tedious process. Therefore, it is necessary to have techniques that may do with needless separation processes. Hence, chemical screening is carried out to enable the localization and targeted isolation of novel or beneficial components with potential activities. The identification of recognized metabolites in extracts or in the initial phases of separation is made possible by this process. For the localization and rapid characterization of natural compounds, phytochemists rely heavily on chromatographic methods as an analytical support.

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CHAPTER 3:

QUANTITATIVE AND QUALITATIVE PHYTOCHEMICAL CONSTITUENTS OF THE RHIZOMES OF *G. PERPENZA* L.

3.1 INTRODUCTION

Botanical medicine, often known as phytomedicine, is the broad term for plants utilized for medicinal purposes. These plants, either whole or in parts, that have therapeutic qualities, are referred to as crude drugs of natural or biological origin (Anoka, 2012). They have endured over time because of their low cost, affordability, availability, acceptability, and low toxicity. Because of their exceptional chemical diversity, secondary metabolites found in medicinal plants are known to offer countless potential for identifying new drug leads (O'Bryan et al., 2008).

In most cases, secondary metabolites are not necessary for the growth, development, or reproduction of the plant. They are created either because of the plant adjusting to its surroundings or as a potential defence mechanism against predators to help the plant survive. The core activities of photosynthesis, glycolysis, and the Krebs cycle serve as the starting point for the lengthy process of producing secondary metabolites (Hakim & Kadarohman, 2020).

Some bioactive substances derived from plants are carotenoids, alkaloids, tannins, steroids, flavonoids, and phenolic compounds (Doughari, 2012). These compounds act as a plant defence strategy against herbivorous animals, insects, and microbial predators (Hakim & Kadarohman, 2020; Kaunda & Zhang, 2019). Terpenoids repel predators with their unpleasant smell and bitter taste. Alkaloids are among the natural products that deter and repel predators, while occasionally also poisoning them (Chowański et al., 2016).

According to Africander & Storbeck (2018), breast cancer has surpassed lung cancer as the second most common cause of cancer-related deaths in women. Moreover, it is also a growing

health problem in sub-Saharan Africa (Sylla & Wild, 2012) and comprises approximately 10% of all new cancer diagnoses worldwide (Krishnamoorthy & Sankaran, 2016). Chemotherapy, radiation, hormone therapy, and surgery are among the available therapies for breast cancer (Tao et al., 2014), yet the issue of indiscriminate toxicity and severe side effects still exists (Mfengwana et al., 2019; Ogbole et al., 2017). The negative adverse effects from synthetic drugs motivated researchers to look for new effective drugs, with an emphasis on going back to natural sources of medicine (Mfengwana et al., 2019). Many researchers from around the globe have confirmed that products derived from plant have effective activity against cancer with efficacy and acceptable toxicity to cancer patients (Shammugasamy et al., 2019). Efforts are being made by African scientists to search for new drugs from among their most affordable resources, which are medicinal plants. Several plants from the flora of Africa have been found to be active against various types of cancer cells. Even if not reported in the scientific literature for their antiproliferative potential, several medicinal plants of the African continent contain known antineoplastic compounds (Kuate & Efferth, 2011). African flora have enormous potential in terms of phytochemicals that are active against multi-drug resistant (MDR) cancer cells and have unstudied anticancer potential, since they include an impressive arsenal of bioactive compounds (Kuate & Efferth, 2015; Kuate et al., 2017).

The most important bioactive constituents of plants that are beneficial to humans are steroids, alkaloids, triterpenes, terpenoids, lignans, tannins, flavonoids, and phenolic compounds that are usually found in plants, and these can play a significant role in breast cancer and cancer treatment (Kamble & Gacche, 2019). Published scientific evidence has revealed that phenolic compounds possess several biological properties, such as anti-oxidant, anti-inflammatory (Mfengwana et al., 2019), antitumor (Islam et al., 2018), antifungal, antiviral (Daboor & Haroon, 2012), antimutagenic (Kabir et al., 2013), and anticarcinogenic activities (Manduna et al., 2014). Natural substances with anti-oxidant activity have been shown to reduce breast cancer and cancer side effects and stimulate the immune system (Aiyegoro & Okoh, 2009; Ramos, 2007).

In this respect, this study attempted qualitative and quantitative phytochemicals analyses carried out on MeOH and water (H₂O) extracts of the roots of *G. perpensa*. The total phytochemical content in the plant sample was also investigated to assess a wide polarity window of the phytochemical profile of the roots of *G. perpensa*.

3.2 MATERIALS AND METHODS

3.2.1 Plant collection, authentication, and preparation of extracts

For the purposes of plant collection, an export permit from the Ministry of Tourism, Environment and Culture, Lesotho, and import approval (NC.553/2017) from the Department of Economic Development, Tourism, and Environmental Affairs, South Africa, were obtained. Fresh *G. perpensa* L. roots were harvested from the Mohale's Hoek district in Lesotho, and Thaba 'Nchu in the Free State, South Africa. Plant materials were authenticated by a scientist at the National Botanical Garden in Bloemfontein, South Africa, Mr Lufuno Nenungwi. Plant material voucher specimen PHM02 for *G. perpensa* L. was deposited to the National Botanical Garden's herbarium.

The collected roots were thoroughly washed under running tap water, chopped, and air-dried to constant weight at room temperature (25 °C), and subsequently milled (model MS-223; Miller) into a fine powder. Following drying, 200 g of the material was extracted in 400 mL of MeOH and distilled H₂O using the maceration extraction technique. The mixture that resulted was filtered. The H₂O filtrate obtained was lyophilised (Martin Christ Alpha 1-2 LDplus Freeze Dryer, Separations, Johannesburg, South Africa) at -40 °C and the MeOH extract was concentrated with the rotary evaporator [Buchi Rotavapor® R-300, Labotec (Pty) Ltd., Johannesburg, South Africa]. The resulting crude extracts were kept at -20 °C for later usage. The filtrate was then restored in H₂O and dimethyl sulphoxide (DMSO), respectively, to make a stock solution of 50, 100, 200, and 400 mg/kg body weight dose levels.

3.2.2 Qualitative phytochemical analysis of *G. perpensa* L.

Adopting the procedures described by Sofowora (2006), Prashant et al. (2011), and Al Ghasham et al. (2017) with some slight modifications, the MeOH and H₂O extracts of *G. perpensa* were subjected to qualitative phytochemical screening. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

3.2.2.1 Detection of alkaloids

For the screening of alkaloids, the powdered extract was dissolved in 2 mL of 1% hydrochloric acid and then filtered. The filtrate obtained was thereafter subjected to Wagner's reagent (2 g of iodine and 6 g of potassium iodide in 100 mL of water) treatment. The formation of a brown/reddish precipitate confirmed the presence of alkaloids.

3.2.2.2 Detection of anthraquinones

Chloroform (2 mL) was added to 0.2 g of the extract and the resulting mixture was vigorously shaken for five minutes prior to filtration. Equal volumes of the filtrate were obtained, and 10% ammonia solution was thoroughly mixed. The formation of a bright pink colouration in the aqueous layer of the mixture confirmed the presence of anthraquinones.

3.2.2.3 Detection of glycosides

The smallest quantity of extract was diluted in 5 mL of distilled H₂O and filtered. The filtrate was hydrolysed with 10% aqueous hydrochloric acid, and then treated with 2% sodium nitroprusside in pyridine and 20% sodium hydroxide. The formation of pink to blood red or reddish-brown precipitation indicated a positive test for cardiac glycosides.

3.2.2.4 Detection of flavonoids

The extract (50 mg/mL) was treated with few drops of 10% sodium hydroxide solution. The formation of intense yellow precipitation, which becomes colourless with the addition of dilute acid, indicated the presence of flavonoids.

3.2.2.5 Detection of phenols

Three to four drops of 10% ferric chloride solution were added to 0.5 g of *G. perpensa* H₂O extract in order to screen phenols. The formation of a dark blue or bluish-black precipitate confirmed the presence of phenols

3.2.2.6 Detection of saponins

The powdered extract (2.0 g) was boiled in 20 mL of distilled H₂O for five minutes and filtrated. The filtrate (10 mL) was mixed with 5 mL of distilled H₂O in a graduated cylinder and shaken vigorously and left for 15 minutes for persistent frothing. The froth was thereafter mixed with three to four drops of olive oil and shaken again for observation of an emulsion layer, which signified the presence of saponins.

3.2.2.7 Detection of tannins

10 mL of distilled H₂O was used to mix the extract (0.5 g) separately before it was filtered. 10% sodium chloride was added in a few drops. The formation of a dark blue or greenish grey precipitate confirmed the presence of tannins.

3.2.2.8 Detection of triterpenes

The *G. perpensa* extract was treated with 2 mL chloroform and filtered. The filtrate was then treated with a few drops of concentrated sulphuric acid, shaken, and allowed to stand. The appearance of a golden yellow colour was an indication of triterpenes.

3.2.2.9 Detection of phytosterols

A gram of the crude extract was dissolved in a few drops of acetic acid, then treated with 3 mL acetic anhydride treatment, and then boiled and cooled. Following the addition of the concentrated sulphuric acid, the formation of a brown ring at the layer junction indicated the presence of phytosterols.

3.2.3 Quantitative phytochemical analysis of *G. perpensa* L.

Elemental analysis is a systematic examination of provided facts from materials like compounds, soil, inorganics, waste water, and bodily fluids to determine their elemental components or to learn and analyse their isotopic components. Quantitative data describes the overall number of elements present.

The total phenolic content (TPC) was determined by the Folin-Ciocalteu colorimetric method using Gallic acid as the standard, and various concentrations of the extract solutions were measured at 760 nm. The total flavonoid content (TFC) was determined by an aluminium chloride colorimetric assay. Quercetin was used as the standard, and the absorbance was measured at 450 nm.

3.2.3.1 Determination of total phenolic content (TPC)

The TPC of the organic extracts of *G. perpensa* was determined using the Folin-Ciocalteu method as described by Makkar (2006), with slight amendments. Briefly, 25 μ L of crude plant extract (0.5 mg/mL) was treated with 250 μ L Folin-Ciocalteu reagents for five minutes. The reaction was stopped by adding 750 μ L 20% anhydrous sodium carbonate. The volume was made up to 5 mL with distilled H₂O and incubated in the dark at room temperature for two hours. After incubation, the absorbance was read at 760 nm with a spectrophotometer. The phenolic content was determined from a standard curve of different concentrations of Gallic acid in DMSO. The results were expressed as mg/g Gallic acid equivalents (GAE).

3.2.3.2 Determination of total flavonoid content (TFC)

The TFC of the organic extracts was determined using the method of Yadav and Agarwala (2011), with a slight modification. Crude extracts at 0.5 mg/mL (100 μ L) were dissolved in 300 μ L MeOH, to which 20 μ L 10% aluminium chloride was added. A further 20 μ L of 1 M sodium acetate was added to the solution. The resultant solution was made up of 1 mL with distilled H₂O. This was incubated at room temperature for 30 minutes. After incubation, the absorbance was read at 450 nm in a microplate reader. Quercetin (10 mM) was used as the standard. The flavonoid content of each extract was expressed as mg/g quercetin equivalent (QE).

3.3 RESULTS

3.3.1 Qualitative evaluation

The results of the qualitative phytochemical analysis of the MeOH and H₂O extracts of *G. perpensa* L. are presented in Table 3.1 for ease of interpretation and data comparison. A positive sign (+) was used to indicate the presence of a phytochemical, while a negative sign (-) was used to indicate the absence of a phytochemical.

From the table, it can be seen that alkaloids, flavonoids, phenols, tannins, saponins, phytosterols, etc. were detected in the MeOH root extracts of *G. perpensa*. Flavonoids were absent only from the MeOH root extracts of *G. perpensa* L.

3.3.2 Quantitative evaluation

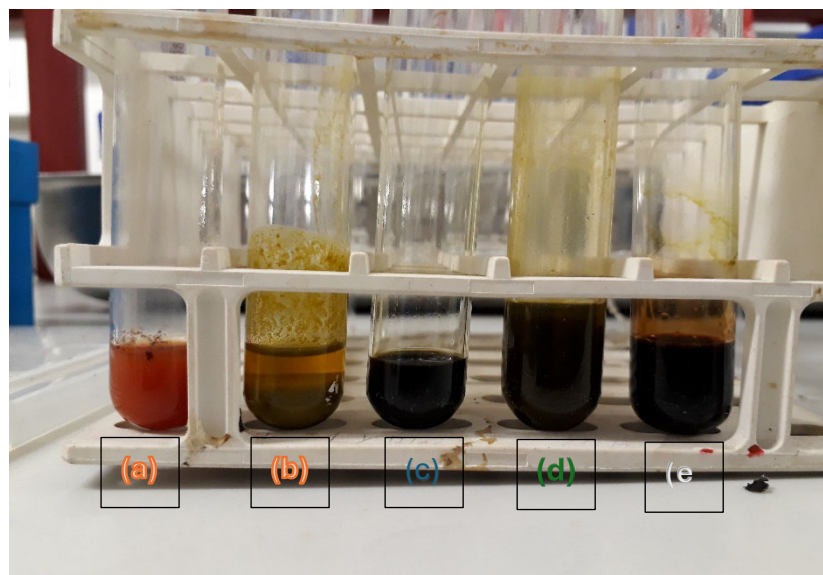
3.3.2.1 TPC and TFC

Phenols and flavonoids represent the phytochemicals or secondary metabolites that are abundantly available in the plant kingdom. The TPC and TFC obtained in this study for the organic extracts of *G. perpensa* L. are presented in Table 3.2 and Figures 3.2 and 3.3 for ease of interpretation and data comparison. The results revealed that *G. perpensa*, which is used

for almost every ailment by the Basotho in Lesotho, does indeed have various phytochemical compounds.

Table 3.1: Phytochemical screening analysis of *G. perpensa* L.

<i>G. perpensa</i> L.	TEST								
	Alkaloids	Anthraquinones	Glycosides	Flavonoids	Phenols	Saponins	Tannins	Triterpenoids	Phytosterols
MeOH extract	+	+	+	-	+	+	+	+	+
H ₂ O extract	+	+	+	+	+	-	+	+	+



**A: Brownish
red (alkaloids)**

**B: Yellow
(flavonoids)**

**C: Dark blue
(phenols)**

**D: Greenish
grey (tannins)**

**E: Dark red
(glycosides)**

Figure 3.1: Positive colour change results for aqueous extract of *G. perpensa* L. indicating the presence of different secondary metabolites

Phenols and flavonoids represent the phytochemicals or secondary metabolites that are abundantly available in the plant kingdom. The TPC of *G. perpensa* extracts was determined by the Folin-Ciocalteu method using Gallic acid as the standard to set up a calibration curve (see Figure 3.1). The TPC of the extracts was calculated from the regression equation of calibration curve ($Y=0.1942x - 0.0174$; $R^2 = 0.951$) and expressed as mg GAE in mg/g extract (Singleton & Rossi, 1965).

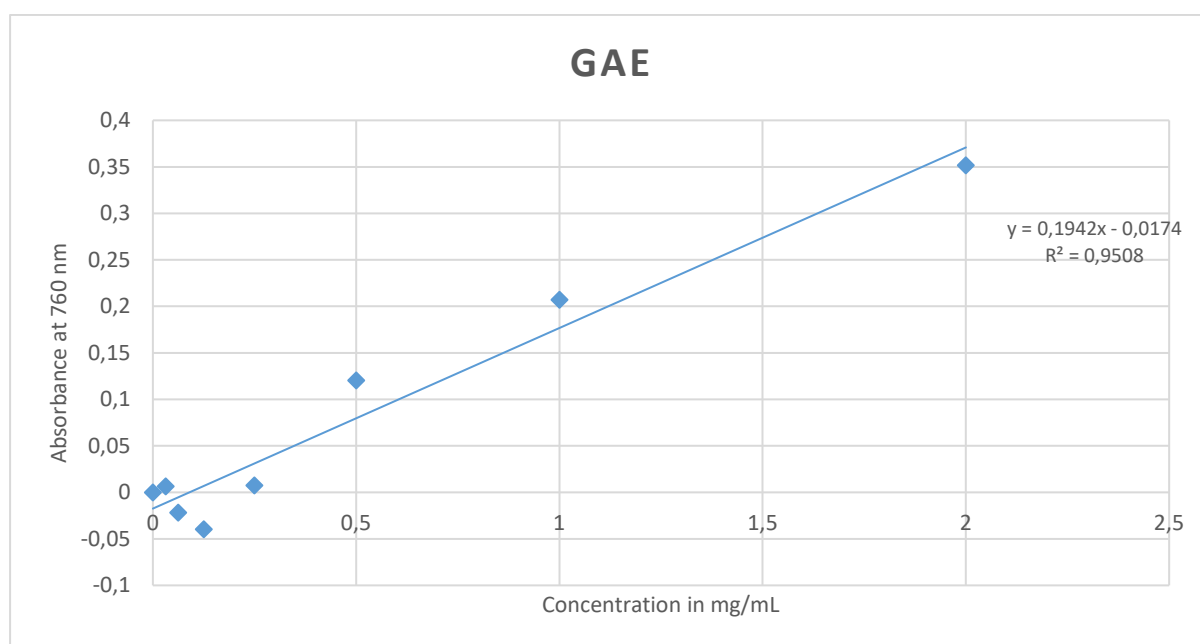


Figure 3.2: Gallic acid standard curve for the estimation of *G. perpensa* phenol content

The TFC of the extract was calculated from the regression equation of the calibration curve ($Y=0.6961x - 0.0046$; $R^2 = 0.997$) and expressed as mg QE per gram of extract in dry weight (mg/g). The calibration curve was obtained by plotting absorbance at 760 nm against the Gallic acid standard concentration, as shown in Figure 3.2.

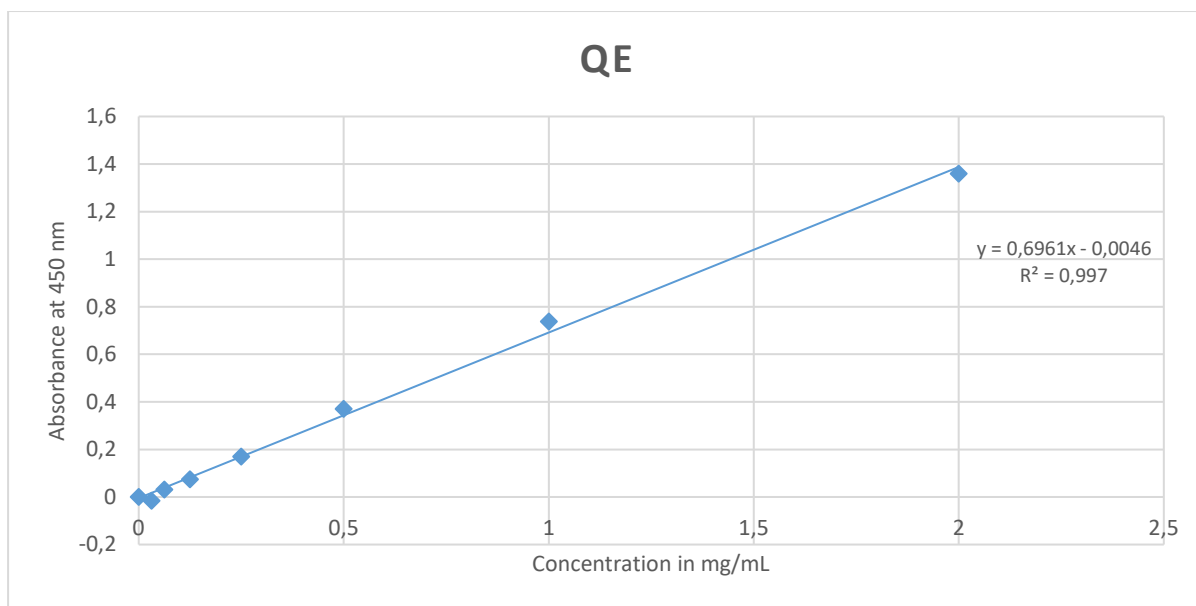


Figure 3.3: Quercetin standard curve for the estimation of *G. perpensa* flavonoid content

The TPC and TFC obtained in this study for the organic extracts of *G. perpensa* L. are presented using tables and figures for ease of interpretation and data comparison.

Table 3.2: TPC and TFC of methanol (MeOH) and water (H₂O) extracts of *G. perpensa* L.

Phytochemicals	<i>G. perpensa</i> extracts
Total phenol (mg Gallic acid g ⁻¹) (MeOH)	17.11±0.036
Total phenol (mg Gallic acid g ⁻¹) (H ₂ O)	16.02±0.012
Total flavonoid (mg quercetin g ⁻¹) (MeOH)	10.33±0.01
Total flavonoid (mg quercetin g ⁻¹) (H ₂ O)	19.69±0.05

*Values are expressed per gram of plant extract and are represented as means ± standard deviation. Tests were repeated in triplicate and the mean was determined.

The *G. perpensa* H₂O extract showed more flavonoids than the MeOH extract. The quantification analysis of the TFC coincided with the phytochemical screening test results. It is evident from these results that many flavonoids in the H₂O extracts of *G. perpensa* indicated a strong presence of flavonoids. This was followed by a decline in the methanolic crude extract 10.33±0.01 mg quercetin g⁻¹, which suggests the absence of flavonoids.

3.4 DISCUSSION

Phytochemicals have a diverse range of pharmacological activities associated with health promotion and disease prevention. Phytochemical examination of the plant extracts showed the presence of constituents known to exhibit medicinal and physiological activities. The phytochemical screening of the crude MeOH and H₂O extracts of the root samples of *G. perpensa* revealed the presence of some secondary metabolites such as alkaloids, anthraquinones, glycosides, flavonoids, phenols, saponins, tannins, triterpenoids, and phytosterols. These phytochemical constituents, also known as secondary metabolites, are known to have important medical benefits for humans.

The *G. perpensa* MeOH and H₂O extracts revealed the presence of alkaloids. Alkaloids have been reported as a powerful poison and many alkaloids derived from medicinal plants show biological activities such as having anti-inflammatory (Souto et al., 2011), antimalarial (Dua et al., 2013), antimicrobial (Benbott et al., 2012), cytotoxic, antispasmodic, and pharmacological effects (Ameyaw & Duker-Eshun, 2009; Thite et al., 2013). Alkaloids make up most of the drugs used pharmacologically as anaesthetics and central nervous system stimulants (Madziga et al., 2010).

Although environmental, genetic, and physiological factors contribute to the overall phytochemical profile of plants, harvest time is a critical factor for the proper agricultural practice of herbal medicine. This is accurate, as the study's findings on *G. perpensa* showed that the H₂O extracts include flavonoids, but not its MeOH extracts. It should be noted that the phytochemical data on flavonoids fully concur with data reported by Mfengwana et al. (2019). It is only present in flavonoids extracted by MeOH and not in H₂O extracts (Simelane et al., 2012; Chigor, 2014; Mammo, 2018). The presence of phenols was detected in the H₂O and MeOH extracts of *G. perpensa* roots. The presence of phenols in *G. perpensa* supports the traditional medicinal use of this plant in the treatment or management of various ailments, especially where access to Western healthcare facilities is limited. Phenolic compounds are

among the major groups of plant metabolites (Lin et al., 2016). Several biological activities such as anti-apoptosis, anticarcinogenic, anti-inflammatory activities, cardiovascular strengthening, and cell proliferation in most plants have been associated with these classes of compounds (Uddin et al., 2014). Mfengwana et al. (2019) revealed the importance of phenols for the treatment of inflamed tissues and as a good candidate for potent MDR bacteria and anti-oxidant drug development. The presence of flavonoids and phenolics also contributes to anti-inflammatory and anti-oxidant activity (Kar, 2007; Walch et al., 2011).

The H₂O extracts of *G. perpensa* generally showed the presence of all tested phytochemicals except saponins, while the MeOH extract showed the presence of all tested phytochemicals except flavonoids. The H₂O extract in this study almost certainly justifies the belief in traditional medicine about the safety of H₂O as the main solvent. Alkaloids have been coupled with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Agidew, 2022). Glycosides lower blood pressure according to many reports (Raquel, 2007). Secondary metabolites such as alkaloids, saponins, phenols, glycosides, and terpenoids, singly or in combination, show potential defence mechanisms against various pathogens (Teoh, 2016).

The term “phenolic compound” or “phenolic” is applied to several classes of aromatic compounds such as simple phenols, phenolic acids, phenylpropanoids, flavonoids, coumarins, quinones, xanthenes, anthrones, lignans, stilbenes, and tannins (Kuate, 2013). They fulfil important functions in plants, such as defence mechanisms against pathogens and herbivores and can thus be considered potential candidates for the management of pathogenic infections (Doughari, 2012). Plants with high levels of phenols can be considered interesting for therapeutic use and their pharmacological properties are worth further in-depth investigation.

The most significant natural phenolics are most likely flavonoids, one of the most varied and abundant classes of natural chemicals (Roy et al., 2022). Tung et al. (2007) reported their

wide spectrum of chemical and biological activities, including radical scavenging properties. For example, flavonoids protect against chronic diseases due to their capacity to transfer electrons to free radicals (Harbone & Williams, 2000; Okwu & Josiah, 2004).

The total phenolic and flavonoids contents of the MeOH and H₂O extracts of *G. perpensa* are presented in Table 3.2. This study revealed the highest TFC of 10.33±0.01 mg QE/g and 19.69±0.05 mg QE/g for the MeOH and H₂O extracts respectively. It should be noted that the H₂O extracts of *G. perpensa* showed more TFC when compared to the organic extract of the same plant species. This discovery indicates that the active ingredients in *G. perpensa* are rich under aqueous conditions; thus supporting its traditional use for medicinal benefits. While water has long been a commonly used and available solvent to prepare medicinal extracts, these findings provide scientific validation for its effectiveness in extracting the plants beneficial compounds.

Elsewhere, a similar trend has been reported. The findings of Kheoane and Mohale (2022) reported the TPC of 146.40±1.94 mg GAE/g and the TFC of 26.54±2.07 mg QE/g for the MeOH extract of *G. perpensa* (Kheoane & Mohale, 2022). This is in agreement with the findings reported in this study, the TFC of the MeOH extracts showed low flavonoid content, which was different from the higher total phenolic content. It seems that *G. perpensa* contains compounds (Phenolics) that are thought to offer a range of health benefits, including preventing cancer, lowering cholesterol levels, and supporting both the cardiovascular and immune system (Singh et al., 2022; Mihaylova et al., 2024).

3.5 CONCLUSION

This chapter presented the results of the qualitative and quantitative phytochemical analyses. The presence of alkaloids, anthraquinones, glycosides, phenols, triterpenoids, and phytosterols may justify its observed anticancer activity. The results were displayed as figures and tables. Although the phenolic and flavonoid contents of *G. perpensa* were reported in this

chapter, there is a need to investigate further and isolate the individual compounds, which may singularly or in combination explain the biological activity of the reported plant species.

The next chapter describes the isolation of the bioactive constituents from the crude extract, which involved detailed analysis of the extract's chemical composition by chromatographic-spectroscopic techniques and subsequent activity or toxicity evaluation.

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CHAPTER 4:

FRACTIONATION, ISOLATION, AND STRUCTURAL ELUCIDATION OF SECONDARY METABOLITES FROM *G. PERPENZA* L. (GUNNERACEAE)

4.1 INTRODUCTION

The use of traditional medicines and natural poisons have been closely associated with medicine and natural products since the beginning of time (Grabley & Thiericke, 1998; Newman et al., 2000; Martinez et al., 2003). Early medications such as aspirin, digitoxin, morphine, quinine, and pilocarpine were primarily derived from plants and were developed based on clinical, pharmacological, and chemical investigations of these traditional medicines (Sneader, 1996; Mann, 2000) (see Figure 4.1).

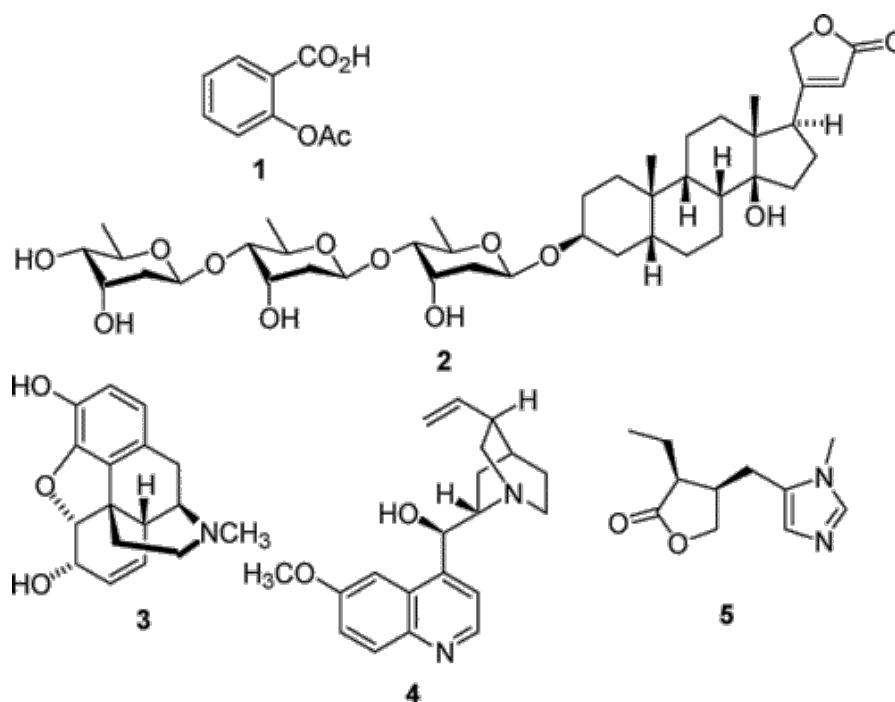


Figure 4.1: The chemical structures of aspirin (1), digitoxin (2), morphine (3), quinine (4), and pilocarpine (5)

Source: Adapted from Dias et al. (2012)

Natural products, whether pure compounds or standardised extracts, provide unlimited opportunities for new drug discovery and are unrivalled in the availability of rich chemical diversity (Cos et al., 2006). Traditionally used medicinal plants contain a diverse range of substances that are used to treat infectious and chronic diseases alike.

Drug discovery is the process of finding possible therapeutic agents by screening compound libraries, isolating naturally occurring bioactive metabolites, and creating synthetic compounds that target particular pharmacological targets (Nature, 2018). According to Grifo et al. (1997), at least 119 compounds that are produced from 90 plant species are important prescription drugs that are used by one or more countries. 74% of these 119 drugs were found by chemical research directed at separating the active ingredients from plants used in traditional medicines (Grifo et al., 1997).

Biologically active compounds can be discovered through the process of extraction, biological screening, isolation, and characterisation of bioactive compounds, toxicological evaluation, and clinical evaluation (Sasidharan et al., 2011).

The most important and first step in separating and purifying the active compounds from plants is the extraction of plant materials. Even though the plant material should be dried in a controlled laboratory environment to avoid chemical changes, plant extraction techniques may employ fresh or recently harvested and dried plant tissues (Harborne, 1998). The solvent used during extraction is the second crucial element in extraction. The quantity and kind of chemicals that must be extracted are greatly influenced by the extraction solvent. Lipids and steroids, for example, will be extracted in large quantities during non-polar solvent extraction, whereas in polar solvent extraction, sugars will be present in large amounts (Verpoorte, 1998). Many kinds of extraction techniques have been developed; some focus on particular compounds, while others extract in general.

The identification and characterisation process of bioactive compounds still poses a great challenge because plant extracts are usually a combination of various bioactive

compounds/phytochemicals with different polarities that are difficult to separate. Therefore, a variety of separation techniques are used to isolate the bioactive compounds. High-performance thin-layer chromatography (HPTLC), preparative thin-layer chromatography (PTLC), column chromatography, thin-layer chromatography (TLC), and vacuum liquid chromatography (VLC) are among the techniques used to extract pure bioactive compounds. The characterisation of these compounds is then achieved through Fourier-transform infrared spectroscopy, high-resolution mass spectrometry (HRMS), and a variety of nuclear magnetic resonance (NMR) experiments.

This study aimed to separate and isolate *G. perpensa* constituents, which might also yield the discovery of a new anticancer lead compound.

4.2 MATERIALS AND METHODS

4.2.1 Extraction, fractionation, and isolation

Extracts of *G. perpensa* L. were prepared in the same manner as described in Section 3.2.1. Proton nuclear magnetic resonance spectroscopy (^1H -NMR), carbon nuclear magnetic resonance spectroscopy (^{13}C -NMR), and two-dimensional spectra were recorded on a Bruker Avance 500 Megahertz (MHz) spectrophotometer (solvent deuterated chloroform). Gas chromatography–mass spectrophotometry (GC-MS) analyses were recorded on a Hewlett-Packard 6890 gas chromatograph equipped with an HP-5 non-polar capillary column (30 m x 0.25 mm id x 0.25 mm film thickness) and interfaced with a Hewlett-Packard 5973 mass selective detector operating in EI (70 eV). The injector and detector temperatures for the gas chromatograph were 250 °C and 280 °C respectively. The column temperature was initially kept at 60 °C for five minutes, then gradually increased to 280 °C at a 16 °C min⁻¹ rate, and finally held for 10 minutes at 280 °C. Silica gel was used for VLC. Silica gel GF₂₅₄ plates (Merck) were used for TLC analysis. Spots were visualised using ultraviolet (UV) light (254 and 365 nm), by spraying vanillin and 50% sulphuric acid, followed by three minutes at 110 °C.

4.2.2 Chemical analysis

4.2.2.1 *Thin-layer chromatography (TLC) fingerprinting*

A straightforward technique for identifying active compounds in an extract and reaction product is TLC fingerprinting. The most diagnostically important information from this technique, apart from retardation factor (R_f) comparisons, comes from differences or similarities in the colour of the bands that make up the species' fingerprint (Rogers et al., 1999).

(a) Procedure

MeOH: Dichloromethane (DCM) (1:1, v/v) (1 mL) was used to dissolve 50 mg of crude extract. using three different solvent systems, the TLC analyses were performed on silica gel-coated aluminium plates (Merck TLC Silica Gel 60 F₂₅₄). The crude extract solutions were applied to the TLC plate 1 cm away from the edge. Three different solvent systems were used to create the plates as the eluent. The compounds on the developed plates were visualised using visible and UV light (254 and 366 nm, Camag, Universal UV Lamp TL-600).

The following three solvent systems used were:

- Solvent System 1: Hexane: Ethyl acetate (EtOAc) (7:3, v/v) for the separation of non-polar constituents.
- Solvent System 2: Chloroform: EtOAc: Formic acid (5:4:2, v/v) for the separation of constituents of intermediate polarities.
- Solvent System 3: DCM: EtOAc: MeOH: Formic acid (90:10:30:1, v/v) for the separation of polar constituents.

To further see the compounds that are not visible under UV light, the visualisation staining reagent vanillin is frequently used. Vanillin (800 mg), glacial acetic acid (40 mL), and concentrated sulphuric acid (2 mL) make up the reagent. The sprayed plate was incubated at 110 °C for three to five minutes

4.2.2.2 Vacuum liquid chromatography (VLC)

VLC is a widely used technique for fractionating crude extracts because it is simple to apply and prevents excessive congestion in the profile of the chemical mixture, which can cause compound overlap and result in unresolved separation (Pelletier et al., 1986).

(a) Procedure

In an effort to isolate the active fractions, the 30 g MeOH extract of the *G. perpensa* roots was further used. Following their dissolution in MeOH, the methanolic roots extracts were mixed with silica gel (Sigma-Aldrich kieselgel 60, 230-400 mesh ASTM), dried, and then packed with pure TLC-grade silica gel in a sintered glass Buckner funnel (20 cm x 15 cm) that was connected to a vacuum line. Toluene/EtOAc, Chloroform/EtOAc/formic acid, DCM/MeOH, and DCM/EtOAc/MeOH/formic acid were the solvent mixtures used in a gradient solvent system. The procedure began with a solvent composition of 7:3, increased the EtOAc by 5%, and ended with a solvent composition of 5:5. A total of 23 fractions of 200 mL each were collected and subjected to TLC analysis.

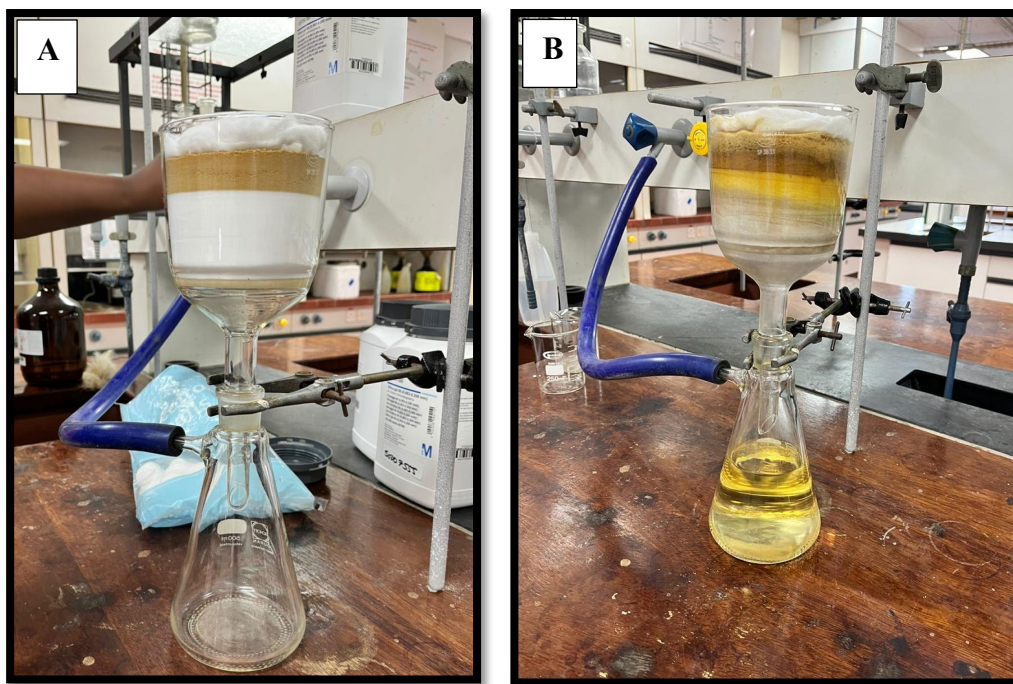


Figure 4.2: VLC setup; A: setup, B: after collecting a few fractions

Source: Researcher

4.2.2.3 Analytical method of thin-layer chromatography (TLC) and fraction grouping

The evaluation of the fractions was conducted using TLC aluminium-coated plates (Merck) to analyse the results through UV light (short and long wavelength) and vanillin spray reagent to identify similar fractions (Bladt, 2009). Six main fractions (F1 to F6) were obtained by combining chromatographically comparable fractions, evaporating them under nitrogen gas until they were dry, and then calculating their weights. Due to its high crystalline quality and well-resolved component as shown in TLC, Fraction 6 (VLC F10 to F21) was recrystallised in diethyl ether, forming a white solid single compound that was identified as Z-venusol, 7.042 g. Finally, to determine the difference in the chemical composition of the MeOH extraction of the rhizomes of *G. perpersa*, PTLC was used to separate single compounds with comparable R_f values, as an indication. Figure 4.3 provides an illustration of the procedure.

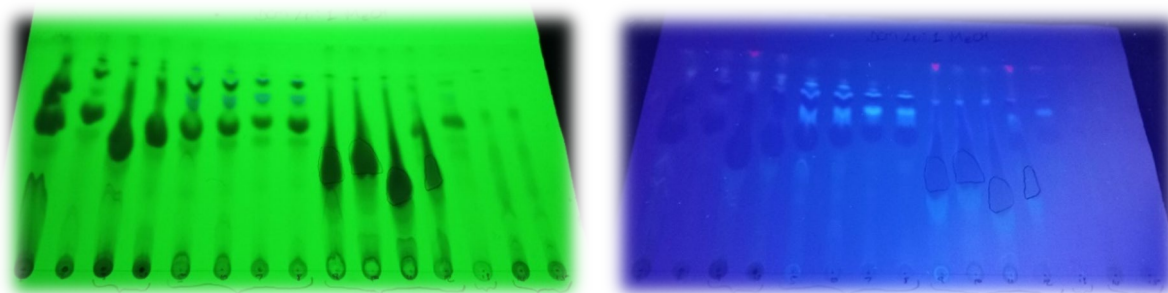


Figure 4.3: The TLC viewing under short- and long-wavelength UV light of *G. perpersa* VLC fractionation TLC analysis

Source: Researcher

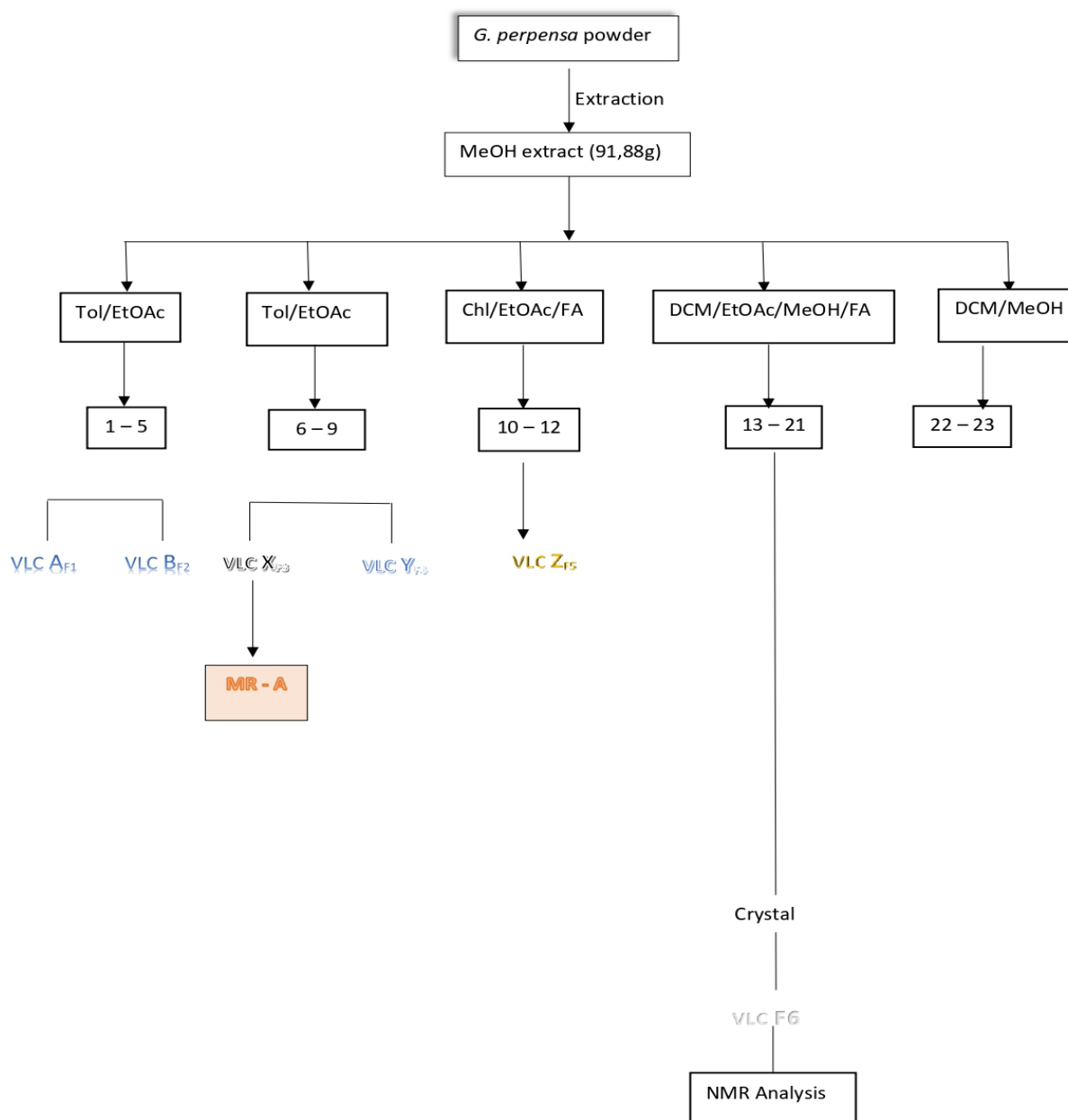


Figure 4.4: Schematic diagram showing the isolation of compounds from the MeOH extract of *G. perpensa*

Source: Researcher

4.2.2.4 Preparative thin-layer chromatography (PTLC)

The use of PTLC has been a widespread isolation technique for a long time, mainly because it is accessible to all researchers studying natural product chemistry. This popularity has decreased recently because of HPTLC's success. Although this technique is similar to TLC, it

is capable of isolating larger quantities of compounds than TLC (milligram to gram) (Colegate & Molyneux, 2007), which is enough for structural elucidation purposes.

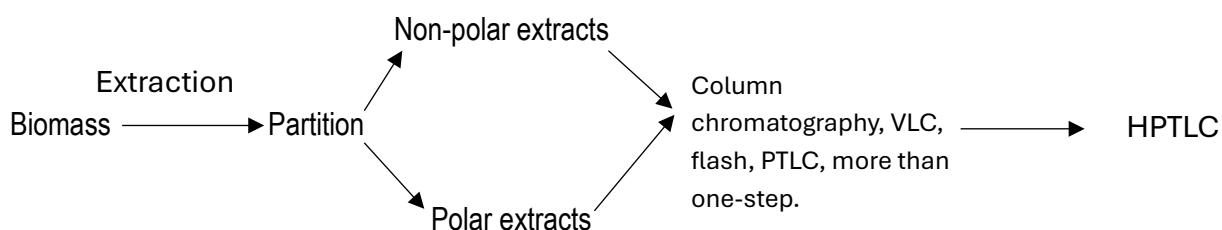


Figure 4.5: A brief description of the general approaches to the extraction and isolation of bioactive compounds

Source: Adopted from Odeleye (2010)

A specific fraction was dissolved in a small amount of solvent (often MeOH) to develop the plates. A 3 cm band of the dissolved fraction was then spotted on the baseline of the TLC plate, 1 cm from the bottom of the plate. The developed plates were visualised using visible light and UV light at two different wavelengths, namely 254 and 366 nm (Camag, Universal UV Lamp TL-600), and by staining the plate with vanillin spray reagent, and then heated for three to five minutes at 110 °C to allow for the development of colour. The marked compounds in the silica gel were removed and then rinsed with MeOH. Pure and dry compounds were obtained after the solutions were filtered, and the MeOH was evaporated from the filtrate.

4.2.2.5 Characterisation and structural elucidation of isolated compounds

^1H -, ^{13}C -, distortionless enhancement by polarisation transfer (DEPT), correlation spectroscopy (COSY), and heteronuclear single quantum coherence (HSQC)-NMR spectroscopy were recorded at room temperature either using deuterated methanol or deuterated chloroform as a solvent on a Bruker ULTRASHIELD™ 400 MHz / 54 mm magnet system with tetramethylsilane as an internal standard. The chemical structures of the isolated compounds were determined by comparing the assignment of their ^{13}C signals with the literature values of the corresponding known compounds.

The Attenuated Total Reflectance–Fourier Transform Infrared TENSOR 27 spectrophotometer, fitted with a Diamond/ZnSe internal reflectance element with single bounce mode, was used to record the infrared spectra. The characteristic peaks were recorded in wave members. The liquid chromatography–mass spectrometry (LC-MS) and HRMS analysis was conducted by the Central Analytical Facilities at Stellenbosch University on a Waters Synapt G2 Instrument, where the sample was introduced by electrospray ionisation (ESI) probe and detected by ESI positive source with 15 V cone voltage.

4.3 RESULTS

The yields of the isolated fractions from the MeOH root extract of *G. perpensa* are shown in Table 4.1. Fractions F1, F3, and F4 had the highest percentage yields (0.079 g, 0.087 g, and 0.076 g respectively). In addition, F6 formed a crystal when concentrated and dried (see Figure 4.15).

Table 4.1: Yield of fractions labelled as F1 to F6

Fraction	Mass of fraction (g)
F1	0.079
F2	0.066
F3	0.087
F4	0.076
F5	0.318
F6	7.042

4.3.1 Isolation of single compounds

4.3.1.1 *Z*-methyl lespedezate (MR-A)

The NMR spectral data of MR-A is summarised in Table 4.2. In the ¹H-NMR spectrum, the *p*-distributed aromatic ring (δ_{H} 6.80 d, J = 8.2 Hz, 2H; 7.77, d, J = 8.3 Hz, 2H), an anomeric carbohydrate signal (δ_{H} 5.11, d, J = 7.6 Hz, 1H), and an olefinic proton (δ_{H} 7.01 s, 1H) were observed. These values agree with those of methyl lespedezate by comparing them to the literature. The structure of the isolated Compound MR-A was assigned as methyl lespedezate.

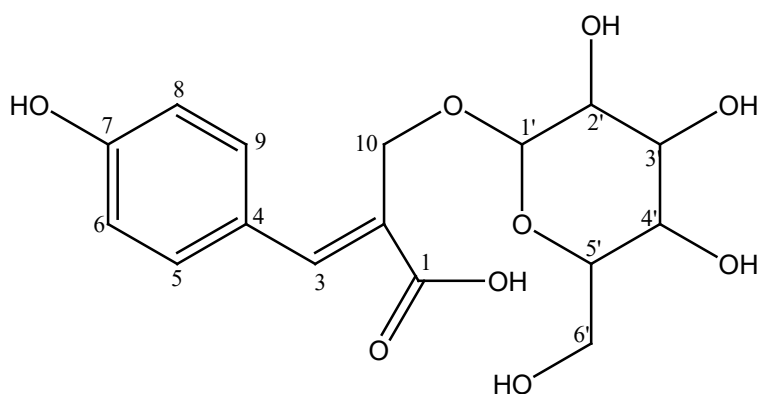


Figure 4.6: Proposed molecular structure of the compound (MR-A)

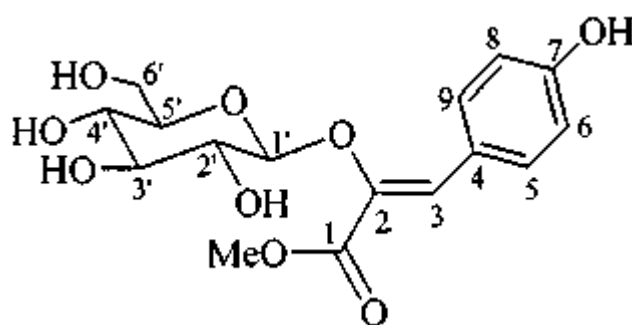


Figure 4.7: Chemical structure of Z-methyl lespedezate

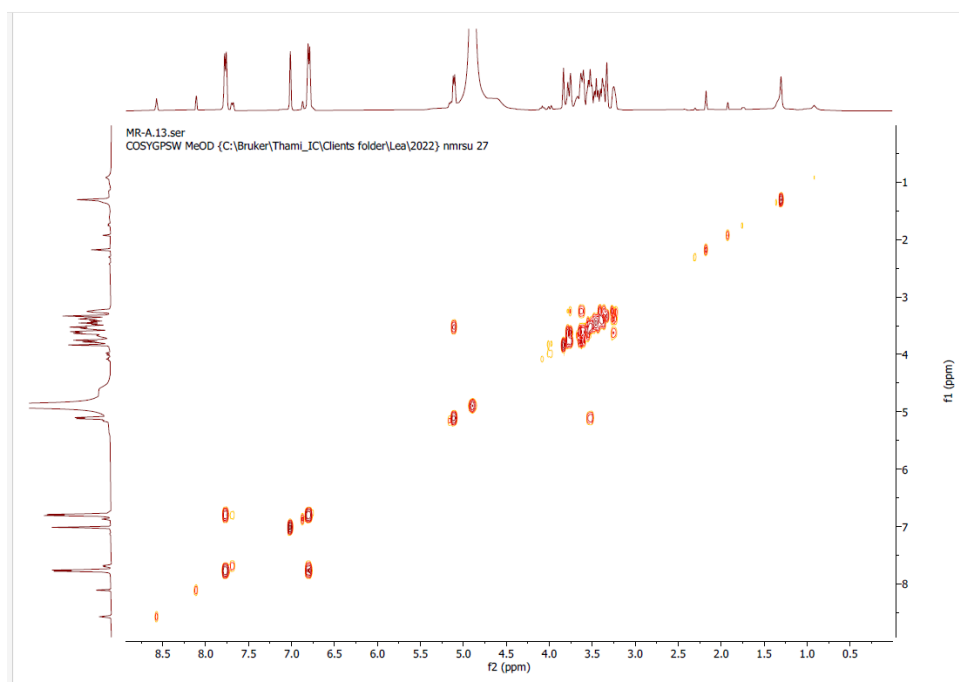


Figure 4.8: $^1\text{H} - ^1\text{H}$ COSY NMR spectrum (deuterated methanol, 500 MHz) of Compound MR-A showing resonances in the phenyl region

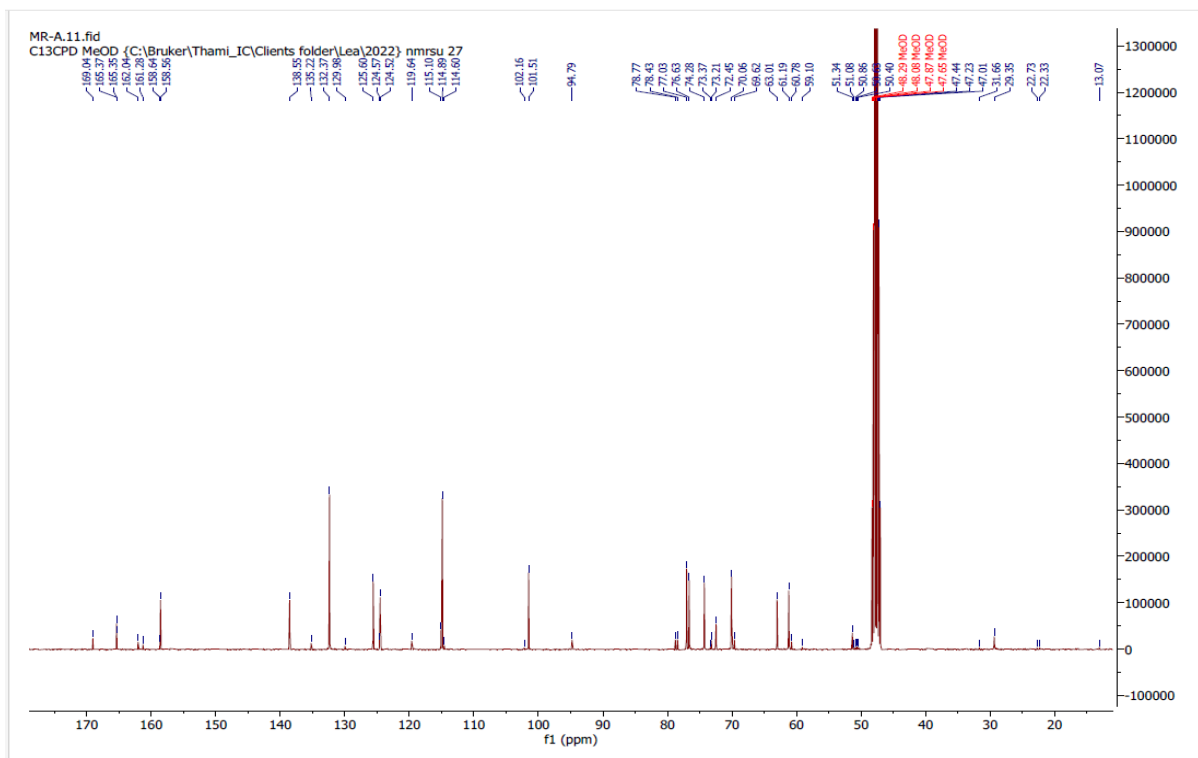


Figure 4.9: ^{13}C NMR spectrum of Compound MR-A isolated from *G. perpersa* root extract

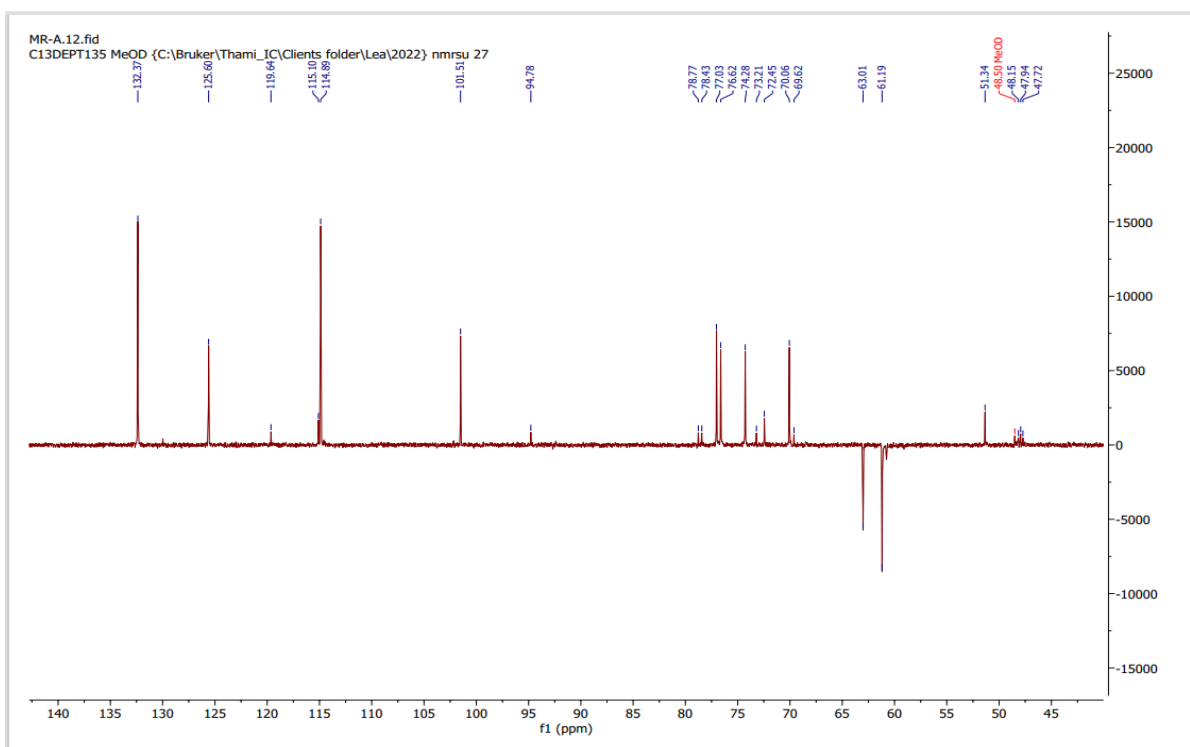


Figure 4.10: Distortionless enhancement by polarisation transfer (DEPT) spectrum of Compound MR-A isolated from *G. perpersa* root extract

Table 4.2: ^1H -NMR and ^{13}C -NMR of Compound MR-A with those observed in the literature

Compound MR-A					Literature	
Position	δ_{C}	δ_{H}	COSY	HMBC	δ_{C}	δ_{H}
1	165.4	-	-	-	166.6	-
2	138.5	-	-	-	139.9	-
3	125.6	7.01 (s, 1H)	-	-	125.9	6.98
4	124.5	-	-	-	126.7	-
5	132.4	7.77 (d, J = 8.3 Hz, 2H)	6.80	-	133.7	7.74
6	114.9	6.80 (d, J = 8.2 Hz, 2H)	7.77	-	116.2	6.77
7	158.6	-	-	-	159.9	-
8	114.9	6.80 (d, J = 8.2 Hz, 2H)	7.77	-	116.2	6.77
9	132.4	7.77 (d, J = 8.3 Hz, 2H)	6.80	-	133.7	7.74
10	63.0	3.62 (d, J = 11.2, 1H) 3.50 - 3.56 (m, 1H)	-	-	-	-
O-Me	-	-	-	-	52.6	3.81
β -glucose	-	-	-	-	-	-
1'	101.5	5.11 (d, J = 7.6 Hz, 1H)	-	-	102.8	5.10
2'	74.3	3.50 - 3.56 (m, 1H)	-	-	75.7	3.48
3'	76.6	3.45 (t, J = 8.0, 8.0 Hz, 1H)	-	-	78.0	3.41
4'	70.0	3.36 - 3.40 (m, 1H)	-	-	71.4	3.34
5'	77.0	3.28 - 3.20 (m, 1H)	-	-	78.4	3.21
6'	61.2	3.77 (d, J = 12.5 Hz, 1H), 3.62 (d, J = 11.2, 1H)	-	-	62.5	3.59 3.73

4.3.1.2 Structural elucidation of Z-venusol (MR-B)

Compound MR-B was isolated as a brown resin and by incorporating the unaccounted carbon atom (δ_c 161.2). The structure of this compound was identified as a venusol. The structures of this compound are illustrated in Figures 4.11 to 4.16. The NMR spectral data in deuterated methanol (500 MHz) are shown in Table 4.3.

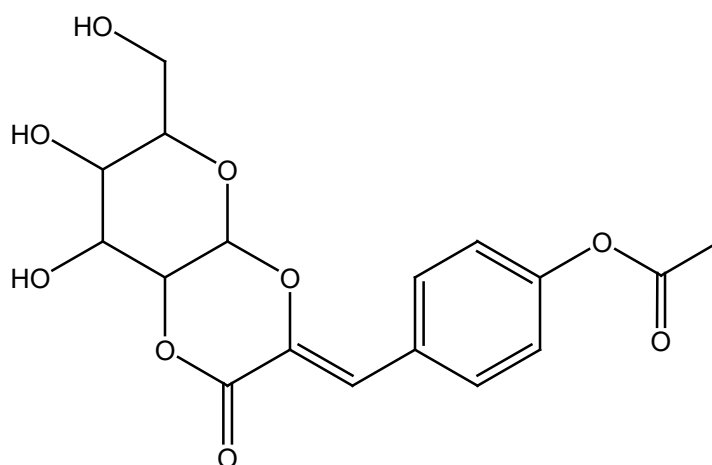


Figure 4.11: Proposed molecular structure of the compound (MR-B)

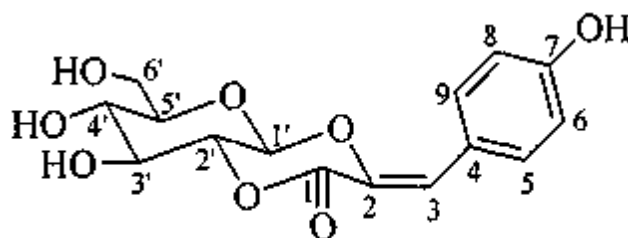


Figure 4.12: Chemical structure of Z-venusol

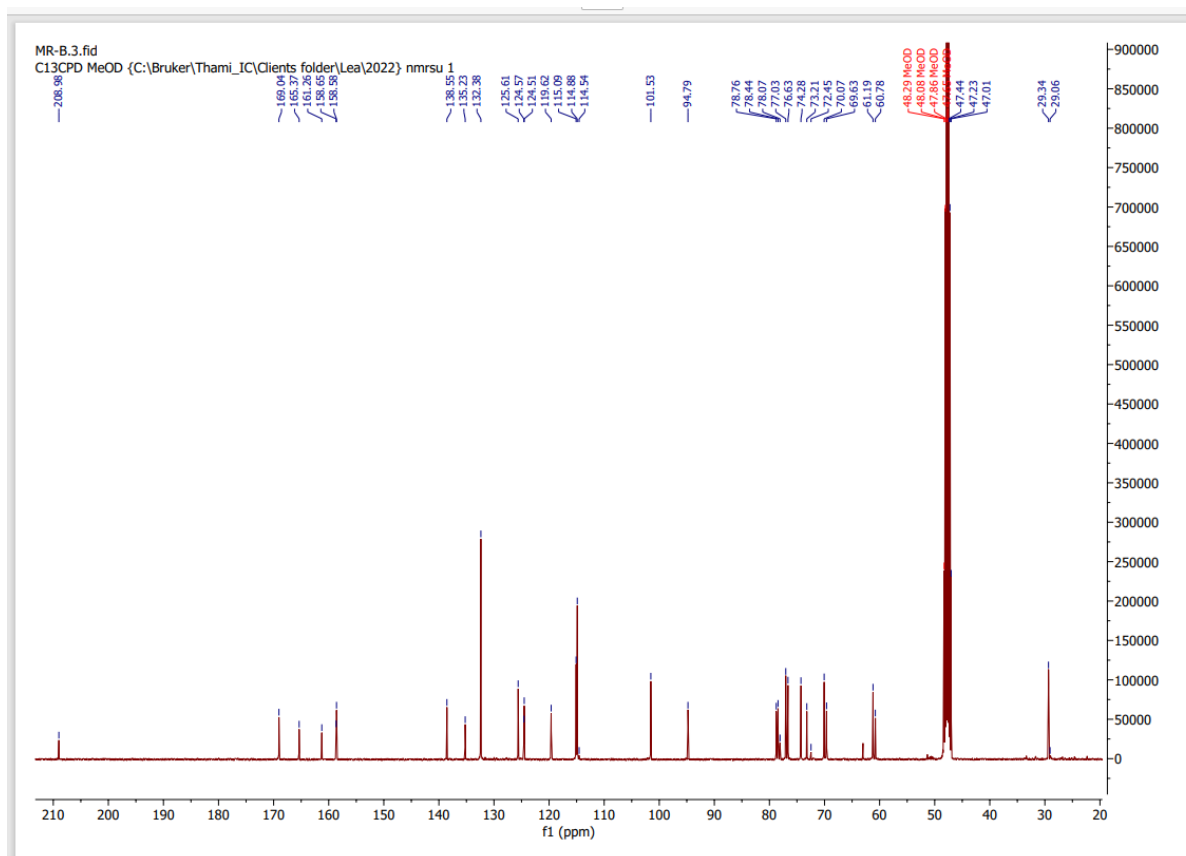


Figure 4.13: ^{13}C NMR spectrum of Compound MR-B isolated from *G. perpensa* root extract

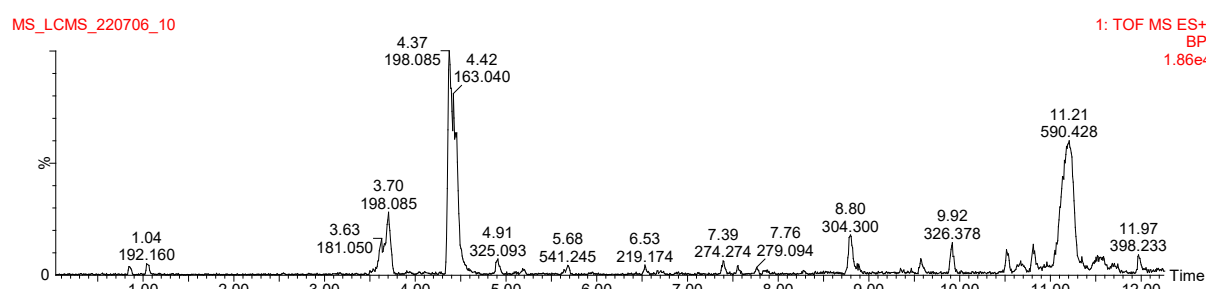


Figure 4.14: Molecular ion of Z-venusol (MR-B) at mass spectrometry (MS) positive mode

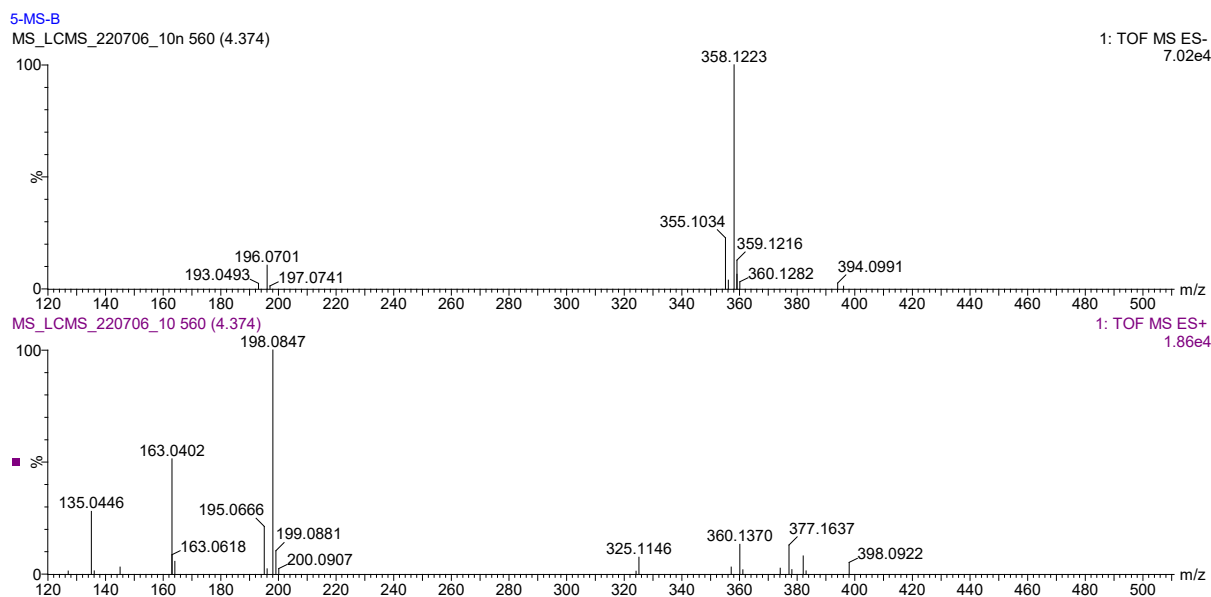


Figure 4.15: Mass spectrum of Compound MR-B isolated from *G. perpensa* root extract matched the library of 7,8-Dihydroxy-6-(hydroxymethyl)-3-((Z)-(4-hydroxyphenyl) methylidene) - tetrahydro-4aH-pyrano (2, 3-b) (1, 4) dioxin-2-one

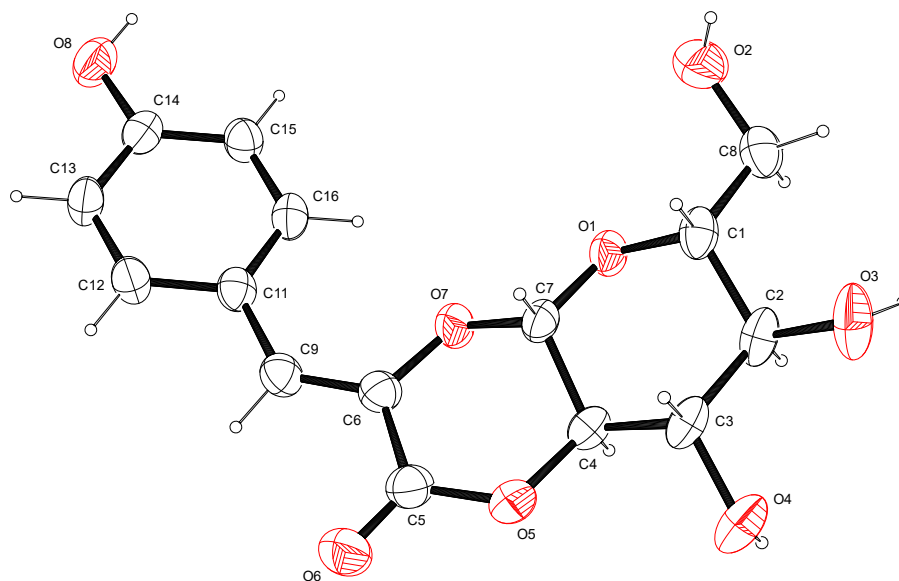


Figure 4.16: The crystal structure of 7,8-Dihydroxy-6-(hydroxymethyl)-3-((Z)-(4-hydroxyphenyl) methylidene) - tetrahydro-4aH-pyrano (2, 3-b) (1, 4) dioxin-2-one, $C_{15}H_{16}O_8$

Table 4.3: ^1H -NMR and ^{13}C -NMR of Compound MR-B with those observed in the literature

MR-B			Literature	
Position	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	161.2	-	162.5	-
2	135.2	-	136.6	-
3	119.6	6.86	120.9	6.84
4	124.6	-	125.9	-
5	132.4	7.68	133.7	7.66
6	115.1	6.79	116.4	6.77
7	158.6	-	159.9	-
8	115.1	6.79	116.4	6.77
9	132.4	7.68	133.7	7.66
COOCH_3	29.3	2.19	-	-
COOCH_3	169.0	-	-	-
β -glucose	-	-	-	-
1'	94.8	5.15	96.2	5.12
2'	78.8	4.08	80.1	4.05
3'	73.2	3.76	74.6	3.72
4'	69.6	3.52	70.9	3.58
5'	78.4	3.76	79.8	3.48
6'	60.8	3.99	62.1	3.79
		3.83		3.95

4.4 DISCUSSION

In this study, chromatographic analysis/purification of extracts and successive fractions led to the isolation of two compounds. The structures of the isolated compounds were elucidated using the techniques as described in section 4.2.2.5, and the compounds were identified as Z-methyl lespedezate and Z-venusol.

In order to optimise the separation of the compounds from the MeOH root extract, TLC was performed to view which mobile phase could best separate the compounds of this extract. The MeOH root extract was exposed to different mobile phases. The Toluene:EtOAc at a ratio of 70:30 and slowly increasing the polarity of EtOAc to 50:50 held most of the compounds at the baseline and allowed the separation of two bands. The chloroform:EtOAc:formic acid at a ratio

of 50:40:1 initiated the movement of some compounds. The DCM:EtOAc:MeOH:folic acid at a ratio of 90:10:30:1 began the actual separation for most of the compounds. The DCM:MeOH mobile phase showed poor separation and allowed the compounds to run too quickly to the solvent front. A similar principle was applied to the VLC as these solvents showed the potential to separate the compounds of the MeOH root extract.

A total of 21 fractions were obtained and chromatographically similar fractions were combined. Figure 4.2 showed the most active fractions pooled with a similar TLC profile. The combined fractions were concentrated on VLC to afford six major sub-fractions. The yields of these fractions were showed in Table 4.1. Two pure compounds were isolated and one of the fractions isolated from the MeOH root extract formed a crystal. Fraction 6 was chromatographed using VLC with the solvent system of 10% MeOH in DCM and the collected fractions yielded colourless crystals upon evaporation, and Compound 6 was obtained. The molecular structure of 7,8-Dihydroxy-6-(hydroxymethyl)-3-((Z)-(4-hydroxyphenyl)methylidene) - tetrahydro-4aH-pyrano (2, 3-b) (1, 4) dioxin-2-one was established unambiguously by single crystal X-ray diffraction analyses.

Non-polar and semi-polar fractions of *G. perpensa* (roots) gave one bioactive compound each, namely Compound MR-A and Compound MR-B. The compounds were identified based on high-resolution MS, MS/MS, and UV data compared with published data and confirmed by the injection of standards for some compounds

Compound MR-A exhibited a yellow amorphous powder. ¹H-NMR (500 MHz) the *p*-distributed aromatic ring (δ_{H} 6.80 d, *J* = 8.2 Hz, 2H; 7.77, d, *J* = 8.3 Hz, 2H), an anomeric carbohydrate signal (δ_{H} 5.11, d, *J* = 7.6 Hz, 1H), and an olefinic proton (δ_{H} 7.01 s, 1H) were observed. COSY (1H-1H) and HMBC correlations (1H-13C) were then joined to confirm the structure. These results were consistent with those of *Z*-methyl lespedezate (see Figure 4.6). The NMR spectra were also compared to previous literature data.

Venusol (MR-B) was transformed into methyl lespedezate (MR-A) upon standing in MeOH. The methyl lespedezate that was isolated was most likely an artefact formed during extraction. Z-methyl lespedezate is common in woody and herbaceous monocotyledons (Harborne, 1964). The potassium salt of lespedezic acid isolated from *Lespedeza cuneata* was proven to be biologically active and responsible for the leaf-opening mechanism in night-closing plants (Mensor et al., 2001).

Compound MR-B was collected as a brown crystal. In the ^{13}C -NMR spectrum, six signals (δ_{c} 60.8 to 94.8) characteristic of a glucopyranoside moiety, four aromatic signals [δ_{c} 124.6, δ_{c} 132.4(2C), δ_{c} 115.1(2C), δ_{c} 158.6] a non-protonated carbon at δ_{c} 135.2 (C-2), an olefinic CH at δ_{c} 119.6, and an additional non-protonated carbon δ_{c} 161.2 were observed. An HSQC experiment allowed the correlation of all protonated carbon atoms with well-resolved protons. The NMR spectra were compared to previous literature data (see Table 4.3). From these data, Compound MR-B was determined as Z-venusol (7,8-Dihydroxy-6-(hydroxymethyl)-3-[(Z)-(4-hydroxyphenyl) methylidene]-tetrahydro-4aH-pyrano (2, 3-b) (1, 4) dioxin-2-one] and the molecular formula was $\text{C}_{15}\text{H}_{16}\text{O}_8$.

African traditional healers use it for the treatment of many maternal/foetal ailments, and other conditions such as painful joints, cancerous sores, rheumatism, and psoriasis. The benefits of the traditional use of *G. perpensa* extracts have been reported in the literature. Khan et al. (2004) identified Z-venusol, a phenylpropanoid glycoside, as the major constituent of *G. perpensa*, which was able to stimulate uterine muscle contractility. Moreover, Mathibe et al.'s (2017) *in vitro* study reported that Z-venusol has anticancer properties. The traditional claim that *G. perpensa* extracts are beneficial therefore has credence. Peter (2007) reported the following compounds from *G. perpensa* rhizomes collected in the Gauteng province: Z-venusol, methyl lespedezate, 4-O- β -D-glucopyranosyl-3, 3', 4' -tri-O-methylelagic acid, and punicalagin. The compound, Z-venusol was also isolated from *G. perpensa* rhizomes collected from the KwaZulu-Natal province in studies by Mathibe et al. (2017) and Webb (2017). Even though Z-venusol is a known compound from *G. perpensa*, its crystal form is

reported for the first time in this study. Chapter 5 reports on the *in vitro* anticancer and toxicological evaluations of *G. perpensa* isolated compounds.

4.5 CONCLUSION

The analytical techniques of the MeOH extract aided with the process of identifying different fractions present in the respective solvent and further isolated single compounds. From the MeOH extract of *G. perpensa*, two compounds were isolated, namely Z-methyl lespedeazate (MR-A) and Z-venusol (MR-B). Moreover, the crystal structure of Z-venusol was also reported (7,8-Dihydroxy-6-(hydroxymethyl)-3-[(Z)-(4-hydroxyphenyl) methylidene]-tetrahydro-4aH-pyrano (2, 3-b) (1, 4) dioxin-2-one] and the molecular formula was C₁₅H₁₆O₈.

Further study is necessary to support traditional use or claims that *G. perpensa* extracts or pure components are useful in the treatment of cancer. The next chapter focuses on the anticancer effects of crude, fractions, and isolated compounds. From the results, Fractions 3 to 5 were selected for further testing of their anticancer activity because these fractions have sufficient weight and because of their colour intensity.

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CHAPTER 5:

***IN VITRO* ANTICANCER ACTIVITY AND TOXICOLOGICAL EVALUATION OF *G. PERPENS* L. (GUNNERACEAE) ISOLATED COMPOUNDS**

5.1 INTRODUCTION

Considering that many plants can present toxic effects, and that genetic toxicity is linked to an elevated risk of cancer generally (Hagmar et al., 1998), it is essential to do research to assess the danger of using plants with medicinal potential for human health. At the same time, it is also crucial to find plants that offer chemical protection against genotoxic agents to decrease the overall risk of cancer and to protect against secondary cancers caused by traditional treatments with chemotherapy (Meadows et al., 1985).

Many dangerous illnesses, some of which were informally classified as "cancers" based on signs of abnormal swelling, such as that found in tumors, calluses, abscesses, and polyps, have long been treated traditionally with plants (Cragg & Newman, 2005). Most indigenous medicinal plants, especially in Africa, are still widely used in unprocessed form, without any scientific validations for the safety and efficacy thereof (Fennell et al., 2004; Ndhlala et al., 2013). There are approximately 1 000 known poisonous plant species worldwide, containing 750 toxic substances, which occur out of a pool of 150 000 plants' secondary metabolites (Wink & Van Wyk, 2008).

To evaluate compounds that are likely to fail later on in the drug development process, it is of paramount importance to test for toxicity. Furthermore, the past few decades have seen increased interest in the development of natural product-based nanoparticles as novel drug delivery systems due to their increased bioavailability, improved solubility, and high target selectivity (Khan & Gurav, 2018). These "phyto-nanoparticles" have raised serious safety concerns regarding their impact on human health; hence the need to adequately evaluate their potential toxicity during early drug discovery (Boczkowski & Hoet, 2010; Boraschi et al., 2011).

The diverse medicinal uses of *G. perpersa* have been described and confirmed in several publications using a variety of cell types (Iwalewa et al., 2007; Koduru et al., 2007; Drewes et al., 2005; Lall & Meyer, 1999). However, this study further explored cell viability using the cytotoxicity test as well as the toxicity effects of the compound using a colony-forming assay. The *in vitro* cytotoxicity was assessed using fluorescence or absorbance measurements after toxicant exposure and incubation with a colorimetric dark-coloured formazan dye (Alamoudi et al., 2018). This potentially leads to misinterpretation of particle toxicity, which suggests that this assay is not enough/sufficient for quantitative assessment of toxicity. Despite these limitations, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is largely considered the pillar of cytotoxicity assays and is frequently used for high-throughput screening of medicinal plant extracts (Van Tonder et al., 2015).

In this study, the clonogenic test was used as an alternative approach that did not need the use of any colorimetric or fluorescent indicator dyes; removing the danger of interactions and allowing genuine cytotoxicity evaluation. The clonogenic assay, also called colony-formation assay, is an *in vitro* cell survival-based assay that measures the ability of a single cell to form a colony (Herzog et al., 2007). The clonogenic assay has been used over the past decades for a large variety of studies with many types of cells, using improved complex culture media, which have partly eliminated the need for feeder cells. The assay detects all cells that have retained the capacity for producing many progenies after treatments that can cause cell reproductive death because of the damage to chromosomes, necrosis, plasma membrane alterations, and apoptosis (Brown & Attardi, 2005). Clonogenic assays have also been developed for stem cells in various tissues by *in vivo* techniques (Hall, 1993). In addition to methods for cells in cell culture in normal tissues, clonogenic assays have also been developed for cells from tumours in animals (Reinhold, 1966).

Toxicity is defined as either resulting from general cytotoxicity mechanisms caused by interference with cellular basic structures or functionally important components (cell membrane permeability, metabolism, ion regulation, cell division, oxidative stress) or resulting

from specific cell, organ, or system toxicity mechanisms (affecting a particular ion channel, central nervous system, or a cardiac receptor). Most chemicals with specific effects on target organs were classified by the *in vitro* assay as acutely toxic by basal cytotoxicity (Prieto et al., 2019), confirming general toxicity as an important determining factor of acute system toxicity. The clonogenic assay appeared to be very useful to the extent that harvested cells can be cultured directly and quantitatively *in vitro* to produce clones and provide information on the effectiveness of treatments *in vivo* (Franken et al., 2006). The application to the toxicology of these clonogenic assays provides an essential tool for better understanding *in vivo* observation (experimental and clinical).

Chapter 4 focused on the isolation of methyl (Z)-3-(4-hydroxyphenyl)-2-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyprop-2-enoate and 7,8-Dihydroxy-6-(hydroxymethyl)-3-((Z)-(4-hydroxyphenyl) methylidene)-tetrahydro-4aH-pyrano (2, 3-b) (1, 4) dioxin-2-one from the roots of *G. perpersa*. This chapter investigates the *in vitro* cytotoxicity of the MeOH extract of the medicinal plant and the active fractions and single isolated compounds on human tumour cells and Vero cell line.

5.2 MATERIALS AND METHODS

5.2.1 Cytotoxicity activity using using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

5.2.1.1 Cell lines

MDA-MB-231 is a breast adenocarcinoma cell line that was derived by pleural effusion of a 51-year-old Caucasian female with metastatic breast adenocarcinoma (Cailleau et al., 1974). MCF-7 is a cultured human breast cancer cell line that is frequently employed in the study of breast cancer biology. The cell line was originally derived from the pleural effusion of a 69-year-old woman with metastatic disease at the Michigan Cancer Foundation (Osborne et al., 1987). The cytotoxicity screening was done as a first step to find out how harmful *G. perpersa*

is to human breast cancer cell lines (MCF-7 and MDA-MB-231) obtained from the American Type Culture Collection (ATCC) (Rockville, MD, United States of America). This study investigated the cytotoxicity of *G. perpensa* through an MTT assay using *G. perpensa*-derived fractions.

Vero is a normal African green monkey kidney epithelial cell line (also obtained from ATCC). Vero cells were used as control cells for this particular section of this study because it had to be assured that the fractions were not toxic to normal cells.

5.2.1.2 Cell regeneration and maintenance

All cell regeneration and culture maintenance were conducted in an aseptic environment using a laminar flow cabinet (Scientific Engineering), and 70% ethanol. Prior to conducting every experiment, the laminar flow cabinet was sterilised using 70% ethanol, together with the materials required for a particular experiment. Cells were grown in a complete growth medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum. They were kept at 37 °C and in a 5% carbon dioxide environment in a humidified incubator where the cells could grow.

The capacity of the cells to cleave the tetrazolium salt MTT was used to assess the cell viability. This was done using the mitochondrial enzyme succinate dehydrogenase, as detailed in the procedure below.

5.2.1.3 MTT

The cytotoxicity impact of *G. perpensa* extract, fractions, and compounds were evaluated in this study using the MTT test technique established by Mosmann (1983). MTT is a yellow tetrazolium salt that is converted by dehydrogenase enzymes in viable cells (see Figure 5.1). These enzymes cleave the tetrazolium ring. The end result is a reduction of the yellow dye to formazan crystals, which are purple in colour in DMSO and sodium dodecyl sulfate. The

amount of purple formazan that is extracted from the cells is proportional to the number of viable cells. The resulting purple solution can be measured spectrophotometrically. The increase or decrease in the number of cells therefore depends on the quantity of formazan produced, which in turn can denote the degree of growth inhibition exhibited by the test drug (Berridge & Tan, 1993). It has been shown that plant extracts with strong antioxidant activity can also convert MTT to purple formazan (Muraina et al., 2009).

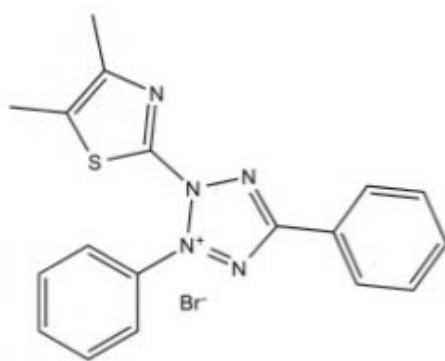


Figure 5.1: The structure of MTT

Source: Adopted from Stockert et al. (2012)

The MTT assay was performed to determine the cytotoxicity effect of the extract, fractions, and compounds as previously described. DMSO was used to dissolve the samples and was also tested as a negative control instead of plant extract, while doxorubicin (DXR) was used as a positive control due to its potential as a DNA-damaging agent.

Cells were harvested after reaching 80% confluence and were grown in 96 well-microtitre plates for 24 hours. The cells were then treated with 100 μ L of prepared sample in different concentrations series (1.5625-100 μ g/mL) at 37 $^{\circ}$ C for 24 hours in a gas-controlled environment of 5% carbon dioxide. Thereafter, the medium was removed and replaced with fresh medium, and the cells were subjected to MTT assay after 48 hours of incubation. After the 48-hour incubation, the supernatants were removed from the wells and 30 μ L of the MTT solution was added to each well. The plate was then incubated for four hours at 37 $^{\circ}$ C and the MTT medium was removed from the wells. Then 50 μ L of 100% DMSO was added to each well to dissolve the insoluble formazan crystal. Finally, the plate was gently shaken on a

microtitre plate shaker for five minutes and then read immediately on a microtitre plate reader at 540 nm, and cell viability was calculated. Each experiment was carried out in triplicate, and the number of viable cells was calculated using the following formula:

Percentage cell viability:

$$= \frac{\text{Absorbance of treatment} - \text{Absorbance of media}}{\text{Absorbance of negative control} - \text{Absorbance of media}} \times 100\%.$$

5.2.2 Selectivity index (SI)

The Vero and normal human breast cancer cell lines were used to measure the SI. The SI was established by calculating the ratio between the IC₅₀ values obtained for the Vero and MCF-7 cells; where (SI ≤ 1.5) was classified as non-selective (toxic) and (SI ≥ 1.5) was considered safe (non-toxic).

5.2.3 Toxicity evaluation

5.2.3.1 Sample preparation

The compounds were reconstituted in DMSO to give a final concentration of 100 mg/mL. Stock solutions were sonicated, if necessary, where solubility was an issue. All samples were then stored at 4 °C.

5.2.3.2 Toxicity evaluation using colony-forming assay

The MTT assay was applied as previously described and used to evaluate the cytotoxicity of the extract and Compound MR-B. Exponentially growing cells were harvested and seeded into six-well flat-bottom polystyrene microplates at a density of 800 cells/well for the MCF-7 cells. Each well contained 2 mL cell culture medium. Cells were allowed to attach for ~16 hours.

After the attachment period, the cell carpet was gently washed with 3 mL of phosphate buffered saline and treated with 2 mL of the compound prepared in Roswell Park Memorial Institute 1640 complete medium supplemented with 10% foetal bovine serum to final concentrations of 0 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, and 1 000 µg/mL, which were renewed every three days. Exposure concentrations were chosen to be comparable to previous studies on toxicity (Herzog et al., 2007). The cells were incubated for 12 days, and exposed to the extract and Compound MR-B for 12 days. The number of colonies was scored by observing them under an inverted microscope, and the surviving fraction obtained using cells treated only with diluents as control was normalised at 100% (Crowley et al., 2016). The colonies were defined as cell clusters composed of ≥ 50 cells.

Before colonies were counted, particle solutions were removed, and cells were washed with 3 mL phosphate buffered saline. Fixation and staining of clones were done with MeOH, and 0.5% crystal violet for 30 minutes. The dishes were rinsed with distilled H₂O and left to dry at room temperature. The clones were counted the following day.

5.2.4 Statistical analysis

All experiments were performed in triplicate and the data is shown as the mean $\pm$ standard deviation (SD). The collected data was imported to a Microsoft Excel spreadsheet and analysed and processed using GraphPad Prism 5 for IC₅₀ determination. For the clonogenic assay, the survival rates were calculated as: (mean plating efficiency of treated cells/mean plating efficiency of control cells) $\times$ 100%, where the plating efficiency was the number of colonies divided by the number of inoculated cells. Microsoft Excel Graph was used to produce the bar charts.

5.3 RESULTS

5.3.1 *In vitro* screening of MTT assay and selectivity studies

A quantitative evaluation of cell viability and proliferation was performed to demonstrate that the viable cells after screening were capable of forming formazan crystals around them by the cleavage of the tetrazolium salt. After incubation of the cells submitted to this assay, a dye formed, which was measured under a microplate reader, as shown in Figure 5.2.

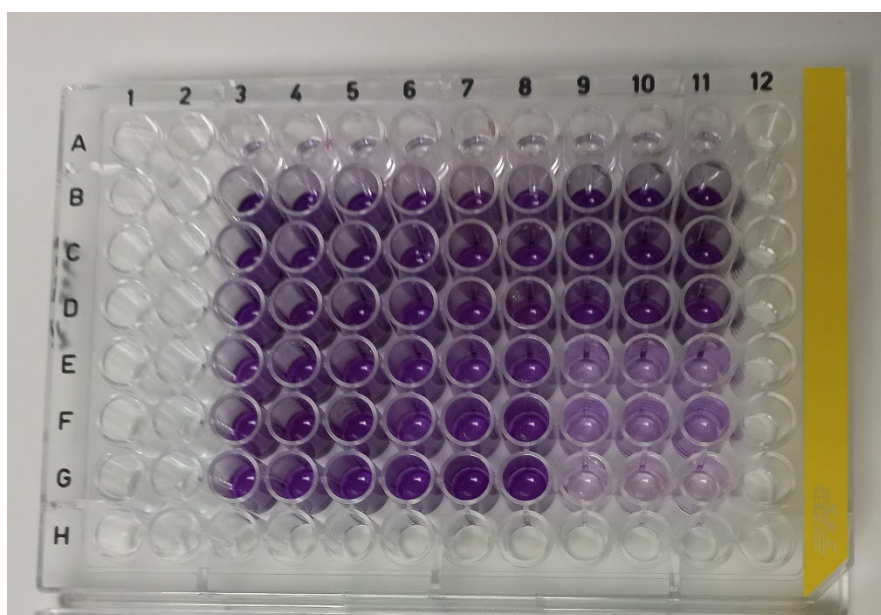


Figure 5.2: A 96-well plate used in the MTT assay method after incubation, after a dye solution formed

Source: Researcher

Cytotoxic studies were determined in breast adenocarcinoma cells (MDA-MB-231) and MCF-7 and Vero cells were treated with different sample concentrations and MTT for four hours at room temperature as described in the materials and methods section. The MTT assay results of the extract, fractions, and isolated compounds of *G. perpensa* against the tested cancer cells displayed moderate to low cytotoxicity. Potential cytotoxicity was observed for the crude extract against the Vero cells. On the other hand, the crude extract displayed no cytotoxicity or a low percentage of cytotoxicity on MCF-7 (see Figure 5.3). The results obtained

for the Vero cell line showed a concentration-dependent cytotoxicity of the plant on the cells. The highest concentration (1 000 $\mu\text{g/mL}$) of the plant extract showed a significant decrease in viable cells.

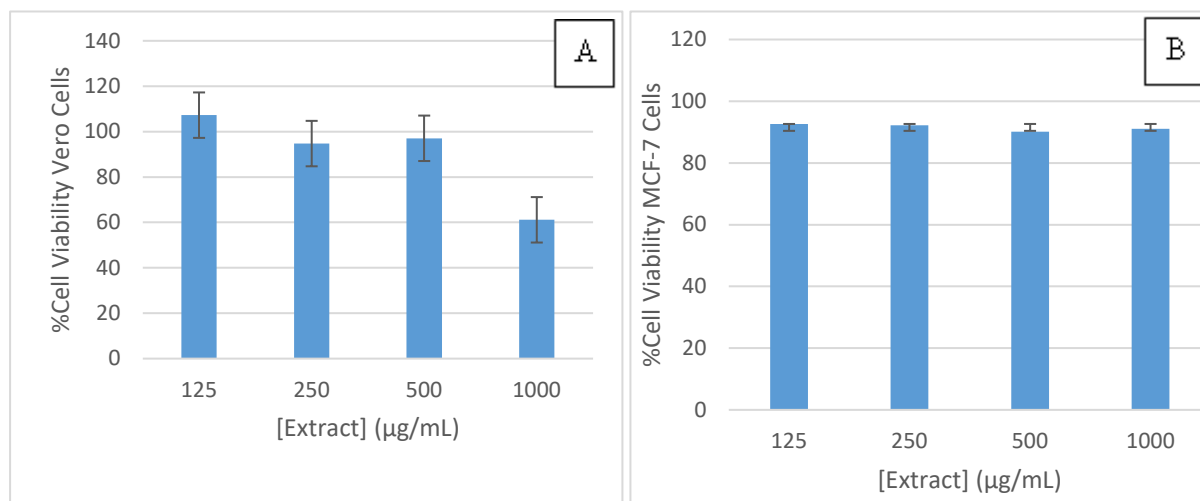


Figure 5.3: Percentage cell viability of (A) Vero cells and (B) MCF-7 treated with the extract of *G. perpensa*

Figure 5.4 indicates the 48-hour exposure of the Vero and breast cancer cell lines to different concentrations of fractions of *G. perpensa* (6.25 to 100 $\mu\text{g/mL}$) and were used for the calculation of IC_{50} values using GraphPad Prism 5 software. At the lowest concentration (6.25 $\mu\text{g/mL}$) for both the Vero and MCF-7 cells, the results showed no significant toxicity to the cells, since more than 80% of the cells were still viable post-treatment. However, the lowest concentration (1 $\mu\text{g/mL}$) against MDA-MB 231 displayed that Fractions 3, 4, and 5 and DXR had no significant toxicity to the cells. An increase in the concentration series of the fractions showed a decrease in relative cell viability.

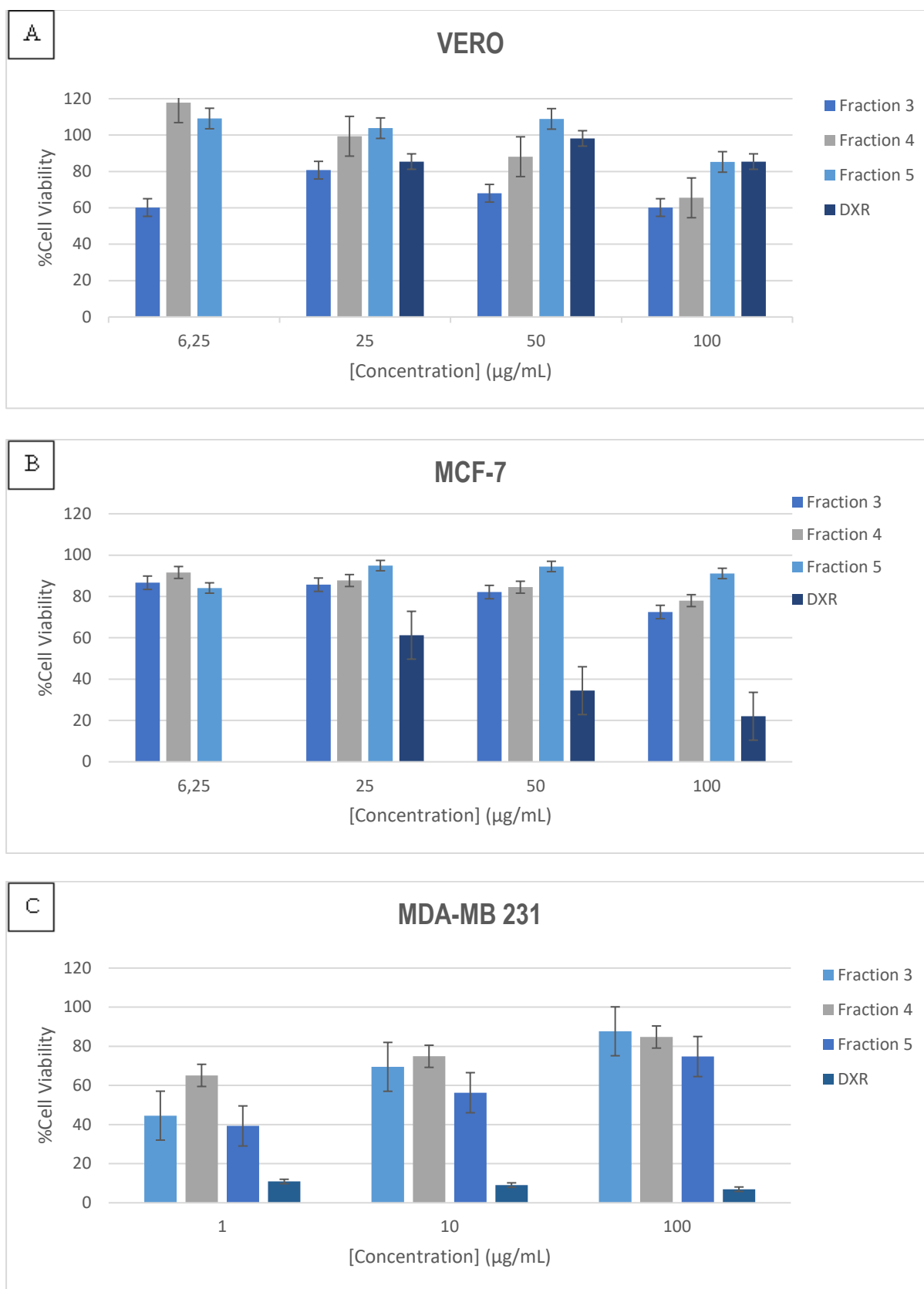
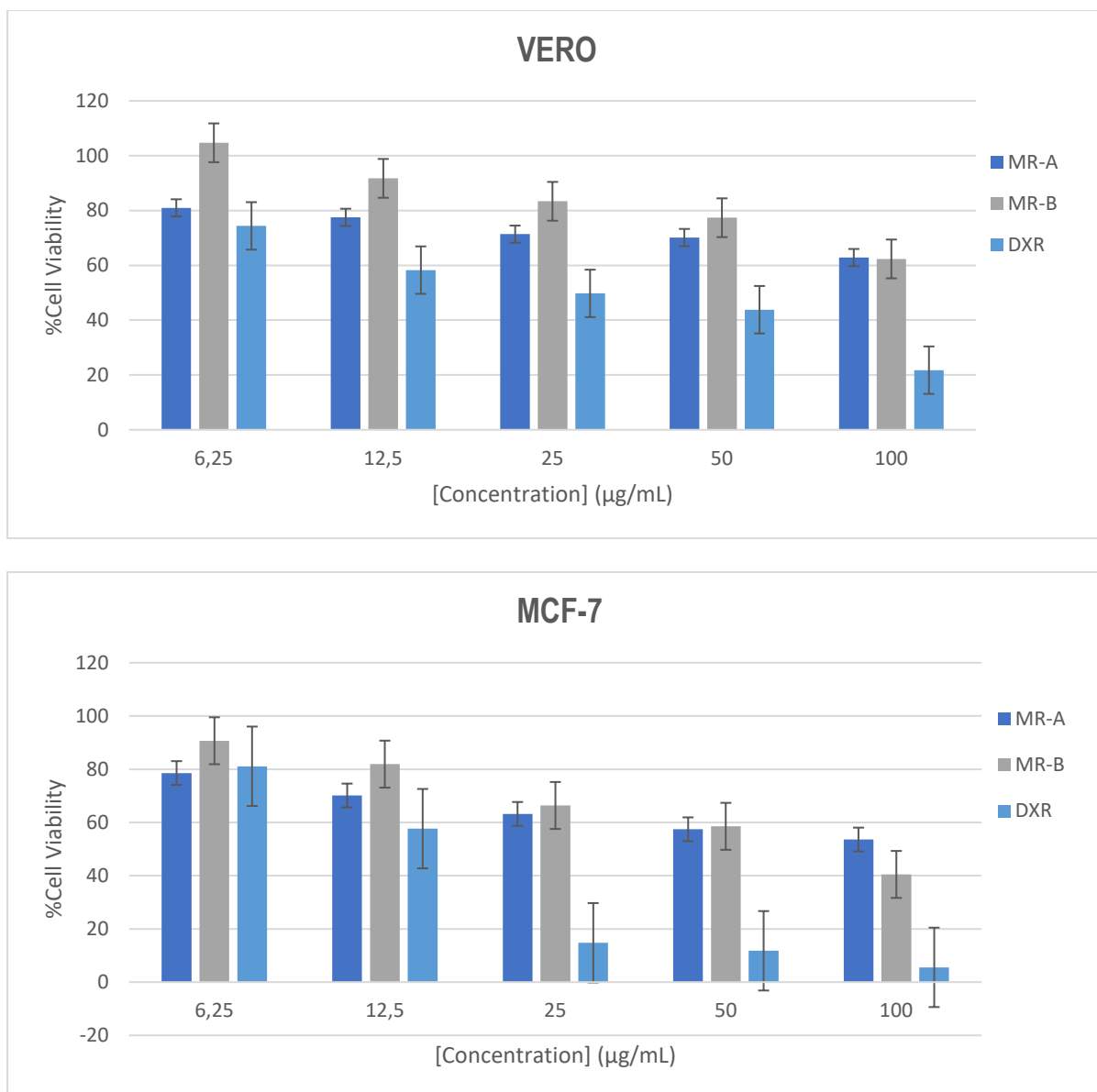


Figure 5.4: Percentage cell viability of (a) Vero, (b) MCF-7, and (c) MDA-MB 231 cells treated with the fractions of *G. perpensa*



Values are expressed as mean $\pm$ SD.

Figure 5.5: Percentage cytotoxicity results of the isolated compounds of *G. perpensa* against the MCF-7 cell line (44-hour exposure)

The isolated compounds were found to have more cytotoxicity potential against the tested MCF-7 cancer cell line. The highest cytotoxicity potential was recorded for Compound MR-B, followed by moderate activity of Compound MR-A (see Figure 5.5). From these results, it is clear that the isolated compounds of *G. perpensa* are not as strong when compared to the activity of the organic crude extract of *G. perpensa*. This means that using the full-plant extract where all of these naturally occurring compounds are modulating naturally will provide the best

therapeutic benefit, which suggests that a full-plant extract will have a better effect than individual fractions or an isolated single compound of *G. perpensa*.

5.3.2 Selectivity towards non-cancerous cells

The SI was determined as the ratio of IC₅₀ value in the non-cancerous Vero cells, divided by IC₅₀ in the cancer cell lines. The obtained IC₅₀ values and SI are summarised in Table 5.1. By calculating the SIs in brackets, the values in bold mean that the test drug is safe (non-toxic), and “N.d.” means “not determined”. The study found that the crude extract had poor selectivity (SI ≤ 1), and the isolated Compound MR-A also had poor selectivity for all cancer types. On the other hand, Compound MR-B displayed acceptable selectivity with an SI value of 1.5 for MCF-7 but was not determined for other cancer cells. Fractions had SI values (SI ≤ 3.3 SI ≤ 31.8) for MDA-MB 231 cells towards non-cancerous Vero cells, which means tested Fractions 3, 4, and 5 can selectively kill cancer cells without causing harmful effects to normal cells.

Table 5.1: Cytotoxicity effects of pure tested Compound X and reference drug (doxorubicin [DXR]) against cancer cells and non-cancerous Vero cells using MTT assay

Cell lines	Extract	Fraction 3	Fraction 4	Fraction 5	Compound MR-A	Compound MR-B	DXR
		IC ₅₀ values (in μM), and SI* (in brackets)					
Vero	0,35	8,54	59,59	6,98	17,64	22,30	4,85
MCF-7	0,4 (0.8)	4,9 (1.7)	121,9 (0.5)	N.d.	61,21 (0.3)	15,49 (1.5)	2,02 (2.4)
MDA-MB 231	N.d.	2,02 (4.2)	1,87 (31.8)	2,08 (3.3)	N.d.	N.d.	0,71 (6.8)

(*): The SI was determined as the ratio of IC₅₀ value in the non-cancerous divided by the IC₅₀ in the cancer cell lines.

5.3.3 Clonogenic assay

Compound MR-B showed weak antiproliferative effects; it was therefore excluded from the determination of a radioprotective effect. The dilutions of MCF-7 cells showed a cell concentration-dependent response treatment to *G. perpensa* extracts, which indicated that the decrease in dose rate induced greater viability (see Figure 5.6a). The crude extract was reported earlier as possessing anticancer activity.

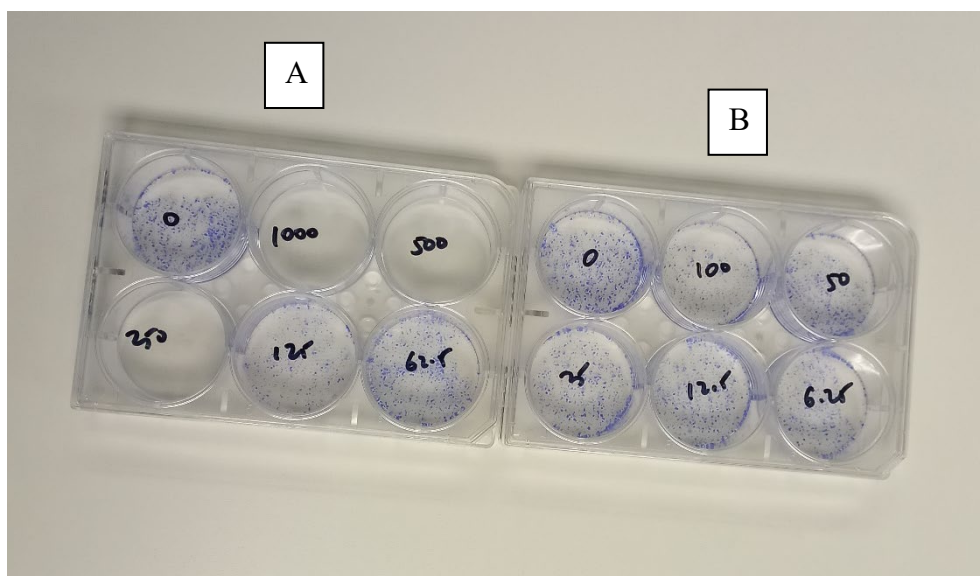


Figure 5.6: Clonogenic assay performed in six-well plates, with clones produced by MCF-7 breast adenocarcinoma cell lines: (A) Treated controls with *G. perperisa* extracts, formed after seeding with 800 cells and (B) treated with Z-venusol (MR-B) isolated compound

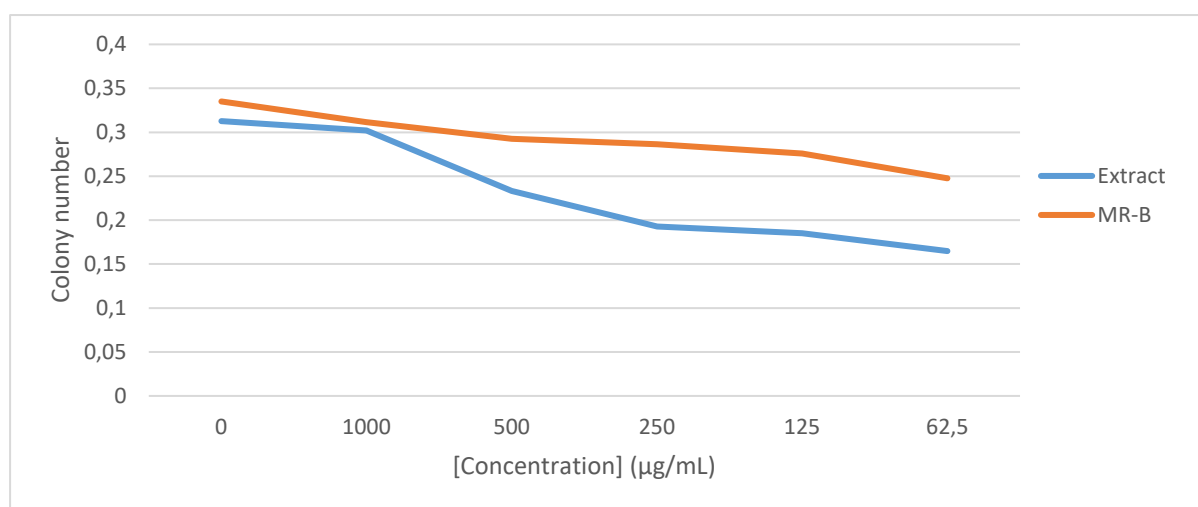


Figure 5.7: Survival curves derived from clonogenic assay experiments

5.4 DISCUSSION

There are many ancient plants and herbs that can have an effect on cancer. *G. perperisa* is a medicinal herb that grows in South America, Asia, and Central and Southern Africa. This is one species of plant out of hundreds of thousands that is spreading across the world and with good reason. *G. perperisa* is so versatile that it also has antibacterial activity (Mfengwana

et al., 2019), antimicrobial activity (Mfengwana et al., 2019; Muleya et al., 2014), antifungal activity (Buwa & Van Staden, 2006), anticancer (Mammo, 2018), antinociceptive, and anti-inflammatory properties (Nkomo et al., 2010), anti-oxidant activity (Mfengwana et al., 2019; Muleya et al., 2014), toxicological evaluations (Mwale & Masika, 2011), and cytotoxicity (Simelane et al., 2012).

In this chapter, the MeOH crude extract and various fractions from *G. perpensa* were identified and screened for their potential *in vitro* cytotoxicity. MeOH extracts are normally used for anticancer screening for two reasons: (1) the presence of MeOH may enhance the nature of biologically active components (alkaloids, flavonoids, essential oil, terpenoids, etc.), and (2) the larger extraction capability of MeOH may create a bigger number of active compounds responsible for anticancer activity (Ghosh et al., 2008). The individual compounds were also evaluated further for their potential anticancer activity using MTT. Based on the ability of the cellular mitochondrial dehydrogenase enzyme in living cells to convert the yellow, water-soluble substrate MTT into a dark blue/purple formazan product that is insoluble in water, the MTT assay is a sensitive, quantitative, and trustworthy colorimetric test (see Figure 5.2) (Riss et al., 2016). According to the criteria of the National Cancer Institute and the Geran protocol, extracts with $IC_{50} \leq 20 \mu\text{g/mL}$ = highly cytotoxic, IC_{50} ranged between 21 and 200 $\mu\text{g/mL}$ = moderately cytotoxic, IC_{50} ranged between 201 and 500 $\mu\text{g/mL}$ = weakly cytotoxic, and $IC_{50} > 500 \mu\text{g/mL}$ = no cytotoxicity (Grever et al., 1992; Goldin et al., 1981; Geran, 1972).

The MTT findings showed concentration-dependent anticancer efficacy and cytotoxicity in the cell lines employed in this investigation, as shown in Figure 5.4. In the MDA-MB-213 cell line, fraction 5 showed considerable cytotoxicity at doses of 1 and 10 $\mu\text{g/mL}$. As a result, its use against cancer is restricted at these levels. Several studies have found that *G. perpensa* does not cause any harmful effects on cells. Brookes et al.'s (2003) *in vitro* study reported that *G. perpensa* extracts with an IC_{50} value $> 500 \mu\text{g/mL}$ on Vero (monkey kidney epithelial) cells. Khan et al. (2004) isolated Z-venusol compound from *G. perpensa*. The compound has been

reported to stimulate the uterus smooth muscle and ileal muscle. Drewes et al. (2005) isolated 1,4-benzoquinones, phenylpropanoid glucoside, and Z-venusol from *G. perpensa*. These compounds have been reported to have antimicrobial activity. Additionally, Z-venusol was also isolated from the roots of *G. perpensa* in a study by Mathibe et al. (2017). An *in vitro* test was performed in which the compound was found to be cytotoxic against human breast tumour (MCF-7) cells, with $IC_{50} = 53.7 \mu\text{g/mL}$, which indicates that Z-venusol has anticancer properties. In our study, the isolated compounds of *G. perpensa* Z-venusol (MR-B) showed strong cytotoxicity ($IC_{50} = 15.5 \mu\text{g/mL}$), while Compound MR-A had moderate cytotoxicity ($IC_{50} = 61.2 \mu\text{g/mL}$) (see attached appendix). Our observation is in agreement with studies of Khan et al. (2004) and Simelane et al. (2017). Interestingly, anticancer efficacy could once again be achieved when the *in vitro* test was performed against MCF-7 cell lines.

The primary goal of cancer chemotherapy is to specifically target cancer cells without displaying toxicity towards normal cells. However, many anticancer drugs fail to meet this criterion, which make them cytotoxic not only to cancer cells, but also to normal cells. Selective toxicity must therefore be taken into consideration in the discovery of leads for cancer treatment (Wong et al., 2012). Vero cell lines were used as a normal cell model for the calculation of the SI. The SI value was calculated by dividing the IC_{50} value of the Vero cell line with the IC_{50} value of the breast cancer cells (MDA-MB 231 and MCF-7) (Aljewari et al., 2010). To determine whether bioactive compounds will be cytotoxic to cancer cell lines, SI values less than 1 are classified as non-selective (toxic), between 1 and 10 is weakly selective, and an SI higher than 10 is considered safe (non-toxic) (Ondo et al., 2012; Ogbale et al., 2017).

As presented in Table 5.1, the SI was determined. The *G. perpensa* crude extract had poor selectivity ($SI \leq 1$) and the Z-methyl lespedezate (MR-A) isolated compound from the MeOH root extracts of *G. perpensa* also had poor selectivity for all cancer types (MCF-7 and MDA-MB 231). Similarly, Fractions 3, 4, and 5 displayed acceptable selectivity (except for MCF-7

cells), which suggests that these fractions possess bioactive compounds. On the other hand, Z-venusol (MR-B) was the only compound that had acceptable selectivity (1.5) and is considered non-toxic, which suggests that this compound can selectively kill cancer cells at IC_{50} without causing harmful effects to non-cancerous cells. The cytotoxicity and the selectivity of Z-venusol (MR-B) make it a potential cytotoxic agent that can be used to fight against human carcinoma. This compound was therefore further tested for *in vitro* studies (colony-forming assay) on cancer cells for 10 days to evaluate the toxicity on cell growth.

Over the last 10 years, a lot of work has been done to determine if *in vitro* cytotoxicity studies may be used to predict the acute toxicity of chemicals *in vivo*. Cytotoxicity tests undoubtedly have a contribution to make in several areas of toxicity testing (Garle et al., 1994). In this respect, the clonogenic assay, also called a colony-formation assay, serves as a useful tool to test whether a given cancer therapy can reduce the clonogenic survival of tumour cells. It is used as a fallback method to determine the survival of cancer cells or cancer cells that have been exposed to radiation. It may therefore be considered a kind of reference assay for cell viability measurement. By using the clonogenic assay instead of these widely used absorbance or fluorescence-based assays, any adsorptive interactions between the nanomaterial and indicator dyes might be avoided, allowing for a more accurate and realistic assessment of toxicity. Even though the use of the clonogenic assay as a high-throughput assay is limited, this assay offers the possibility for *in vitro* assessment of long-term toxicity, which makes it highly sensitive and valuable. Additionally, the MeOH extracts' isolated compound bioactivity and toxicity score analyses provided very promising results.

It has been demonstrated that the number of colonies decreases at low concentrations, and that the reduction of colonies varies throughout a range of concentrations. Z-venusol had the highest number of colonies in the highest concentration. Here, too many colonies were overlapping after seeding 800 cells. On the other hand, the crude extract of *G. perpensa* (Gunneraceae) showed the lowest number of colonies formed. Dose-response

curves show that the human breast cancer cell lines MCF-7 lose their clonogenicity while preserving the function of healthy tissue. The colony pattern of test compounds observed in this study was consistent with the results observed under cell proliferation.

The clonogenic assay is a long-term test that is used to demonstrate survival, which implies the retention of regenerative capacity, typically measured by plating efficiency through clonogenic cells. In contrast, the MTT assay is a metabolic test that provides an immediate or short-term response to an insulting agent. Therefore, a clonogenic cell may produce offspring that grow into a colony big enough to be seen with the naked eye, demonstrating that it is not only a live cell but also—and perhaps more importantly—that it has maintained its ability to reproduce.

5.5 CONCLUSION

Investigating the cytotoxic activity or cell proliferation of *G. perpensa* (root extracts, fractions, and isolated compounds) on the MCF-7 and MDA-MB-213 breast cancer cell lines in contrast to the non-cancerous Vero cells was the goal of this chapter. Activity was found in Z-venusol (MR-B) as isolated from *G. perpensa* crude extract. The compound was found to be cytotoxic against human breast tumour cells, which indicates that Z-venusol has anticancer properties. Moreover, the safety of the compound for normal cells was determined through the SI. The results indicated that the Z-venusol (MR-B) compound can selectively kill cancer cells at IC_{50} without causing harmful effects to non-cancerous cells.

This study's goal was to examine the toxicity assessment of compounds isolated from *G. perpensa*. The clonogenic assay study revealed the reduction of colonies, and that the number of colonies being reduced decreased in low concentration. The results suggest that the human breast cancer cell lines MCF-7 lose their clonogenicity while preserving the function of healthy tissue.

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CHAPTER 6:

CONCLUSION AND LIMITATIONS

6.1 CONCLUSION

This work evaluated valuable insight into the *in vitro* cytotoxicity and antiproliferative evaluation of isolated compounds from *G. perpersa* L. using thorough data collecting, thorough analysis, and critical interpretation. After demonstrating the cytotoxic properties of *G. perpersa* (MeOH crude extract, fractions, and single compounds), the study assessed the clonogenic activity of the compounds to determine their toxicity. The clonogenic test is very sensitive and helpful since it offers the possibility for the *in vitro* assessment of long-term cytotoxicity. The results of this study showed dose-response curves that indicate that the human breast cancer cell lines MCF-7 lose their clonogenicity while preserving the function of healthy tissue. The colony pattern of crude extract observed in this study was consistent with the results observed under cell proliferation. The majority of medicinal plants come in the form of crude extracts, which are a combination of multiple ingredients and active principles that don't work as intended when separated out. This implies that the activity of the extract is the synergistic effect of its various components.

This special interest in toxicity against isolated compounds led to conducting this study to provide up-to-date information on these important compounds so as to benefit the scientific community. It is my honour and privilege to serve as the author of this special research. I sincerely hope that the reviewers will enjoy marking it half as much as I deeply enjoyed writing it.

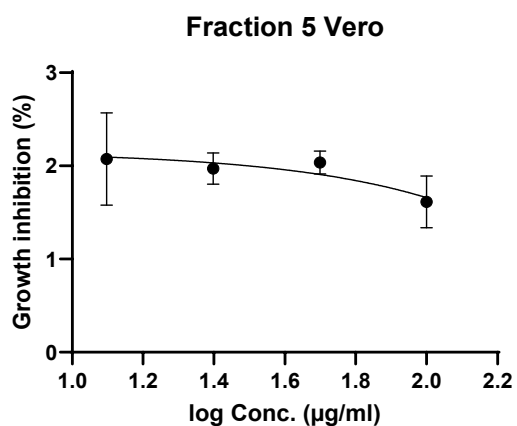
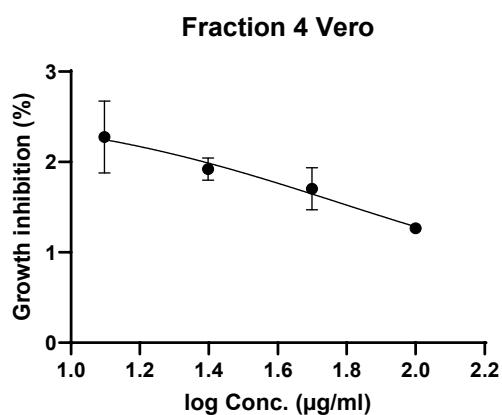
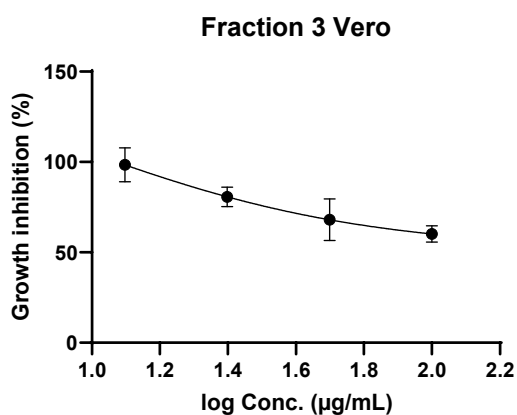
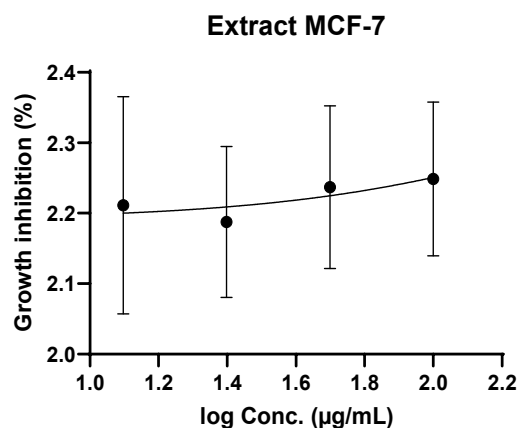
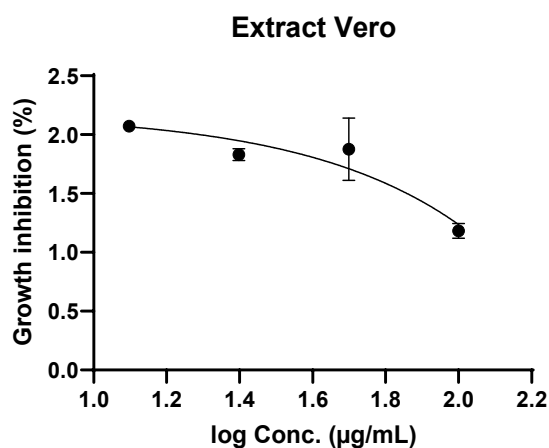
This study gives credence to the claim that the *G. perpersa* plant is a rich source of anticancer compounds.

6.2 LIMITATIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

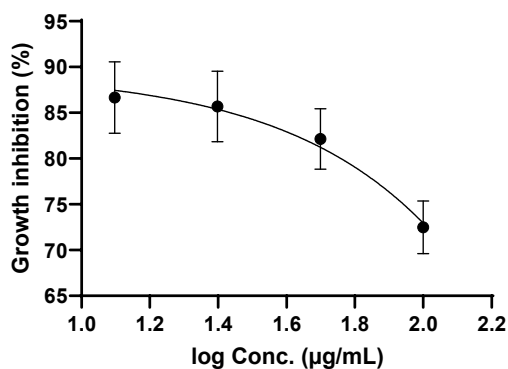
This study explored and analysed various aspects of the topic, shed light on important findings, and contributes to knowledge. The limitations of this study are that the mechanisms of action of the compound were not established. It is thus necessary to extensively investigate the compounds. To the best of my knowledge, the toxicity of *G. perpensa* isolated compounds on rats has not been scientifically investigated and there is thus no documented evidence. I do, however, recommend that further research (*in vivo* and *in silico*) towards the isolated compounds be conducted. More compounds from the plant must be isolated to confirm their traditional usage and their likely use as natural (cancer) medicine sources. It is also necessary to screen for their safety in humans.

APPENDIX:

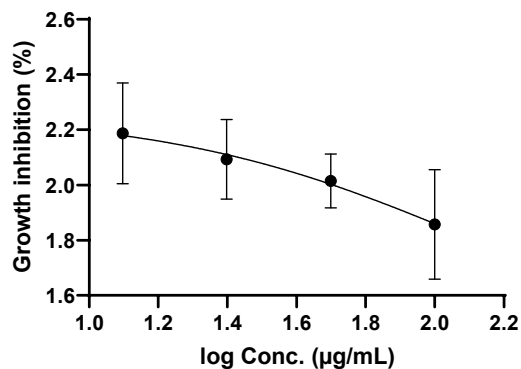
LOG CURVES OF THE CANCEROUS CELLS TREATED WITH THE EXTRACT, FRACTIONS, AND COMPOUNDS



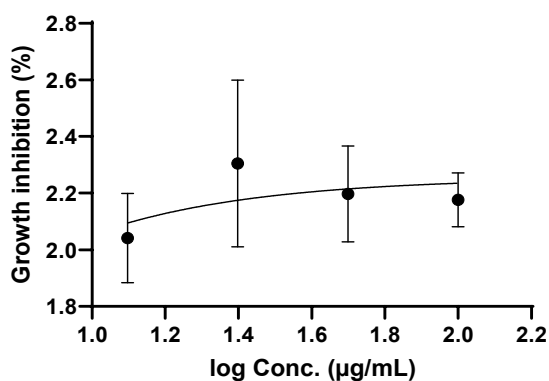
Fraction 3 MCF-7



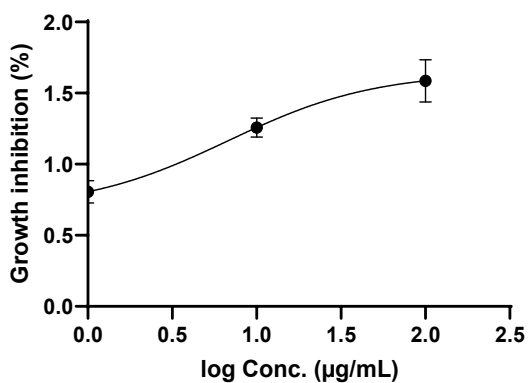
Fraction 4 MCF-7



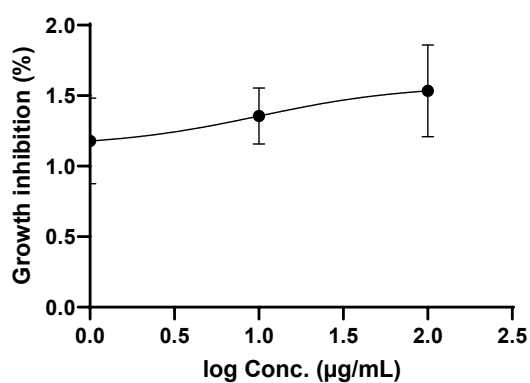
Fraction 5 MCF-7



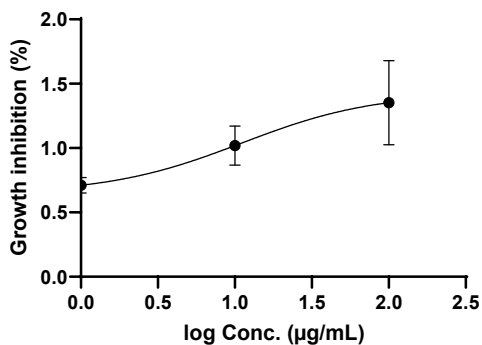
Fraction 3 MDA-MB 231



Fraction 4 MDA-MB 231



Fraction 5 MDA-MB 231



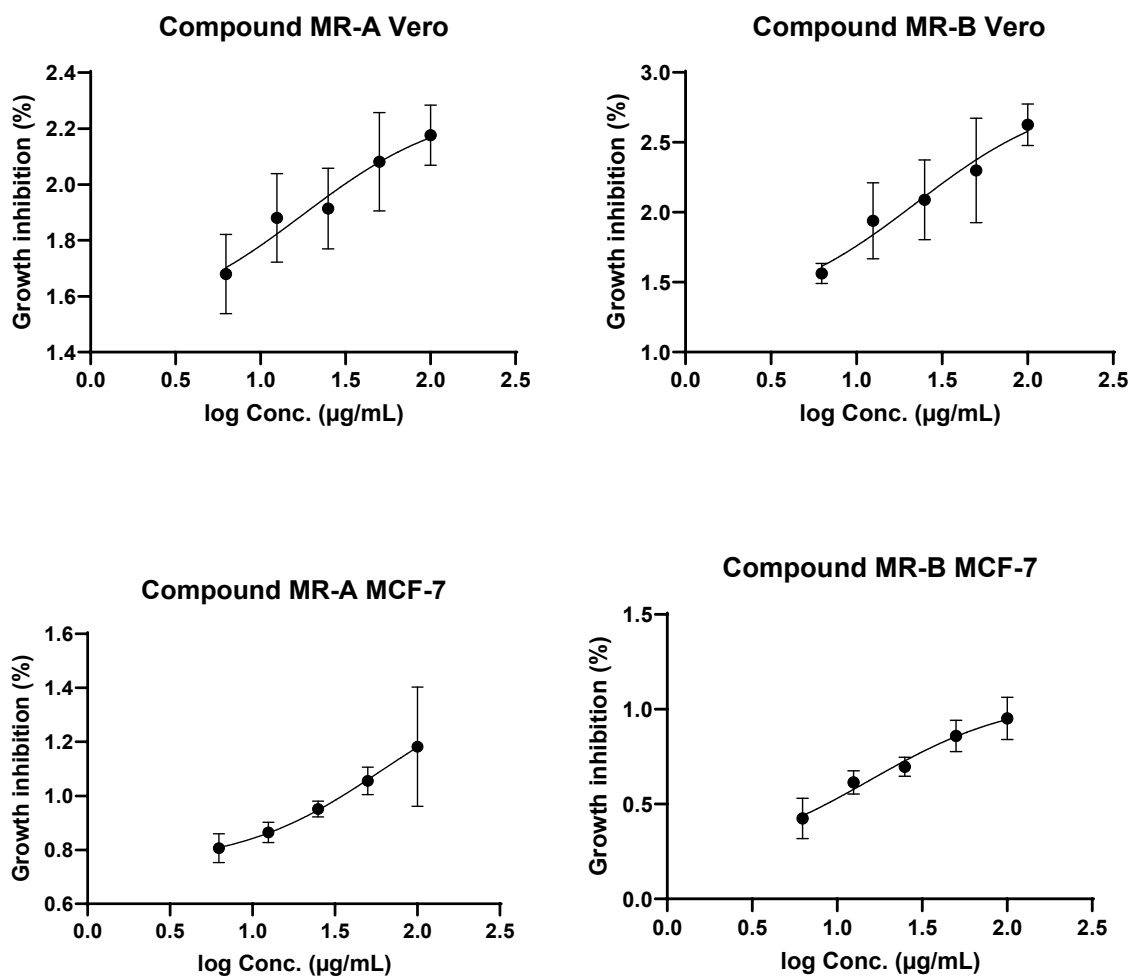


Figure A1: Dose-response curves of the breast cancer cells treated with DXR, extract, and Fractions 3, 4, and 5 single compounds of *G. perpensa*