

**UNDERSTANDING THE PHARMACOGENETICS OF DRUG RESISTANCE IN THE
TREATMENT OF PROSTATE CANCER**

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DECLARATION OF INDEPENDENT WORK

i, Paballo Motloun, hereby declare that the dissertation submitted by me for the M.H.SC degree in the Department of Health Sciences at the Central University of Technology is my own work and has not been previously submitted by me at another university or faculty for admission to a diploma or degree. Furthermore, I cede copyright of the dissertation to the Central University of Technology.

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SUPERVISOR'S DECLARATION

I, Professor MP Sekhoacha, the supervisor of the master's research dissertation entitled: Understanding the pharmacogenetics of drug resistance in the treatment of prostate cancer, hereby certify that the work in this project was done by Paballo Motloun at the Department of Health and Environmental Sciences, Central University of Technology.

I so give my approval for the thesis to be presented and declare that it has never before been submitted, in whole or in part, to the assessors, to this institution, or to any other institution for admission to a degree program or other qualification.

DEDICATION

The dissertation is dedicated to my family, especially my mother, for their constant support, having faith in me, and their words of encouragement throughout the research project.

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ABBREVIATIONS

ABC – ATP BINDING CASSETTE

NBD – NUCLEOTIDE BINDING DOMAIN

TMD – TRANSMEMBRANE DOMAIN

MDR – MULTIDRUG RESISTANCE

P-gp – P GLYCOPROTEIN

MRP1 – MULTIDRUG ASSOCIATED RESISTANCE PROTEIN 1

BCRP – BREAST CANCER RESISTANCE PROTEIN

BBB – BLOOD BRAIN BARRIER

TKI – TYROSINE KINASE INHIBITORS

NGS – NEXT GENERATION SEQUENCING

PSA – PROSTATE SPECIFIC ANTIGEN

ddNTP'S – DIDEOXYNUCLEOTIDES

MSD – MEMEBRANE SPANNING DOMAINS

CYP-A – CYCLOSPORINE A

MTT – 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5DIPHENYLTETRAZOLIUM BROMIDE

DOX- DOXORUBICIN

CDDP- CISPLATIN

DPBS- DULBECCO'S PHOSPHATE BUFFERED SALINE

FBS – FETAL BOVINE SERUM

DNA – DEOXYRIBONUCLEOTIDE ACID

DMEM – DULBECCO'S MODIFIED EAGLES MEDIUM

WHO – WORLD HEALTH ORGANISATION

CANSA – CANCER ASSOCIATION OF SOUTH AFRICA

MXR – MULTIXENOBIOTIC RESISTANCE

PCR – POLYMERASE CHAIN REACTION

RT-qPCR – REVERSE TRANSCRIPTASE QUANTITATIVE POLYMERASE CHAIN
REACTION/REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION

IC₅₀ – HALF MAXIMAL INHIBITORY CONCENTRATION

DMSO – DIMETHYL SULFOXIDE

SDS-PAGE – SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL
ELECTROPHORESIS

MeOH – METHANOL

T_m – MELTING TEMPERATURE.

T_a – ANNEALING TEMPERATURE.

CT – THRESHOLD CYCLE

C_q – QUANTIFICATION CYCLE

NTC – NO TEMPLATE CONTROL

NRT – NO REVERSE TRANSCRIPTASE CONTROL

AMPLICON – PCR PRODUCT

RIN – RNA INTEGRITY NUMBER

RT – REVERSE TRANSCRIPTASE

ABSTRACT

INTRODUCTION

Drug resistance has been identified as one of the major causes of chemotherapeutic treatment failure in prostate cancer patients. One of the Multidrug resistance mechanisms for cancer resistance, explored in this study, is the overexpression of transport proteins involved in transporting chemo drugs in and out of the cells. The study analyses the expression of the ABC (ATP-binding cassette) transporter genes, ABCC1 and ABCB1 genes and their relative protein products (mrp-1 and p-gp respectively) involved in MDR in prostate cancer, and explores them as targets for combating chemo resistance. ABC transporters are trans-membrane proteins that facilitate the translocation of different substrates across membranes as well as non-transport-related biological functions including RNA translation and DNA repair by using the energy of adenosine triphosphate (ATP) breakdown. Medicinal plants are important sources of therapeutic agents and are good sources of anticancer agents; they can be also used for the restoration of sensitivity in resistant cancer cells and reverse drug resistance *in vitro*. *Ruta graveolens* and *Malva parviflora* are medicinal plants reported to exhibit antioxidant, antibacterial and anti-inflammatory effects but their anticancer activity has not been fully elucidated. The study aimed to assess the anticancer and potential drug resistance reversal potential of these plant species against prostate cancer cell lines, and whether their mechanism of action involves upregulation of ABCC1 and ABCB1 genes and their relative protein products. This is the first study to report the anticancer effects of the DCM extract of *R. graveolens* and that of DCM+MeOH and MeOH extracts of *M. parviflora*.

METHODOLOGY

The DU-145 prostate cancer cell line was cultured according to established procedures. Resistant sublines were established by continuously exposing the DU-145 cell line to increasing concentrations of Doxorubicin and Cisplatin for 6 months. Cell viability and chemosensitivity was assessed by MTT assay. RT-qPCR and western blot were used to

determine the mRNA and protein expression levels of the ABCC1 and ABCB1 gene and their relative protein products, mrp-1 and p-gp. Calcein AM assay was used to determine the function of the ABC transporters in the drug resistant cells, DU-145/DOX and DU-145/CDDP cells. *R. graveolens* and *M. parviflora* were authenticated and subjected to extraction by different solvents.

RESULTS AND DISCUSSION

Acquisition of drug resistance by DU145 prostate cancer cell line was confirmed. DU-145/CDDP and DU-145/DOX were 2,156 and 54,23-fold resistant to Cisplatin and Doxorubicin respectively, when compared to the DU1-45/WT cells. mRNA expression levels of ABCC1 and ABCB1 gene were successfully quantified by RT-qPCR, protein products of ABCC1 and ABCB1, mrp-1 and p-gp were below detectable levels when determined by western blot. Function of the ABC transporters in the drug resistant prostate cancer DU-145 cells was successfully determined by the Calcein AM assay, the results obtained were comparable to that of positive controls verapamil and cyclosporine A. *R. graveolens* DCM extract exhibited >50% growth inhibitory effect at 5µg/ml on the DU-145 wild type cell line. In addition to the anti-proliferative activity against the DU-145 wild type, *M. parviflora* DCM+MeOH and MeOH extracts exhibited activity against DU-145/DOX at a concentration of 5µg/ml and 10µg/ml respectfully, and the *M. parviflora* DCM+MeOH exhibited growth inhibitory effect at 10µg/ml for DU-145/CDDP.

CONCLUSION

This study unequivocally confirms that *Ruta graveolens* and *Malva parviflora* have the potential to provide anticancer lead compounds for further development. This is proven by the biological activity observed. Further studies to identify the bioactive compounds in the extracts that exhibited anti-proliferative activity and the mechanism of action are recommended. Moreover, combination studies are necessary to validate the potential drug resistance reversal activity of *M. parviflora*.

Publication



molecules



Review

Prostate Cancer Review: Genetics, Diagnosis, Treatment Options, and Alternative Approaches

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Simple Summary: Prostate cancer affects men of all racial and ethnic groups and leads to higher rates of mortality in those belonging to a lower socioeconomic status due to late detection of the

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CHAPTER 1

INTRODUCTION

1.1.BACKGROUND

Cancer has been ranked the second-leading cause of death in many countries, after coronary heart disease and stroke; with prostate cancer as one of the leading causes of cancer deaths in men, after lung cancer (Iqbal, 2017; Sung, 2021). According to global cancer statistics (GLOBOCAN), an estimated 19.3 million new cancer cases and 10 million cancer deaths were recorded, with an estimated almost 1.4 million new cases and 375,000 deaths recorded worldwide for prostate cancer (Ferlay, 2019; Sung, 2021). Cancer is a disease that has distinctive and acquired capabilities which enable cancerous cells to grow and multiply uncontrollably. The two most common cancer enabling traits include tumour promoting inflammation and mutation (Hanahan, 2000; Marco, 2013; Hanahan, 2022).

Cancer can occur anywhere in the body and there are 100 types of cancers that affect humans. A cancer that affects only males, which is cancer of the prostate, is the focus of this study.

A number of treatment options for cancer, such as surgery, radiation therapy and chemotherapy, are available. Selection of a treatment option is based on the nature of the tumour, the grade and stage, as well as the origin of the cancer (Termini, 2020). Chemotherapy is one of the most widely used treatments for cancer. Chemotherapy is characterized by the killing of cancerous cells through the use of a specific drug or anticancer agent. Despite its wide use, there are major drawbacks associated with chemotherapy, which hinder its effectiveness. These include the expression of intolerable side effects, a lack of specificity, and the development of drug resistance by the cancer cells, which ultimately leads to a decline in the quality of life of patients, treatment failures, and increasing mortality rates (Rizzo, 2021).

Drug resistance has been identified as one of the major causes of chemotherapeutic treatment failure in prostate cancer patients. Multidrug resistance is defined as the cross-resistance or insensitivity of cancer cells to the cytotoxic action of various anticancer drugs, which are structurally and functionally unrelated, and with different molecular targets (Gottesman, 1993; Ullah, 2008). Different processes have been found to be responsible for mdr in cancer cells.

One of the mechanisms for cancer resistance, which is explored in this study, is the overexpression of transport proteins involved in transporting chemo drugs in and out of the cells. Proteins involved in transport are found on membranes' lipid bilayer.

Strategies to overcome this mechanism of drug resistance in cancer cells, have been by either blocking or deactivating the transporters and individual targeting of the transporters, which can be achieved by the use of MDR modulators, chemosensitizers and inhibitors (tyrosine kinase inhibitors, verapamil, and cyclosporine A) (Perez-Thomas, 2006; Nabekura, 2010; Eid, 2015).

However, the use of MDR modulators/inhibitors/chemosensitizers alone, or in conjunction with other chemotherapeutic agents, has resulted in toxicity, adverse side effects, and reduced efficacy in cancer patients (Nabekura, 2010; Szakacs, 2006; Amdukar, 1999; Eckford, 2009). The current study analyzes the expression of the ABC transporter genes and their protein products involved in MDR in prostate cancer, and explore them as targets for combating chemo resistance.

While there is scientific merit in the proposed strategy, it is also necessary to continue with efforts to discover and develop new effective, affordable, safe and easily accessible chemotherapeutic agents to combat chemo resistance. Most cancer patients, if not all, rely on the use of herbal medicine for primary health care (Hassan, 2020). Various anticancer agents in use today were derived from plants. Not only are medicinal plants good sources of anticancer agents, they can also be used for the restoration of sensitivity in resistant cancer cells and can reverse drug resistance in tumour cells (Nabekura, 2010; Hassan, 2020).

1.2 Problem statement

Acquired multidrug resistance in prostate cancer is associated with the overexpression of the two ABC transporters. In conventional prostate cancer treatment, chemotherapy is still the preferred therapy for cancer treatment, but may result in undesirable side effects and toxicities. Another obstacle to successful chemotherapy in clinical settings is chemotherapeutic drug resistance (Shafiei-Irannejad et al., 2018). This phenomenon is known as 'multidrug resistance' (MDR) (Kumar et al., 2019). The most frequent mechanism in MDR is the efflux of drugs and overexpression by a group of membrane transporters known as ABC transporters. Three ABC transporters play a significant role in chemoresistance: ABCB1 (MDR1/P-glycoprotein); ABCC1 (MRP1/multidrug resistance associated protein); and ABCG2 (BCRP/breast cancer resistance protein) (Fletcher et al., 2016). In prostate cancer, the MRP-1 is expressed at early stages of the disease (Sullivan, 1998) and increases with disease progression, resulting in decreased sensitivity to cytotoxic agents (van Brussel, 2001). The expression levels of the transporters, and a patient's response to treatment, depend on the individual's genetic makeup.

The study utilizes in vitro models to answer the research question of whether medicinal plants have the ability to restore sensitivity of drug resistant cancer cells to chemo agents, so as to combat multidrug resistance and treatment failure, specifically targeting their effects on the expression of multidrug resistant P-glycoprotein and multidrug resistant protein 1.

1.3 Aim

Investigate the anticancer potential of *Ruta graveolens* and *Malva parviflora in vitro*, specifically targeting their effects on the expression of multidrug resistant P-glycoprotein and multidrug resistant protein 1.

1.4 Objectives

1. Expose the parental (chemo sensitive) prostate cancer DU-145 cell line to increasing concentrations of the chemotherapeutic agents, doxorubicin (DOX) and cisplatin (CDDP), to induce resistance and to confirm induction of resistance in cancer cells.
2. Screen the *Ruta graveolens* and *Malva parviflora plant* extracts for their cytotoxic activity against the wild type prostate cancer DU-145 cell line, and the resistant strains.
3. Assess gene expression levels of ABCB1 and ABCC1 genes in wild type (DU-145) and drug resistant cells (DU-145/DOX and DU-145/CDDP) with RT-qPCR, to investigate the involvement of ABC transporter proteins coded by these genes, in the acquired resistance.
4. Analyze protein expression in parental (DU-145) and drug resistant (DU-145/DOX and DU-145/CDDP) prostate cancer cells using the western blotting technique, after exposure to plant extracts.

1.5 Research questions

- (a) What is the phytochemical composition of *Ruta graveolens* and *Malva parviflora*?
- (b) What is the biological activity of the plant species' extracts against the sensitive DU-145 cell line?
- (c) What is the biological activity of the plants species' extracts against the drug resistant DU-145 cell line?
- (d) What is the role of ABC transporters, ABCB1 and ABCC1, in prostate cancer resistance?
- (e) What is the effect of the plants species' extracts on the expression level of ABCB1 and ABCC1, and are the plants extracts able to reverse the acquired resistance in the resistant strains of the DU145 prostate cancer cell line?

CHAPTER 2

LITERATURE REVIEW

SECTION A: PROSTATE CANCER AND MULTI DRUG RESISTANCE

2. CANCER

2.1 EPIDEMIOLOGY OF CANCER

Cancer is the second-leading cause of deaths after cardiovascular disease, globally (O'Connor, 2016; Sung, 2021). An estimated 18.1 million new cases and 9.6 million deaths were recorded in 2018, worldwide (Ferlay, 2019). GLOBOCAN recorded close to 19.3 million new cancer cases and approximately 10 million cancer deaths in 2020 with 70% of cancer deaths occurring in low and middle income countries (Sung, 2021). The South African National Cancer Registry data reported that one-in-nine women and one-in-eight men are at risk of developing cancer in their lifetimes, while the global cancer burden is anticipated to be 28.4 million cases in 2040 (Sung, 2021).

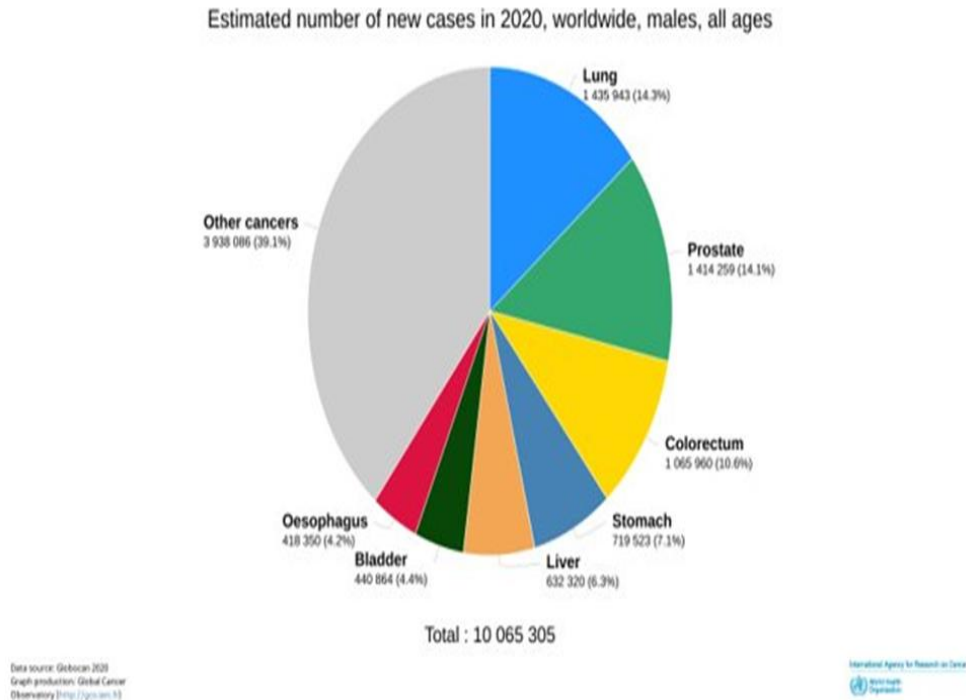


Figure 1: Globocan statistics showing the estimated number of cancer cases in men in the year 2020. (Globocan cancer statistics 2020, Sung, 2021)

2.2 EPIDEMIOLOGY OF PROSTATE CANCER

Amongst known cancers, prostate cancer is the second-leading cause of death in men globally, after lung disease, accounting for 7.3% of all diagnosed cancers (Bray, 2010; Iqbal, 2017; Ferlay, 2019; Sung, 2021). Prostate cancer is one of the commonly diagnosed cancers in men (Moodley, 2016). In sub-Saharan Africa, incidence rates of prostate cancer have increased annually, from 2% to 10%, in nine countries (including Zimbabwe, South Africa, Mozambique, Kenya and Uganda). On average, 4200 South African men are newly diagnosed with prostate cancer every day. The mortality rates related to cancer cases in South Africa has increased from 7.3% to 7.8% in 2011-2012 and 8.3% in 2013 (Moodley, 2016). New cancer cases are predicted to increase by 40% by 2030 (Moodley, 2016).

2.3 CANCER DESCRIPTION

Cancer is a complex disease that can be described as the abnormal and uncontrollable proliferation of normal cells, which leads to the formation of masses of cells known as tumours, and the accumulation of tumours in tissue, until finally the body's normal mechanisms are halted or become faulty (Salehi, 2019). Cancer is a constellation of over 200 different diseases which possess similar characteristics, but are very different from one another (Hassan, 2020). Cancer is not homogenous. There are different types of cancer cells in tumours, with each cancer type being different in different individuals due to observed differences in genetic makeup.

Cancer can be initiated through various mechanisms, such as avoidance or suppression of apoptosis; activation of oncogenes (*MYC*, *RAF*, *Bcl-2*, *RAS*); the deactivation or suppression of tumour suppressor genes (*p53*, *NF1*, *NF2*, *RB* and biological breaks); the development of mutations in DNA repair genes (*p21*, *p22*, *p27*, *p51*, *p53*) (Iqbal, 2017), and the activation of pro-onco genes.

Pro-oncogenes are genes that regulate the formation of tumours. These genes are involved in many cellular processes in the body, including cell division; growth; differentiation; and the formation of proteins that regulate DNA repair systems, apoptosis, and mitosis (Knudson, 2001; Croce, 2008; Sarkar, 2013). Genetic mutations and other genetic alterations occurring in proto-oncogenes result in a defective and malfunctioning gene, known as an oncogene, through a process referred to as activation (Torry, 1991; Hnisz, 2016).

There are two types of cancer/tumour cells, which are referred to as cancerous cells (malignant) and non-cancerous cells (benign). Benign cells do not spread to other body parts but may cause discomfort due to bleeding; while malignant cells have the potential to spread to nearby body parts, thereby destroying normal cells or disturbing normal cellular processes (Mbele, 2017). Malignant cells have the ability to spread to other tissues and organs away from where the tumour originated, through the bloodstream or

lymphatic system, in a process known as metastasis (Mbele, 2017). This process is one of the main reasons for death in most cancer patients.

Several factors contribute to the increased risk for cancer in humans. These include internal factors (mutations; inheritable genetic risks; hormonal disorders; and the body's immune system) and external factors (certain bacterial and viral infections such as hepatitis A and B; and human papillomavirus (HPV); race; age; obesity; alcohol consumption; tobacco use diet; the use of anabolic steroids; environmental pollutants a lack of physical activity; and exposure to radiation) (CANSa, 2016; Iqbal, 2017). These factors may lead to genetic mutations that may inhibit the induction of apoptosis and changes in other regulatory mechanisms (Mbele,2017).

2.4 THE PROSTATE: ANATOMY AND PHYSIOLOGY

The prostate gland is a small walnut-sized organ situated between the rectum and the base of the penis and rectum just below the bladder. It is an important part of the male reproductive system. It secretes semen, a fluid that protects and nourishes the sperm. The semen's alkalinity neutralizes the more acidic environment in the female's vagina, therefore, prolonging the lifespan of the sperm (Hoofman, 2014; Cansa, 2014; Rebello, 2021).

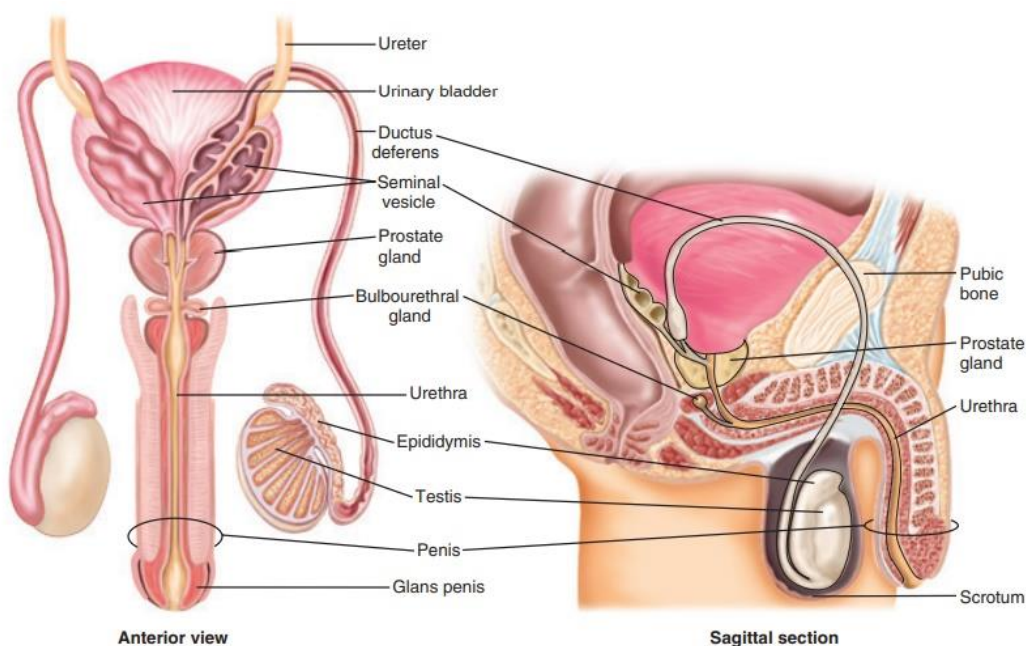


Figure 2: Anatomy and physiology of the male reproductive system (<https://www.pharmacy180.com/article/anatomy-of-the-male-reproductive-system-3707/>)

2.5 SYMPTOMS AND RISK FACTORS OF PROSTATE CANCER

Family history, heredity and age have been identified as the major risk factors associated with prostate cancer, with the incident rate increasing exponentially with age (Aslam, 2015; Plourde, 2018; Malik, 2020). Heredity and a family history of cancer play a key role in the development of prostate cancer, due to the presence of gene mutations which, in some cases, are inherited. It has been reported that men with a family history of prostate cancer have a higher incidence and mortality rate compared to men with no family history. Men with relatives (first degree) have a two-fold greater risk of developing prostate cancer (Das, 2019; Salehi, 2019). Inherited mutations which predispose cancer are present in the BRCA1 and BRCA2 genes, which are also related to the development of breast and ovarian cancers. Individuals with mutations present in the DNA repair system genes (BRCA1, BRCA2, PALB2, ATM and CHEK2) are at risk of developing prostate cancer and the presence of some of the genes have been associated with an aggressive disease (Das, 2019). Other factors include race; lifestyle; alcohol consumption; tobacco use; cadmium exposure; use of anabolic

steroids; infectious agents; hormones; occupational exposure to carcinogens; and environmental pollution. These cancer-causing factors vary from one individual to another (Bostwick, 2004; Patel, 2009; Plourde, 2018). Common symptoms of prostate cancer are dysuria stranguria, which is described as the slow, painful flow of urine; loss of bladder control; blood in the semen and urine; erectile dysfunction; painful ejaculation; and swollen legs or pelvis. When the cancer has metastasized, symptoms may include unexplained weight-loss; swelling or buildup of fluid in the feet and legs; pain in the thighs, hips, back or shoulders; a change in bowel movements; and fatigue. These symptoms differ from patient-to-patient (<https://www.cancer.org/cancer.html>).

2.6 DIAGNOSIS OF PROSTATE CANCER

The standard diagnostic techniques for the detection of prostate cancer include a digital rectal examination and prostate-specific antigen (PSA) level testing; imaging tests (MRI and CT scans); biopsies and grading and staging (Graham, 2008; Graham, 2014; Nguyen-Nielsen, 2016). Other tests are conducted to determine if metastasis has occurred (bone scan). Staging is normally conducted to describe the location of the cancer and any metastasis. It is usually done with DRE or other imaging techniques. Grading measures how quickly the cancer cells are growing (using the Gleason grading system) (Litwin, 2017; Schatten, 2018). Physical palpation of the prostate involves the assessment of the enlargement of the gland, its stiffness and texture. This method has a predictive value in the detection of prostate cancer in about 5-30% of men with a PSA level under 2ng/ml. A digital rectal examination (DRE) can be successfully performed by the insertion of a gloved, lubricated finger inside the rectum to feel for any irregularities, such as hard areas or bumps. If cancer is suspected, a DRE can detect if the cancer is one-sided or on both sides of the prostate; or if it has spread to nearby organs. After a DRE, more tests are suggested.

The prostate specific antigen (PSA), is a protein produced by both normal and cancerous cells in the prostate. PSA is normally found in the semen, and a small amount can also be detected in the blood. PSA tests are recommended for patients who

display no symptoms, and they are one of the tests conducted in men who present symptoms caused by prostate cancer, which is measured in ng/ml (Graham, 2008). There is no significant cutoff range for PSA, but most clinicians or physicians use a 4ng/ml cutoff. Men may have a PSA level below the normal cutoff and may not have prostate cancer; and men with a PSA level of 4ng/ml may also not have prostate cancer. The PSA level can be used together with other diagnostic tests, physical examination results and tumour grading, which is determined during the prostate biopsy, to help decide if more tests are required, such as CT scans or bone scans (Conran, 2016). Measuring the PSA level complements prostate cancer detection efforts and is a better predictor of prostate cancer than a DRE. Both tests can be abnormal without prostate cancer concerns (false positives), or they can be normal despite the presence of prostate cancer (false negative). Serum PSA level is the only parameter that can be elevated due to prostate cancer, and also to benign prostatic hyperplasia (BPH) and infection (Merriel, 2018). A prostate biopsy is conducted when the DRE and imaging results are suspicious, or if the PSA level is elevated without any explanation. Another reason for elevated PSA levels may be a non-cancerous (benign) enlargement of the prostate (prostatitis), which is inflammation of the prostate (Merriel, 2018). If the PSA test and DRE and other tests suggest the presence of cancer, then a prostate biopsy will be conducted.

A biopsy is a test where a small sample of the prostate is removed and viewed under a microscope. A core needle biopsy is the main method used to diagnose prostate cancer. There are a number of ways in which a prostate biopsy is conducted. It can be conducted using transrectal ultrasonography (TRUS), which looks for suspicious areas in the prostate with an abnormal DRE or PSA test result, which in some cases may miss the cancer (Merriel, 2018, Schatten, 2018). TRUS helps guide the needle, measures the size of the prostate gland, which determines the PSA density, and can also act as guide in certain treatments, such as internal radiation therapy and cryotherapy. Another useful prostate biopsy method is the MRI, which creates detailed images of soft tissues in the body using radio images and magnets. To increase the accuracy of the MRI, a probe called an endorectal coil is placed inside the rectum for the scan (Mendhiratta, 2016;

Schatten, 2018). Multiparametric testing is a newer technique used to better detect and define areas where the cancer is inside the prostate, as well as giving an idea of where the cancer may possibly grow. It may also help in determining whether the cancer has grown outside the prostate, to other parts of the body (Aslam, 2015; Merriel, 2018; Mendhiratta, 2018; Schatten, 2018).

Other tests, such as bone and CT scans determine if the cancer has spread to the bones or lymph glands (Schatten, 2018). A CT scan can also determine if recurring cancer is growing on other organs (American Cancer Society, 2018; Graham, 2014; Litwin, 2017; Schatten 2018). Genetic tests are also suggested and recommended to men with inherited genes which are linked to prostate cancer. This includes men with a family history of BRCA gene mutation, which normally results in breast cancer (Liu, 2017).

2.7 TREATMENT OPTIONS

There are several treatment options for prostate cancer patients. Treatment options depend on numerous factors, including the nature of the tumour; the stage of the cancer; whether it is localized or has metastasized; the Gleason score; the prostate-specific antigen (PSA) level; and patient preference (Schmitges, 2011, Chen, 2013).

Treatment options include active surveillance or watchful waiting, which monitors disease progression with the intention of reducing overtreatment. It is the preferred method of treatment for patients with less progressive disease – such as patients with a clinical stage of T1c or T2a; a Gleason score of six or less; and a PSA level less than 10ng/ml.

Radiation therapy and surgery are suggested treatments for patients with a more localized disease. However, they result in severe side-effects, including sexual dysfunction and urinary symptoms, which eventually affect the quality of life of prostate cancer patients.

Androgen deprivation therapy is used as a first line of treatment for patients with metastatic or advanced prostate cancer disease (Hwang, 2012; Chen, 2019; Famuyiwa,

2020). Androgen deprivation therapy (ADT) has been widely used by clinicians to exploit the role of hormones in the progression of prostate cancer. Androgens exert trophic effects on prostate cancer cells through the activation of androgen receptors (AR). These effects can be reduced by decreasing levels of androgens or by disrupting the ligand/receptor interaction. ADT can, therefore, be used to slow the progression of prostate cancer by reaching castrate-levels of serum testosterone. The clinical effects of androgen blockade through surgical castration were described by Huggins and Hodges in 1941.

Diethylstilbestrol (DES) was used for the treatment of advanced prostate cancer, but was later found to be associated with high cardiovascular toxicity and was replaced by luteinizing hormone-releasing hormone (LH- RH) agonists. LH-RH antagonists were first developed to prevent the testosterone surge phenomenon associated with LH-RH agonists, which causes hot flashes, loss of libido, and erectile dysfunction. However, despite the effectiveness of ADT, more than half of the reported cases showing that the response to the initial treatment cells becomes less responsive and progresses to castrate-resistant prostate cancer (CRPC) or hormone resistant prostate cancer (Hwang, 2012; Famuyiwa, 2020). In addition, androgen deprivation therapy is associated with toxicity and has unfavorable side effects, such as metabolic changes; decreased bone mineral density; hot flashes; sexual and cognitive dysfunction; and cardiac morbidity.

Chemotherapy is another treatment option offered to metastatic prostate cancer patients. Chemotherapy has been shown to improve patient survival, as opposed to androgen deprivation therapy, when used as a single treatment (Litwin, 2017). Approximately 10% to 20% of prostate cancer cases progress to castration-resistant prostate cancer (CRPC) within five years of diagnosis, and 84% of newly diagnosed castration-resistant prostate cancer (CRPC) cases have metastasized (Cancer.Net, 2021). Eventually, almost all prostate cancer cases progress to castration resistance. Chemotherapy is one of the widely-used treatment options for this type of cancer.

2.7.1 Chemotherapy in Prostate Cancer

Chemotherapy involves the use of chemotherapeutic agents, given over a period of time, with specific cycles to destroy the cancerous/tumour cells, while inducing apoptosis in cancer cells (Xiang, 2019). The first biggest drawback of chemotherapy is that it not only kills cancerous cells, but normal cells as well, which results in a range of adverse side-effects, including nausea, hair loss and fatigue (Sugerman, 2013, Hassan, 2019). The second drawback is that the cancer develops tolerance to the drug used, thereby developing resistance to the drug. The development of resistance to anti-cancer agents remains one of the main reasons for unsuccessful and failed chemotherapy (Wu, 2011; Jovelet, 2011, Cort, 2015). There are several standard chemotherapeutic agents used for prostate cancer. These include:

- a) Docetaxel, an antineoplastic agent belonging to a group of plant alkaloids, called taxanes. It is a microtubule stabilizer which inhibits the assembly of microtubules and cell division. It has been reported that docetaxel improves patient survival in metastatic prostate cancer patients (Petrylak, 2004; Tannock, 2004). However, patients receiving docetaxel present with a number of side-effects, including a low white blood cell count, which increases the risk of infection; swelling of the ankles or abdominal area; fatigue and weakness; nausea; diarrhoea; hair loss; mouth sores; vomiting, etc. (Thomas-Charles-2019).
- b) Cabazitaxel is a docetaxel derivative, and a second-generation drug with a similar mechanism of action to docetaxel (de Bono, 2010; Hurwitz, 2018).
- c) Estramustin: Before the approval of docetaxel in 2004 by the FDA (Food and Drug Administration), the approved drug for CRPC was estramustine, which only showed a 30% response rate when used as single treatment. Estramustine is an anti-microtubule agent and is still in use today for the treatment of prostate cancer (Thomas-Charles, 2019).
- d) Mitoxantrone, an anthracenedione, is structurally similar to anthracyclines (such as doxorubicin). The mechanism of action is to generate DNA strand breaks and to stabilize type II topoisomerase-DNA complexes (Nittis, 2009).

Generally, conventional chemotherapy starts with docetaxel and prednisone as a combination treatment. Current studies demonstrate that using a combinatorial therapy of docetaxel and hormonal therapy in newly diagnosed or castration-sensitive metastatic prostate cancer patients significantly helps them live longer, by halting disease growth and metastasis; low red blood cell counts causing anemia; fluid retention, and weight gain (Cancer.Net, 2021).

2.8 Drug resistance in cancer and prostate cancer

A major challenge to successful chemotherapy is the development of drug resistance by tumour cells to anti-cancer agents (Wu, 2011; Hassan, 2020). In cancer/tumour cells, resistance to anti-cancer agents is either acquired or intrinsic. Intrinsic resistance is defined as resistance that was present in the cells before drug treatment, while acquired resistance is observed after exposure to drug treatment (Bronger, 2005; Sanchez, 2009; Housman, 2014). Factors that contribute to the development of drug resistance in cancer cells include the development of mutations in the tumours; limited drug penetration into cells; altered pharmacokinetics; and tumour heterogeneity (Huang, 2012; Gottesman, 2002). Mechanisms that result in drug resistance include inhibition of cell death; drug target alteration; drug inactivation; damage to DNA repair systems; and drug efflux. Drug resistance can also be developed due to the increased cellular metabolism of drug detoxifying proteins, such as glutathione-S-transferase, or alterations in β -tubulin isotypes with different kinetics of microtubule formation, as summarized in Figure 3, below. These mechanisms have been shown to protect cancer cells against cytotoxic drugs (O'Neill, 2011).

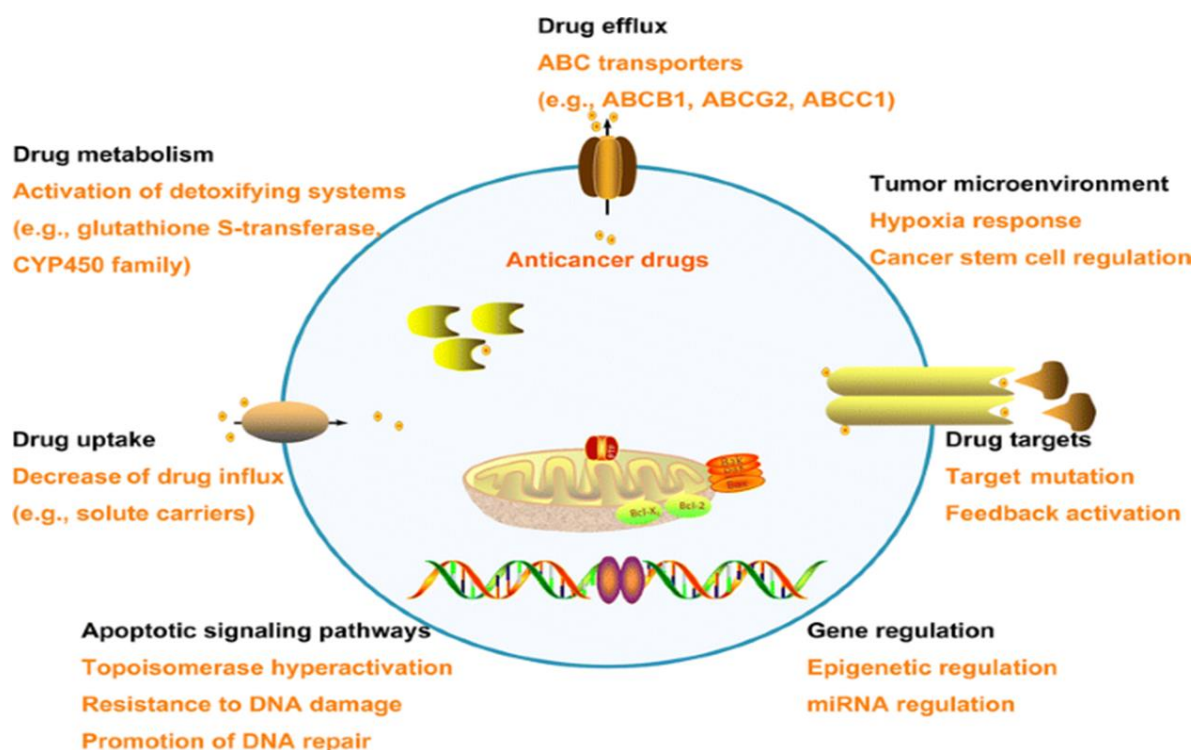


Figure 3: Mechanisms of drug resistance in prostate cancer. Cancer cells become resistant to drugs through different mechanisms, such as the inactivation of the drug; multi-drug resistance; cell death inhibition (apoptosis suppression); changes in the drug metabolism; epigenetic changes; changes in the drug targets; enhanced DNA-repair; and target gene amplification (Li, 2017; Mansoori, 2017).

In clinical settings half of the patients treated with docetaxel fail to respond to therapy and some develop clinical resistance (Floden, 2012). The mechanisms that lead to docetaxel resistance in prostate cancer are not well understood, but well known and studied mechanisms include antagonizing the drug stabilization effect on microtubules; limiting intracellular drug concentration; and overcoming the cytotoxic effects of taxanes through apoptosis escape or alternate pathways (Hwang, 2012).

One well-described mechanism that results in chemotherapeutic resistance is mediated through multidrug conveyers that act as efflux pumps to minimize the intracellular concentration of chemotherapeutic drugs inside cells or tumours. These transporter pumps are associated with an MDR phenotype which results in resistance to multiple functionally- and physically-unrelated agents. The overexpression of MDR proteins is an adaptive mechanism for cell survival (Sanchez, 2009; Hwang, 2012).

Multidrug resistance (MDR) is a phenomenon described as cross-resistance of cancer cells to structurally and functionally different anti-cancer agents with different molecular targets (Gottesman, 1993; Ambudkar, 1999; Choi, 2005; Ullah, 2008; Shafiei-Irannejad, 2017). Multidrug resistance results in low intracellular accumulation of drugs, due to the limited uptake of drugs, or the enhancement of the efflux of drugs. There are several different mechanisms associated with MDR, such as modulation of apoptotic pathways; activation of DNA repair; reduction in drug influx; alteration of target molecules; and enhanced drug efflux (ABC Transporters) (Gottesman 2001&2002; Yousefi, 2015; Shafiei-Irannejad, 2017). However, the enhanced drug efflux by ABC transporters is the most-frequently observed mechanism in MDR (Gottesman et al., 2001, Gottesman et al., 2002, Yousefi et al., 2016). Various members of the ABC transporter family have been identified and shown to be responsible for the efflux of xenobiotics, including cancer drugs. These include P-glycoprotein (P-gp); multidrug associated resistance proteins (MRP1; MRP2; MRP3; MRP7 and MRP 10); and the breast cancer resistance protein (BCRP) (Shafiei-Irannejad et al., 2018). Furthermore, according to literature, P-glycoprotein exhibits a high-affinity binding to docetaxel and can effectively pump docetaxel out of treated tumor cells hence decreasing the efficacy of stabilizing microtubules (*de Bono, 2010*).

2.8.1 Multidrug resistance (MDR)

(i) Mechanisms of multidrug resistance:

Various mechanisms have been identified as playing a role in the development of multidrug resistance in cancer cells and are classified as either cellular or non-cellular. Cellular drug resistance includes non-transport or transport-based factors. Non-transport-based mechanisms involve enzymes that do not alter the drug's effective intracellular concentration. Transport-based mechanisms involve the efflux of chemotherapeutic drugs from cancer cells by energy-dependent membrane transporters (Deeley, 1997; Gottesman, 2002; Ullah, 2006; Sharom, 2008; Shafiei-Irannejad, 2017; Robey, 2018).

(ii) Transport-based multidrug resistance

Transport mediated proteins are located on the lipid bilayer of membranes. These particular membrane transporters are major determinants of the pharmacodynamics and pharmacokinetics of several drugs (Krishna, 2000; Eid, 2015). ABC transporters use the energy derived from ATP to drive the movement of various biological products, such as lipids, carbohydrates, steroids and metabolites, across the plasma membrane, against their concentration gradients. Chemotherapeutics that are linked to the membrane transporters include natural amphipathic products; hydrophobic compounds such as taxanes (paclitaxel and docetaxel); vinca alkaloids (vincristine and vinblastine); anthracyclines (doxorubicin, daunorubicin and epirubicin); epipodophylotoxins (etoposide and teniposide); and anti-metabolites (methotrexate, fluorouracil and 6-mercaptopurine) (Sharom, 2008; Eid, 2015).

2.9 ATP BINDING CASSETTE (ABC) TRANSPORTERS

2.9.1 Overview of ABC transporters: structure and function

The ATP binding cassette, abbreviated as ABC, transporters are found in all realms of life. They form one of the largest families of transmembrane protein transporters. These membrane transporters are associated with a broad spectrum of physiological functions, including detoxification; defense against xenobiotics; oxidative stress; absorption; secretion processes; lipid metabolism; and antigen representation, to name a few (Borst, 2002; Fletcher, 2016; Mohammad, 2018; Robey, 2018). The membrane transporters function as active transporters, ion channels, and receptors, where they mediate the transport of a variety of substrates through membranes against their concentration gradients. This process utilizes the energy from ATP hydrolysis (Mohammad, 2018; Kumar, 2019). The basic structure of the transporters is characterized by the presence of two conserved nucleotide-binding domains (NBDs) and two trans-membrane domains (TMDs) (Deeley, 2006; Linton, 2007; Robey, 2018). The membrane-spanning domains form the key structural background of the ABC transporter function. The ABC transporter family is divided into seven subfamilies, from ABCA to ABCG, based on sequence homology, gene structure, and order of domains in the ATP-binding domains (Borst, 2002; Leornard, 2003; Conseil, 2005; Cascorbi, 2006; Linton, 2007; Sharom, 2008, Sharom, 2014; Fletcher, 2016). Mutations of the ABC transporter genes, such as P-gp, MRP1 and BCRP, are associated with acquired drug resistance, which remains a major drawback in successful chemotherapy (Gottesman, 2002; Leornard, 2003; Choi, 2005; Robey, 2018).

2.9.2 ABCB1/P-gp/MDR1 Transporter

P-glycoprotein (P-gp) was the first ABC transporter identified in drug-resistant cells. The protein is encoded by the ABCB1 gene located on chromosome 7q21 and is 170kDa in size. The membrane-bound glycoprotein, P-gp, consists of 28 exons and is made up of 1280 amino acids (Riordan, 1979; Choudhuri, 2006; Moura, 2008; Krishna, 2000; Bruhn, 2008). The protein is identified as a full transporter, meaning it is composed of two homologous transporters, with each having a nucleotide-binding domain (NBD) and

a trans-membrane-binding domain (TMD) (Ullah, 2008; Taiwan, 2009; Krishna, 2000; Domenichini, 2019).

P-glycoprotein is a broad spectrum multidrug efflux pump which binds to the hydrophobic substrate through its trans-membrane domain and ATP hydrolysis, causing conformational change in the transporter, leading to the release of the drug to the outer leaflet or the extracellular space (Gottesman, 2002).

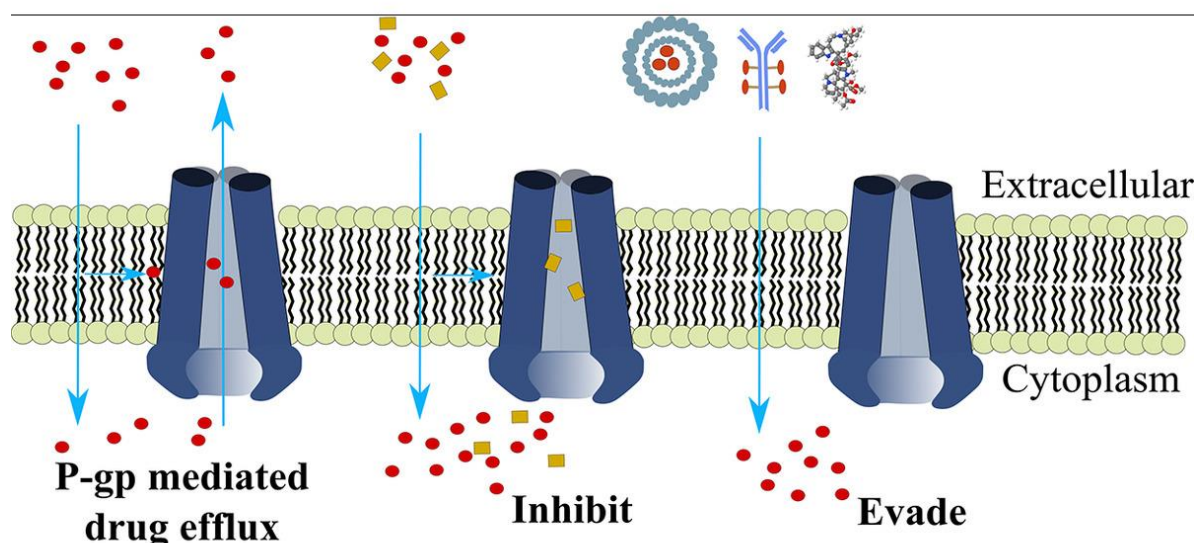


Figure 4: P-gp- mediated transport through the cellular membranes. (Waghray, 2018)

P-glycoprotein mediates the transport of, and resistance to, chemotherapeutic agents such as doxorubicin; vincristine; vinblastine; actinomycin D; daunorubicin; colchicines; paclitaxel; docetaxel; imatinib; and etoposide (Ween, 2015). The protein is expressed in the gastrointestinal tract; kidneys; liver; blood-brain barrier; testis-blood barrier; intestines; hepatocytes and pancreas (Hakaruma, 2002; Uhlen, 2015; Domenichini, 2019). Single nucleotide polymorphisms of ABCB1 have been reported to play a role in the proper functioning and progression survival after drug treatment (Sissung, 2008; Bruhn, 2014).

2.9.3 ABCC1/MRP1 transporter

MRP1 encoded by the ABCC1 gene is the second member of the ABC transporter family involved in multidrug resistance. The 190kDa protein is located on chromosome 16p13.1. The membrane-bound glycoprotein consists of 1531 amino acids (Deeley, 2006; Choudhuri, 2006; Cole, 2014). The protein was first discovered in the doxorubicin-resistant human small cell lung cancer (SCLC) cell line, H69AR (Mirski, 1987; Cole, 1992; Deeley, 2006; Kathawala, 2015). It has a similar structure to p-glycoprotein, with two half transporters (NBD and TMD), but unlike p-glycoprotein, MRP1 has an additional N-terminal segment, TMD0, that connects TMD1 to the linker region (Cole, 2014; Bakos, 1998).

The protein is expressed in many cell types and organs including the pancreas; kidney; testis; colon; breasts; lymph nodes; brain; and blood-brain barrier (Uhlen, 2015; Domenichini, 2019). It is mainly located on the basolateral membranes and normally pumps drugs into the body instead of excreting them into the bile, urine, and gut. Multidrug resistance-associated protein 1 (MRP1) overexpressing cells confer resistance to many chemotherapeutic agents, including vinca-alkaloids (vincristine, vinblastine); anthracyclines (daunorubicin, doxorubicin); epipodophyllotoxins (teniposide, etoposide); camptothecins (irinotecan, topotecan); saquinavir, and methotrexate (Sharom, 2014; Kathawala, 2015). Unlike P-glycoprotein, this protein does not confer resistance to taxanes (Kathawala, 2015; Saraswanthy, 2013; Sharom, 2014).

2.9.4 MDR Proteins in prostate cancer

Two of the commonly found transporters responsible for drug resistance prostate cancer are P-glycoprotein (MDR1) and the multidrug resistance-associated protein (MRP1).

Docetaxel resistance is due to the increased expression of P-glycoprotein (P-gp), the product of the MDR1/ABCB1 gene (Ueda, 1987). Variants of ABCB1 expression have been associated with docetaxel resistance in prostate cancer (Sissung, 2008). The

knockdown of ABCB1 increases the sensitivity of prostate cancer cells (Lombard, 2017). Blocking ABCB1 has shown potential in enhancing docetaxel and cabazitaxel efficacy (Lombard, 2017; Sekino, 2020). One study has reported that P-glycoprotein is weakly expressed in normal prostate, with its expression increasing in tumour cells. MRP1 expression is also increased with the advance of the disease (Sullivan, 1998). The protein has been previously detected in 35% of samples collected by total prostate resection in untreated prostate cancer patients (Sanchez, 2009). In a study conducted by Theyer et al., the expression of P-glycoprotein in the human prostate cancer cell lines DU145 and PC3 was detected, but at low levels (Theyer, 1993). In 1993, another research group reported that MDR1 mRNA was below detectable levels in tested DU145 and LNCaP prostate cancer cell lines (Mickisch, 1993). To test the role of MDR1 and MRP1 in prostate cancer, Zalchberg and colleagues developed resistant DU145 and PC-3 lines by growing parent cells in increased doxorubicin (DOX) concentrations. This latter confirmed that MRP1, and not MDR1, is the dominant mechanism of resistance in prostate cancer (Zalchberg, 2000). MRP1 and LRP are expressed in prostate cancer cell lines such as PC3, DU145 and LNCaP, and the expression of MDR1 was detected after vinblastine, etoposide and doxorubicin treatment (Sanchez, 2009).

2.10 MODULATORS OF ABC TRANSPORTERS

With the discovery of ABC transporters as mediators of drugs resistance in cancer, there has been ongoing research to develop therapies that could block or deactivate ABC transporters to increase the sensitivity of the cells to anti-cancer agents; and enhance drug accumulation, distribution and penetration, thus restoring drug sensitivity in cells (Kathawala, 2005). Three generations of agents were discovered and are currently used as chemo sensitizers to reverse multidrug resistance in cancer (Cort, 2015).

2.10.1 First generation agents

The modulators of ABC transporters that were first discovered were the ‘first generation modulators/inhibitors’, in the 1980s. These include agents such as cyclosporine A; verapamil; quinine; tamoxifen; calmodulin antagonists; anti-arrhythmic quinidine; and antimalarial quinine (Krishna, 2000; Leornard, 2003; Kathawala, 2015). Some of these agents failed in the treatment of cancer due to causing non-specific toxicity in patients in the doses required to inhibit P-glycoprotein (Abdallah, 2015; Kathawala 2015). Verapamil and other calcium channel blockers have been shown to successfully modulate MDR at high levels, but result in increased toxicity in normal cells and cardio-toxicity (Kathawala, 2015).

2.10.2 Second generation agents

Second generation modulators are structural analogs of first-generation modulators, such as verapamil and cyclosporine A. This resulted in agents such as valspador (CSA analog) and biricodar. These agents were reported more to be efficacious, less toxic, and with improved bioavailability, compared to first-generation modulators. However, they lacked significant efficacy in clinical trials (Nobili, 2006; Kathawala, 2015). They also inhibited hepatic and intestinal cytochrome P450 enzymes, decreasing the metabolism and clearance of substrate drugs, resulting in systemic toxicity (Leornard, 2003; Szakacs, 2006). Cyclosporine analogs have reversed MDR in several cancer cell lines.

2.10.3 Third generation agents

Third generation compounds were designed using quantitative structure-activity relationship approaches to obtain molecules with specific physio-chemical traits to circumvent drawbacks encountered with first- and second-generation modulators that function at nanomolar range (Cort, 2015). ABCB1/MDR1 was inhibited by zasquidar, elacridar and laniquidar, with less toxicity. These agents were reported as less toxic than inhibitors of ABCC and ABCG2. Other agents, such as biricodar and timicodar, have dual ABCB1 and Abcc1 blocking effects (Abdallah, 2015).

SECTION B: DRUG DISCOVERY FROM PLANTS

DRUG DISCOVERY AND NATURAL PRODUCTS

2.11 Introduction

Plants are known to be important sources of medicine for the prevention and treatment of diseases (Bulanas, 2005). According to The World Health Organization (WHO), between 65 and 80% of the world's population depends on plants as their source of medicine and primary healthcare (Iqbal, 2017). Plants have medicinal value and have the potential to provide new drug agents, as they are natural reservoirs of bioactive compounds, known as secondary metabolites (Desai, 2008; Kheled, 2013). In plants, secondary metabolites play have a role in metabolism and also have a protective role (Kurrupu, 2019). In humans, secondary metabolites, such as flavonoids, polyphenols, terpenes, alkaloids, and many others, exhibit a wide variety of activities such as anti-inflammatory; antidiabetic; antimalarial; antioxidant; antitumour; anticancer; anti-bacterial; anti-mutagenic; anti-viral; and anti-proliferative activity (Abifarín, 2019; Hassan, 2020). Approximately 50% of the FDA-approved (Food and Drug Administration) anticancer drugs are plant-derived, which further emphasizes the importance of medicinal plants in anticancer drug development (Wang, 2018). Drug discovery and development from natural plant resources is strongly encouraged because it has been estimated that there are approximately 300 000 species of plants that exist in the world, and only 15% have been evaluated for their pharmacological potential (De Luca, 2012; Pone, 2015).

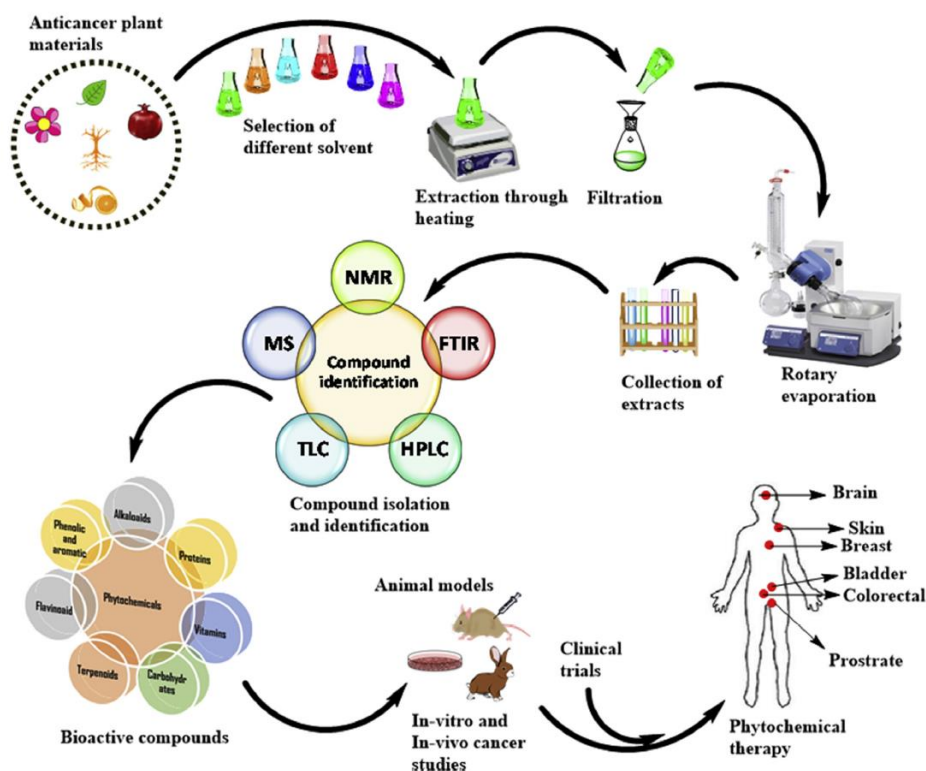


Figure 5: Diagram summarizing the drug discovery and development process (Iqbal, 2017).

2.12 Herbal medicinal plants and cancer

Drug discovery from plants has played a significant role in the treatment and prevention of cancer and prostate cancer, with the most effective anti-cancer agents found in plants (Iqbal, 2017). It has been estimated that approximately 60% of chemotherapeutic agents have been derived from natural products (Varamini, 2009; Pone, 2015; Iqbal, 2017; Termini, 2020). Plant-based medicines are the preferred the treatment for cancer, as they can also be used to alleviate symptoms and the pain caused by cancer, through the synergy of active substances that work on different cancers in several mechanisms and pathways (Hassan,2020).

A number of cancer patients, prostate cancer included, tend to seek alternative and complementary treatments which are safe, affordable and easily accessible, which includes the use of herbal medicinal plants. For example, Chinese patients who were

diagnosed with cancer, but at a much later stage and could not undergo chemotherapy, radiotherapy and surgery, opted for treatment with Chinese traditional medicine. The medicine was able to rein in tumour progression, improved the quality of life, and further extended the survival, of the patients (Wang, 2018). Many Chinese herbal extracts have been proved to inhibit the development of prostate cancer and will be used in the clinical treatment of prostate cancer in the future (Soignet, 2001; Wang, 2018).

Not only can herbal medicine be used for the treatment of cancer and to combat resistance, but it can be used to restore sensitivity in resistant cells for chemotherapeutic treatment, whether used as a single treatment agent or in combination therapy (Hassan, 2020; Sekhoacha, 2022). Many researchers have recently been investigating the active compounds found in plants for the development of MDR modulators and inhibitors with low toxicity and high efficacy to be used in conjunction with other anti-cancer agents to circumvent MDR (Cort, 2015). For example, an active alkaloid extracted from plants, tetradrine, enhanced the anticancer activity of doxorubicin against the resistant MCF-7/dox cells *in vivo*, through the modulation of a p-glycoprotein-mediated drug efflux. Other natural compounds, such as curcumin, have increased the chemotherapeutic activity of vincristine in SGC7901/VCR cells by the suppression of multidrug conveyers, namely P-gp, MRP1 and BCRP proteins (Aung, 2017; Hassan, 2020).

A number of herbal plants have been tested for their activity against prostate cancer cell lines. Mahdavi (2019) reported on the cytotoxic effects of *Juglone* and *Pterocarya fraxinifolia* on PC3 and DU145 cell lines. The study revealed that the plants' methanol extracts showed growth inhibitory effects by reducing cell proliferation and division; activating of the apoptotic process; and repairing damaged DNA and the disruption of the cells' cellular processes, therefore leading to cell destruction and death (Mahdavi, 2019).

2.13 *Ruta graveolens* L.

2.13.1 Plant description

Ruta graveolens L. is a plant species belonging to the Rutaceae family. This plant family is one of the largest plant families with almost 150 genera and 1500 species, largely distributed in the tropical and subtropical parts of the world. The Rutaceae plant family is well known, worldwide, for its citrus fruits, grapefruit, lemons and oranges (citrus family).

Rutaceae plants are used in traditional medicine (Parray, 2012). The most commonly used plant is the *Ruta graveolens* L., commonly known as 'rue' or 'herb of grace' in English and 'wynruit' or 'binnewortel' in Afrikaans. The plant is native to Europe but is also grown in South Africa as a garden plant (Loonat, 2014; Ghramh, 2020). It is a half-shrubby, perennial herbaceous herb growing up to 1 metre in height. The leaves are evergreen, and grayish in colour, with a distinct strong, unpleasant smell. The leaves are irregularly divided into hairless leaflets with translucent glands. The flowers of the plant are yellow in colour, with four petals each, and dry fruit that is hard and round. It has ovoid seeds which are round at the back and flat in the front (Parray, 2012; Loonet, 2014).



Figure 6: An aerial view of *Ruta graveolens* showing its blue-green leaves and arranged pinnate leaves with characteristic yellow flowers. (Asgarpanah, 2012)

2.13.2 Medicinal and traditional uses of *Ruta graveolens* L.

The plant has been used as a seasoning for food such as tea, coffee, butter, cheese and soups. Medicinal preparations, such as oils and infusions, are used as antispasmodics and emmenagogues (Fadlalla, 2012, Ghramh, 2020). Its documented therapeutic uses include in the treatment of anti-inflammatory conditions such as eczema; ulcers; arthritis; fibromyalgia; as an antidote to venoms; as an insect repellent; and as an abortifacient. Traditionally, it is used as a stimulant, diuretic and resolvent; for hypertension and cramps; in hysteria; and in skin and gastrointestinal conditions (Fadlalla, 2012; Parray, 2012). Leaf infusions are taken for fever, epilepsy and hysteria. The leaves are also used for tooth and earache, and to ease childbirth. The plant is also used, traditionally, as an antipyretic; anticancer; antifungal; antibacterial; anti-parasitic; antioxidant and antimalarial agent; as well as a purgative and immune stimulant; a hepatoprotective and hypotensive agent; and in the treatment of multiple sclerosis (Ghramh, 2020; Parray, 2012; Loonat, 2014).

2.14 *Malva parviflora* L.

2.14.1 Plant description

Malva parviflora L is an annual, perennial or biennial medicinal herb belonging to the genus *Malva* under the Malvaceae family. The herb is commonly known as the 'cheeseweed', or 'tika motse' in Sesotho. It grows on waste ground, in rocky soil and on roadsides (Abdel-Ghani, 2013). It grows in all parts of the world, including South Africa. The plant grows to about 40 inches. It has a strong taproot system. The leaves are dark green with between five and seven toothed, rounded lobes (Singh, 2017).



Figure 7: A front view of *Malva parviflora* "little mallow" showing its circular distinctive lobed, alternate leaves (<https://gobotany.nativeplanttrust.org/species/malva/parviflora/>)

2.14.2 Traditional and cultural uses of *Malva parviflora* L

Traditionally, the plant is used for the treatment of various diseases. The leaves are used to draw swollen and inflamed wounds when prepared as a hot poultice. The fresh or powdered leaves of the plant are prepared as a tea to treat bronchitis and dry throats, sores. It is commonly used to treat inflammation; abscesses; pimples; angina; intestinal infections; parasitism; kidney infections; vaginal infections; fever; spider bites; bronchitis and colds; as a hair rinse to remove dandruff; and for tuberculosis (Zayed, 2012; Singh, 2017). Pharmacological activity of the plant has been recorded and includes antibacterial; antifungal; anti-inflammatory; antioxidant; anti-irritant; anti-diabetic; wound healing; analgesic; anti-ulcer genic; neuroprotective and hepatoprotective activity (Singh, 2017). There is no, or very little, literature on anticancer activity recorded for the plant.

SECTION C: DNA TECHNOLOGIES FOR GENOME AMPLIFICATION & PHARMACOGENETICS

2.15 DNA sequencing

DNA sequencing is a process commonly applied in molecular biology to determine the order of nucleotide: adenine (As); thymine (Ts); cytosine (Cs) and guanine (Gs) in a piece of DNA. This identifies the individual genes; full chromosomes; large genetic regions; and whole genomes of an organism. DNA sequencing has advanced over the years and is applied in several fields, such as medicine; virology; biological systematics; biotechnology; medical diagnostics; forensics; evolutionary biology and metagenomics. In medicine and medical diagnostics, the comparison between a normal and a mutated DNA sequence can help in the diagnosis of many diseases, including the different types of cancers and genetic disorders, and it can guide patient treatment (personalized medicine) (Pekin, 2011; Chmielecki, 2014; Heather, 2015). The progress made in DNA sequencing is discussed below.

2.15.1 First-generation sequencing

DNA sequencing techniques were led by two groups in the 1970s. The first group was led by Frederick Sanger in 1975, and the second by Allan Maxam and Walter Gilbert in 1977. The Sanger sequencing, referred to as 'chain termination' sequencing was based on the selective incorporation of radio- or fluorescently-labeled chain-terminating dideoxynucleotides (ddNTP) in a DNA polymerase reaction (Sanger & Coulson, 1975; Sanger, 1977). The Maxam and Gilbert 'chemical sequencing' method is based on the chemical modification of DNA and the cleavage of the specific base on a DNA sequence (Maxam & Gilbert, 1977). The use of gel electrophoresis and radioactive labeling enabled the separation and visualization of the DNA fragments in both Sanger and Maxam and Gilbert sequencing techniques. This was later followed by advances in the automatization of the sequencing reaction and the advanced methods in base detection. After automation, advances in the technique followed, by including fluorescent labeling, automated laser-based fluorescence dye detection, and capillary electrophoresis (Smith, 1986). The Sanger sequencing technique made use of less harmful, less toxic chemicals and less radioactivity, compared to the Maxam and Gilbert method. Sanger's chain termination method was automated and was used to sequence the first human genome in 2001, in the Human Genome Project (Collins, 2003).

2.15.2 Second-generation sequencing

The combination of sanger sequencing and fluorescent dye detection led to second-generation sequencing, also referred to as next-generation sequencing. This sequencing technology allows sequencing reactions to take place simultaneously. Next-generation sequencing technology relies on the principle of sanger sequencing, but at a faster rate and lower cost. Several second-generation sequencing platforms have been introduced and are in use. One, in particular, is the Illumina sequencing platform, which is one of the most popular and commonly used. The technology depends on sequencing by synthesis and reversible chemistry (Bentley, 2008; Quail, 2012). Another popular sequencing platform, Ion PGM from Ion Torrent, relies on similar

sequencing chemistry as the Illumina. It uses semiconductor sequencing technology which detects changes in pH when a nucleotide is incorporated (Quail, 2012).

2.16 The Polymerase Chain Reaction (PCR)

PCR is a technique that was developed by Kary Mullins in the 1980s, which is employed to multiply DNA sequences. This inexpensive and fast technique uses short sequences of DNA primers to select a region on a DNA strand, gene or genome, that needs to be amplified. The sample temperature is frequently raised and lowered to assist the replication the DNA enzyme copy of the target DNA sequence. A PCR reaction can produce a billion copies, referred to as amplicons of the target region, which can be studied and analyzed in molecular and genetic studies. A PCR reaction can be completed in three steps: denaturation, annealing and extension. The sample is first heated to denature the double-strand DNA molecule. This is followed by the synthesis of two new single-strand DNA molecules by the Taq polymerase, which uses the old DNA strand as a template. The duplication of the old DNA strand molecule contains one old and one newly synthesized strand. The denaturing cycle and annealing (synthesis) of DNA can be repeated for 30-to-40 cycles, which will result in billions of copies (amplicons). The PCR process is automated and can be completed in hours. The programmed thermal cycler used in PCR alters the temperature, allowing denaturing and synthesis (annealing and extension). RT-qPCR (real time quantitative reverse transcription PCR) enables the detection and measurement of PCR products generated in PCR cycles. In this technique, a complementary DNA (cDNA) molecule is reverse transcribed from RNA using the reverse transcriptase enzyme. Both PCR and RT-qPCR have a number of applications. PCR is applied in research; molecular diagnostics; cancer diagnostics; the diagnosis of genetic disorders; DNA fingerprinting; evolution studies; and bacteria and virus detection (like HIV-AIDS). RT-qPCR has also been applied in the quantification of gene expression; research; variation analysis; DNA microarray result validation; and bacterial, viral and fungal load counting (Giasuddin, 1995; Peirson, 2007; Kadri, 2019) (<https://www.ncbi.nlm.nih.gov/probe/docs/techqpcr/>).

2.17 Pharmacogenetics

This study focuses on the expression levels of genes that code for proteins that mediate multidrug resistance, and ultimately influence treatment outcomes, which relates to the concept pharmacogenetics. Pharmacogenetics focuses on the effect of a person's genetic profile on their response to drugs (Kalow, 2006). Individuals respond differently to treatment, since multiple genes play a role in drug response, toxicity, and resistance (Mancinelli, 2000; Ozdemir, 2005). Pharmacogenetics combines the knowledge of pharmacology and genetics to tailor drug doses to an individual's genetic makeup, to find the most effective treatment possible for the individual.

Genes that mediate clinical multidrug resistance have been identified. They belong to a superfamily of membrane transporters known as the ATP-binding cassette transporters. The genetic polymorphisms identified in the ABC transporters determine the expression level and activity of the transporters and the response to specific drugs (Bruhn, 2014). Standard chemotherapy in all individual cancer patients produces varying treatment outcomes, from normal, to altered, a cautionary response, or no response at all (Warde, 2012). This emphasizes the need for treatment options to be based on a patient's genetic profile of the genes associated with drug resistance and treatment failure (Ma, 2011).

CHAPTER 3

METHODOLOGY

3.

3.1 Plant collection and preparation

The fresh plants, *Ruta graveolens* (Bethlehem; 28°12'39.5"S 28°19'25.8) and *Malva parviflora* (Bloemfontein, -29.118645,26.21195); were collected from Free State Province, of South Africa. The plant samples were properly cleaned with running water to remove dust and debris. The plant samples were authenticated by a botanist at the Geo Potts herbarium (BLFU), at the University of the Free State, and a voucher specimen was deposited at the herbarium for future reference.

The aerial parts of the *Ruta graveolens* and *Malva parviflora* (leaves, stems) were chopped into small pieces and dried at room temperature, away from direct sunlight, until they were completely dry. The dried plant material was then ground into fine powder using a plant grinder mill and was sieved to remove extraneous material. The plant material was prepared for the extraction process.

3.2 Plant extraction

The plant extraction procedure followed the method described by Harbone (1984) and Ahmed (2017), with minor modifications.

For each plant, approximately 25g of dried plant material was sequentially extracted in 250ml of each solvent, in order of increasing polarity (Hexane, DCM, DCM:Methanol and Methanol) (Merck-Sigma Aldrich, SA and United Scientific) and placed on a horizontal shaker for between 24 and 48 hours. After every 48h the samples were filtered with Whatman™ no1 filter paper and another solvent was added to the plant

material. The filtrates were concentrated using a rotary vacuum evaporator (R300, Buchi). The extraction yield of each solvent was determined in percentages (%), and extracts were stored in the refrigerator at 4°C until further use.

The extraction yield for each solvent was calculated using the equation as follows:

$$\text{Percentage yield} = \frac{\text{mass of crude extract}}{\text{mass of initial powdered sample}} \times 100$$

3.3 Phytochemical analysis

The phytochemical screening of the plant extracts was carried out to determine and identify the presence or absence of the chosen phytochemical constituents by following the protocols of Kotake (2000), Sofowora (1993) and Harbone (1998), with minor modifications.

3.3.1 Phenols and tannins

To test for phenols and tannins, 0.5g plant powder was mixed with 2ml of ferric chloride (FeCl_3), and the appearance of a blue-green or black colour indicated the presence of phenols and tannins.

3.3.2 Flavonoids

The plants were tested for flavonoids by mixing 0.5g of plant powder with 2ml of diluted sodium hydroxide (NaOH). A yellow precipitate indicated a positive flavonoid result.

3.3.3 Saponins (foam test)

An amount (2ml) sodium bicarbonate, and 0.5g of plant powder, were agitated for a few minutes. A positive outcome for saponins was indicated by foam formation.

3.3.4 Glycosides

0.5g of plant powder was dissolved in a solution containing 2ml acetic acid and 2 drops of 10% ferric chloride (FeCl_3). Then 1 ml of concentrated sulphuric acid was added to form a layer. The occurrence of an interphase with a violet or brown ring shows positive results for the presence of deoxysugars.

3.3.5 Phytosterols

To test for steroids, 0.5g of the powdered plant was dissolved in 2ml of chloroform. To the sides of the test tube, a volume of 3ml concentrated H_2SO_4 was properly added to form a layer. The occurrence of an interphase with a reddish brown colour indicated positive results for the presence of steroids.

3.3.6 Triterpenoids

To each 0.5g of plant powder, 1ml of ethyl acetate was added and then mixed into 2ml of chloroform. To the test tube, 3ml concentrated sulphuric acid (H_2SO_2) was properly added to form a layer. The occurrence of an interphase with a reddish brown colouration indicated positive results for the presence of terpenoids.

3.3.7 Alkaloids

To test for alkaloids, 0.2g of plant powder was weighed and 2ml of hydrochloric acid (HCl) was added, followed by the addition of 1ml each of Meyer's reagent and Drangenorff reagent. An orange precipitate was indicative of the positive presence of alkaloids.

3.3.8 Anthraquinones

Twelve milliliters of 10% HCl solution was added to 1g of powdered plant material. The mixture was boiled for five minutes, then filtered and left to cool before 10ml of chloroform was added to the filtrate. The chloroform layer was then placed in a clean test tube and 10 ml of 10% ammonia solution was added to this mixture. The mixture was shaken and the formation of a rose-pink colour in the top layer was indicative of anthraquinones.

3.3.9 Pentose

Forty milliliters of distilled water was added to 2g of powdered plant material. The mixture was then filtered. To the 2ml filtrate, 2ml of hydrochloric acid, containing a little phloroglucinol, was added. The mixture was then heated for five minutes. The formation of a red colour was indicative of the presence of pentose.

3.4 CELL CULTURE

3.4.1 EQUIPMENT

T-25cm³ and T-75cm³ flasks (Highveld Biologicals) were used to grow the cells. A 5% CO₂ incubator (ThermoFisher Scientific) was used in the culturing process of the cells at 37° C. A 96-well microplate was used to seed the cells during assays. A platefuge (Benchmark Scientific) was used to centrifuge the 96-well plates. A multiscanGO ascentplate reader (ThermoFisher Scientific) was used to obtain the photometric measurement performance of the 96-well plate. A centrifuge was used to spin and centrifuge the samples. A cell Countess™ automated cell counter (Life Technologies) was used in the determination of cell viability of the cells and for counting the cells.

3.4.2 Chemical reagents

These were: DMEM (Dulbecco's modified eagle medium, ThermoFisher Scientific); heat-inactivated FBS (foetal bovine serum); cisplatin; doxorubicin hydrochloride; trypsin; DMSO (dimethyl sulfoxide, ThermoFisher Scientific); PBS (Dulbecco's phosphate buffered saline, ThermoFisher scientific); MTT (3-(4,5-dimethylthiazol-2-YL)-2,5diphenyltetrazolium bromide); 1640-RMPI cell culture media; EDTA (ethylenediaminetetraacetic acid).

The DU-145 cell line was used for cell culture procedures. DU-145/DOX; DU-145/CDDP and DU-145 were used for cytotoxicity assay/anti-proliferative assay; chemo-sensitivity assay; calcein AM assay; Western blot and RT-qPCR.

3.4.3 Culturing of cells and induction of resistance

The sensitive prostate cancer DU-145 cell line was donated by a researcher in our department. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (HIFBS), and grown on monolayer cultures at 37°C in an atmosphere of 5% CO₂ and 95% O₂.

All experiments were carried out in a CO₂ air-jacket incubator and horizontal laminar flow cabinet. Cell culture procedures were followed.

The resistant variants DU-145^{DOX} and DU-145^{CDDP} were initiated by growing primary DU-145 cells with increasing concentrations of doxorubicin (from 0.001 to 10µM), and cisplatin (from 0.001 to 10µl/ml) respectively. Cisplatin (CDDP) and doxorubicin (DOX) were added twice a week after reseeding.

The cells were allowed to attach for 24h, after which the flask was removed from the incubator and attachment confirmed by microscopy. The flask was incubated under ideal tissue culture conditions and the growth medium was changed periodically until the cells reached approximately 80-90% confluency.

After reaching confluency, the media was aspirated and cells were washed with PBS. PBS was aspirated and replaced with 0.25% trypsin-EDTA and incubated at 37°C. After incubation, cells were placed in a laminar flow, followed by the addition of a medium to deactivate the trypsin. The detached cells were transferred to a conical tube and centrifuged to separate from the medium-trypsin solution. After centrifugation, the cell pellet was re-suspended in 5ml of complete medium. The cells were mixed to ensure a homogeneous cell suspension, for use in subsequent experiments. The cells were grown for over six months until they developed the resistant phenotype.

3.4.4 Cell harvesting and counting

The cells were counted using a cell counter after culturing (3.3.12). To the suspension of cells (20µl), and an equal amount of trypan blue was added; and 20µl of the coloured cell suspension was placed in the counting chamber. Non-viable cells absorbed the blue dye and viable cells remained uncolored. The viable density was adjusted to cell/100 µM per well with a total plate density of 1×10^5 . Thereafter, 100µM of the cell suspension, together with 100 micromolar of the growth medium, was added to the wells in the plates, except for specific wells which contained only medium and served as blanks. The plates were incubated and allowed to adhere for 24 hours.

Following incubation, the cells were exposed to different concentrations of the plant extracts.

Analysis of *R. graveolens* and *M. parviflora* extracts

3.4.5 Preparation of stock solution

Stock solutions of 10 mg/ml (10mg of plant extract was added in 1ml of DMSO) of *R. graveolens* and *M. parviflora* crude extracts were prepared. Of the DMSO, 50 μ l was used for the reconstitution of each crude extract which was prepared a day in advance. Aliquots of each extract were stored for initial tests and retests, when necessary.

3.4.6 Preparation of working solution

Working solutions were prepared in concentrations of 0.1 μ g/ml; 1 μ g/ml; 5 μ g/ml; 10 μ g/ml; 50 μ g/ml and 100 μ g/ml. by a serial full-dilution of the stock solution using complete growth medium. All working solutions had > 1% DMSO₄, which has been established to be safe on cells.

3.4.7 Addition of crude extracts

After the 24h incubation period, the DU145 cell line was exposed to a 100 μ l/well of the test material (working solution) with concentrations of 0.1 μ g/ml; 1 μ g/ml; 5 μ g/ml; 10 μ g/ml; 50 μ g/ml and 100 μ g/ml. One column contained only complete medium and served as a blank, while the second column contained only cells that were not exposed to any test sample, and this served as a negative control.

The plate was incubated at 37°C for 48h. Each experiment was done in triplicate and was analyzed.

3.4.8 Anti-proliferation/cytotoxicity assay

(i) Reagent preparation

An amount (5mg/ml) of MTT solution was prepared by dissolving 25mg of MTT in 5ml DPBS. The solution was sterilized by filtration through a 0.2 micromolar filter into a sterile, light-protected container.

(ii) MTT Assay

Following a 48 hour incubation, 25 µl of the MTT solution was added to each well and the plate was left to incubate in a CO₂ incubator for four hours at 37°C. Following incubation, the formazan salts appeared as dark crystals at the bottom of each well and the supernatant was gently aspirated from each well. Thereafter, 100 µl of DMSO was added to each well to solubilize the formazan crystals. The absorbance was measured using a microplate reader at a wavelength of 540/570nm.

The absorbance reading correlated with cell viability. To calculate cell viability, the absorbance readings of the blanks were subtracted from readings from test samples, divided by those of the control and multiplied by 100 to give the exact viability percentage:

Sample – Blank

Test samples/control sample x 100 = % cell viability

(iii) Growth inhibition percentage

The half-maximal inhibitory concentration (IC₅₀) values were extrapolated from a dose-response graph to indicate the concentration of the test samples required to inhibit 50% of the cancer cell growth, relative to the control.

3.4.9 Calcein Am retention assay

Drug resistance inhibition was evaluated using the resistant cells (DU-145/DOX and DU-145/CDDP) and the multidrug resistant (MDR) assay (calcein AM) kit. The MDR assay provides a convenient tool when studying the modulation of cellular MDR machinery. The assay employs calcein AM, a cell-permeable, non-fluorescent dye which is cleaved intracellularly to yield the impermeant fluorescent molecule, calcein. The calcein is rapidly excluded from the cells expressing MDR proteins, P-gp or MRP,

and so functions as a probe for the detection of chemical compounds inhibiting MDR proteins. Propidium iodide was used as a stain to mark dead or damaged cells.

Reagent preparation

(i) Cell based buffer

The cell-based buffer was dissolved in 10ml of distilled water

(ii) Calcein AM staining solution (2X)

The staining solution was prepared by adding 2 μ l of cell-based assay calcein AM to 10ml of culture medium containing the wild type and resistant cells which had been treated with *R. graveolens* and *M. parviflora* extracts. The solution was properly mixed away from direct sunlight. Verapamil and cyclosporine A were served as positive controls.

(iii) Analysis of calcein retention

Cells were plated and treated with test samples, as discussed in Section 3.3.17. The culture medium was aspirated from the cells. This was followed by treatment with 100 μ l of culture medium containing test compounds and control drugs (cyclosporine and verapamil) in separate wells and incubation in a CO₂ incubator at 37°C for 30 minutes. Verapamil was diluted in the culture medium in a 1:2000 ratio, and cyclosporine in a 1:1000 ratio. After treatment, 100 μ l of the prepared calcein AM solution (2X) was added to each of the sample wells and incubated for an additional 30 minutes in a CO₂ incubator at 37°C. Following incubation, the plate was centrifuged for five minutes at 400 x g at room temperature. The supernatant from the wells was aspirated and a 200 μ l of ice-cold medium was carefully added to each well, without disturbing the cell layer. The plate was centrifuged for five minutes and the supernatant was aspirated. Thereafter, 200 μ l of ice-cold medium was again added to each well. The samples were immediately analyzed with a fluorescent plate reader. Cells taking up the calcein AM

display strong fluorescence intensity with excitation and emission wavelengths of 485nm and 535nm, respectively.

For the analysis of the percentage calcein retention in parental cells was calculated using the following equation:

$$\% \text{ calcein retention}_{\text{parental cells}} = \left\{ \frac{(\text{calcein fluorescence of treated parental cells})}{(\text{calcein fluorescence of untreated parental cells})} \right\} \times 100$$

And

$$\% \text{ calcein retention}_{\text{MDR cells}} = \left\{ \frac{(\text{calcein fluorescence of treated MDR cells})}{(\text{calcein fluorescence of untreated parental cells})} \right\} \times 100$$

3.4.10 Chemo sensitivity assay

A chemo sensitivity assay is an assay that is used in the laboratory to determine the individualized sensitivity of cells to chemotherapeutics. It can also be used in determining the best treatment for tumours. The DU145 sensitive prostate cancer cell line, resistant DU-145/DOX, and DU-145/CDDP, cells were grown for 24hrs until they reached 80-100% confluency. After reaching confluency, the cells were seeded in a 96-well plate as follows: DU-145/DOX (1×10^4 cells per well), DU-145/CDDP (2×10^4 per well) and DU145 (5×10^4 per well). The cells were grown in normal media and allowed to attach for 24hrs. After 24hrs, the culture media was aspirated, followed by the addition of different drug concentrations (doxorubicin and cisplatin) in microliters (μl) (0.01; 0.1; 1; 10; 100) for 24hrs. After 24 hours, 20 μl of MTT solution was added to each well and incubated for two hours in a 37°C, CO₂ incubator. After the two-hour incubation period, the MTT solution was aspirated. The plate was shaken for a few minutes, followed by the aspiration of the supernatant from each well. Then 20 μl of DMSO was added to each well, solubilizing the formed formazan crystals, and resulting in a purple solution which was incubated for at least 10 minutes. The absorbance of each plate was measured and read at 570nm wavelength using a microplate reader.

In order to determine the sensitivity of the cells against doxorubicin and cisplatin, the fold of resistance was calculated as follows:

$$\text{fold of resistance(Dox)} = \text{IC50 of resistant cell line} / \text{IC50 of parental cell line}$$

The calculation was adopted from McDermott (2014).

3.4.11 Western blotting

(i) Protein extraction

The total protein extracted from the DU145 is necessary for the analysis of expression levels of the MDR proteins, P-gp and MRP1, which are proteins present in drug-resistant tumour cells. Proteins were extracted by using the CellLytic™ M mammalian cell lysis/extraction reagent, following the manufacturer's instructions. The cell lysis reagent enables efficient and rapid cell lysis and solubilization of proteins for both suspension and adherent cells.

Cultured DU145, DU-145/DOX and DU-145/CDDP cells were removed from the incubator. The growth media from the cells to be assayed was removed by careful aspiration. Then 2ml of DPBS was added to the cells to remove the remaining media carefully, without dislodging any cells. After washing the cells, the DPBS was removed. This was followed by the addition of 2ml cell lytic reagent mixture. To the cell lytic reagent, 20µl of protease inhibitor cocktail was added. The cell lytic and protease cocktail inhibitor mixture (T-75cm³ = 2ml, T-25cm³ = 1ml) was added to the cells previously washed with DPBS. The cells were incubated for 15 minutes. After incubation, the lysed cells were removed from the flask and added to centrifuge tubes. The cells were centrifuged for 15 minutes at 12 000 - 20 000 x g to pellet the cellular debris. The protein containing supernatant was removed from the centrifuge tube to a chilled test tube and stored in a freezer at -80°C for further use.

(ii) Protein quantification

Protein samples were quantified using the BCA assay, following the manufacturer's guidelines, on a Glomax multimode plate reader. Protein concentrations of each treatment group, and protein samples, were standardized to 1 mg/ml.

(iii) Protein preparation for SDS-PAGE

The standardized protein samples were added to an eppendorf tube on ice. Laemmli's sample buffer was added to the standardized protein samples at a 1:1 ratio. Samples were heated at 100°C for five minutes on a heat block. Protein samples were then used immediately.

(a) SDS-PAGE

Protein samples were used at room temperature and 10%SDS-PAGE gels were assembled into the electrode assembly with short plates facing inwards. The gel cassette was placed in the SDS-PAGE tank. The inner chamber was filled with cold 1x SDS-PAGE running buffer. Combs were carefully removed from the gel. The molecular marker and the protein samples were vortexed to ensure proper mixing. Using protein loading tips and a loading guide, a molecular marker (10µl) and protein samples (28µl) were added to the respective wells in the gel. Once all samples had been added to the wells, the inner chamber was topped up with cold 1x SDS-PAGE running buffer. The outer chamber was filled with an appropriate amount of cold 1x SDS-PAGE running buffer. The SDS-PAGE tank was placed in a dish surrounded with ice. The lid was placed on the tank and connected to the powerpac. The SDS-PAGE was run at 150V for 1 hour and 30 minutes. After the run was complete, the gel was removed, followed by the addition of a blot of running buffer to the chemidoc to activate the separated proteins.

(b) Transfer step using the Trans blot turbo system

The transfer block cassette was assembled using one fiber pad, a nitrocellulose membrane and SDS-Page gel. Transfer buffer was added on top of the fiber pad and any excess buffer was removed. The transfer block was sealed and placed in the Trans blot (automatically set for three minutes). Following the transfer, the fiber pad and the gel were removed. The nitrocellulose membrane was placed in a plastic container using forceps and 5ml Everyblot blocking buffer was added to cover the membrane. This was placed on a shaker for one hour to block the membrane. After one hour, the Everyblot blocking buffer was discarded. The membrane was washed three times, for 15 minutes each time, with TBST buffer. The primary antibody (1° 1:1000 dilution) was added to the membrane after the wash steps. The membrane was incubated at 4°C overnight. Following incubation, the membrane was placed on a shaker to equalize to room temperature for one hour. After the hour, the primary antibody was removed and placed in sterilin tubes and stored at 4°C. The membrane was rinsed four times with TTBS as a buffer, for 15 minutes each time. The TTBS buffer was discarded after each wash. After the fourth wash, the secondary antibody (1:5000) was added to the membrane, and placed on a shaker for one hour at room temperature. After incubation the secondary antibody was removed, placed in sterilin tubes and stored at 4°C. The membrane was rinsed three times with TTBS wash buffer for 15 minutes each time, and the buffer was discarded after each wash. After rinsing the membrane with TTBS wash, the membrane was rinsed twice with 5ml dH₂O. The membrane was placed on a transparency, and in an eppendorf tube, 200µl of each Bio-Rad ECL viewing substrate was added. Reagents were mixed and added to cover the membrane. The membrane was placed in the chemidoc and viewed following the chemiluminescence protocol.

(c) Membrane blotting buffer preparation

A 5% BSA was made up in 50ml TTBS buffer which prevents non-specific binding i.e. 2.5g BSA was dissolved in 50ml TTBS buffer. All prepared buffers were placed in the refrigerator to ensure they remained cold until used.

(iv) Re-probe membranes

The TTBS wash buffer was added to the membrane to rinse it. Thereafter, 5ml of stripping buffer was added to the membrane to remove the bound antibody, and was placed in an incubator at 37°C for 30 minutes. Following incubation, the stripping buffer was removed. The membrane was rinsed with TTBS wash buffer for 15 minutes, and the buffer was discarded after the wash. Then 5ml of 5% BSA in TTBS buffer solution was added to cover the nitrocellulose membrane, and placed on a shaker for 30 minutes to block the membrane. After 30 minutes, the TTBS buffer solution was removed from the membrane. The next primary antibody (1:1000) was added and steps from the primary antibody incubation were repeated until viewing of the membrane.

(v) Normalization with Beta-actin/GAPDH

Re-probing membrane (v) steps were followed. The membrane was re-probed with GAPDH (1:5000) and incubated for 30 minutes on a shaker at room temperature. The membrane was rinsed twice with TTBS wash buffer for 15 minutes each time, and the buffer was discarded after each wash. The membrane was viewed for 30 seconds (optimal time) to obtain an optimized image for beta-actin/GAPDH. TTBS wash buffer was added to the membrane, with excess buffered removed. It was cling-wrapped and stored at 4°C.

3.4.12 Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

(i) RNA isolation

Total RNA from frozen culture samples was isolated using the RNeasy Mini Kit (Qiagen) in a QIACube (Qiagen) according to manufacturer's instructions, including DNase I digestion. The RNA concentration was quantified using the Quantifluor RNA System and the GloMax Explorer (Promega). Purity was assessed using the NanoDrop 800 spectrophotometer (Thermo Fisher) and integrity was determined using the TapeStation 4200 RNA SceanTape and reagents (Agilent).

(ii) cDNA Synthesis

cDNA was synthesized from total RNA with oligo(dT)18 and random hexamer primers using the Maxima H Minus cDNA Synthesis MasterMix with dsDNase Kit (Thermo Scientific), according to the manufacturer's protocol (Pub. No. MAN0016393, Rev.B.0). The total RNA samples used were: 1 µg of a commercial XpressRef Universal Total RNA (QIAGEN), used for the validation of the primers (sections 4.3); aliquots of 1170, 583, 291, 145, 73 and 36 ng of a total RNA pool comprised of DU-145 cells from each biological group, used to determine the dynamic range of reverse transcription (Section 4.4); and 500 ng total RNA from each sample under study, used for the qPCR analysis to quantify gene expression of each gene in the different samples (Section 4.5). NRT negative control reactions were performed alongside to assess for genomic DNA contamination of the RNA sample. An NTC reaction was also included to assess for reagent contamination.

(iii) Primer Design for qPCR

Primers for the target genes, ABCB1 and ABCC1, were designed using the NCBI primer blast software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), using the parameters shown in Table 1, to result in 80 - 200 bp amplicons. The primers for the reference genes were the same as those used for the previous project (1169PCR).

A set of primer pairs were designed for the target genes, ABCB1 and ABCC1, using the primer design parameters shown in Table 1. The primers for the reference genes were selected from the literature.

Table 1: Primer nucleotide sequences and product lengths for the target genes (ABCB1 and ABCC1) and reference genes (GAPDH, HPRT and HSPCB) amplification by qPCR.

Gene Symbol	Primer Name	Primer Sequence (5' ----- 3')	Amplicon Size	Source
ABCB1	3-abcb1Fp1	GGA CTG TAA CTG ACT GCC TTG C	124 bp	This study
	3-abcb1Rp1	GGC AGT TTG GAC AAG ATG ACT CC		
ABCC1	3-abcc1Fp1	AGC CCA AAG TGG AAT CCG GAA G	80b p	This study
	3-abcc1Rp1	AGT CGG CGG CGT AAT TCT TAG C		
GAPDH	3-GAPDHFp	AGT CCC TGC CAC ACT CAG	123 bp	Nolan et al. 2006
	3-GAPDHRp	TAC TTT ATT GAT GGT ACA TGA CAA GG		
HPRT1	HPRT1F	GAC CAG TCA ACA GGG GAC AT	132 bp	Liu et al. 2015
	HPRT1R	CCT GAC CAA GGA AAG CAA AG		
HSPCB	HSPCBF	TCT GGG TAT CGG AAA GCA AGC C	80 bp	Jacob et al. 2013
	HSPCBR	GTG CAC TTC CTC AGG CAT CTT G		

(iv) Primer Preparation

The qPCR primers were sourced from IDT and were re-suspended in TE buffer (10mM Tris, pH 8.0, 1mM EDTA) to a 100µM stock concentration, as per the manufacturer instructions.

(v) Optimization of qPCR conditions

(a) Optimization of annealing temperature

A gradient PCR (55, 57, 59, 61, 63 and 65°C) was used to test the optimal annealing temperature for each primer pair. Each qPCR reaction consisted of the XpressRef Universal cDNA (1:20 dilution), forward and reverse primers at 500nM, and 1X SYBR Green Mastermix (QIAGEN) in a total reaction volume of 10µL. All the qPCR reactions had three technical replicates. PCR cycling was carried out on a QuantStudio 5 instrument (Applied Biosystems), using the following cycling parameters: 50°C for 2 mins; initial denaturation at 95°C for 2 mins; followed by 40 cycles of 95°C for 15 secs; 55-65°C for 15 secs, and 72°C for 1 min. A dissociation step was included at the end of the PCR run. The mean C_q values were calculated, and the optimal T_a was selected, based on the following criteria: (i) absence of primer dimers; (ii) lowest C_q value – i.e., the T_a resulting in the most sensitive and efficient reaction; and (iii) absence of signal in both the NTC and NRT controls (Nolan et al., 2006).

3.4.13 Optimization of primer concentration

Three different primer concentrations of 300 nM, 500 nM, and 800 nM, of both forward and reverse primers for each gene (ABCB1 and ABCC1), were tested against each other in a 10 µl total reaction volume consisting of the XpressRef Universal cDNA (1:20 dilution); forward and reverse primers; and 1X SYBR Green Mastermix (QIAGEN). All the qPCR reactions had three technical replicates. PCR cycling was carried out on a QuantStudio 12K flex instrument (Applied Biosystems) using the following cycling parameters: 50°C for 2 mins; initial denaturation at 95°C for 2 mins; followed by 40 cycles of 95°C for 15 secs; and 57°C for 1 min. A dissociation step was included at the end of the PCR run.

3.4.14 qPCR reaction and efficiency

PCR efficiency was determined using standard curves generated with two-fold dilutions of the universal cDNA template for each target gene. Each assay was run in triplicate, with each sample consisting of 1 μ L cDNA; both forward and reverse primers at the optimized final concentrations; and 5 μ L 2X SYBR Green Mastermix (QIAGEN) in a total reaction volume of 10 μ L. An NTC was included for each assay as a negative control. Reactions were performed on the QuantStudio 12K Flex PCR System (Applied Biosystems) using the optimized cycling parameters (Section 5.5). A dissociation step was included at the end of the PCR run. Standard curves were generated by plotting the CT values against the log of each serial dilution.

3.4.15 qPCR assays

The reverse transcription reactions for all samples were diluted two-fold. qPCR was performed in a total reaction volume of 10 μ L, consisting of 2 μ L diluted cDNA as template; 5 μ L of 2x SYBR Green I Mastermix (QIAGEN); and both the forward and reverse primers in a final concentration of 800 nM for both ABCB1 and ABCC1, and 500 nM for GAPDH, HPRT, HSCP. NTC reactions were included in all assays as negative controls. Each reaction was run in triplicate. Reactions were performed on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) using the following cycling parameters: 50°C for 2 mins; initial denaturation at 95°C for 2 mins; followed by 40 cycles of 95°C for 15 secs; and 57°C for 1 min. A melt-curve analysis was performed on all the reactions at the end of the PCR run using default parameters. Amplification data were analyzed with Life Technologies QuantStudio 12K Flex Software v1.2.4, applying user-defined thresholds to obtain C_q-values. Outliers in the technical replicate reactions; reactions that showed no amplification; and reactions that showed multiple T_m peaks during melt curve analysis, were removed from the analysis. Data was finally exported into Excel spreadsheets for further analysis.

3.4.16 Data Analysis

Data were subdivided into a control group (Du-145WT Control) vs treatment groups (Dox-R and CDDP-R), as: Du-145WT Control vs Du-145Dox-R vs Du-145Dox-R treated with *Malva* DCM+MeOH (10 µg/ml) vs Du-145Dox-R treated with *Malva* MeOH (10µg/ml) and Du-145WT Control vs Du-145CDDP-R vs Du-145CDDP-R treated with *Malva* DCM+MeOH 10µg/ml vs Du-145CDDP-R treated with *Malva* MeOH 10µg/ml. The stability of the reference genes was then evaluated separately for the Dox-R and CDDP-R experiments, using the web-based tool, RefFinder(<https://blooge.cn/RefFinder/>), which runs four well-established algorithms (GeNorm, BestKeeper, NormFinder and comparative delta-CT) to simultaneously compare and rank all candidate reference genes (from most-to-least stable). The data for ABCB1 and ABCC1 expression were normalized by a combination of two reference genes (GAPDH and HPRT for the Dox-R experiments, and GAPDH and HSCP for the CDDP-R experiments). Fold induction of gene expression was calculated relative to the Du-145WT control, using the Pfaffl Method (Pfaffl, 2001) and was analysed by using one-way analysis of variance (ANOVA), with repeated measurements using Tukey's pairwise comparison test, with the XRealStats add-in for Excel. Data are presented as the mean $\pm$ S.D. Statistical significance among groups was established by one-way ANOVA using Tukey's multiple group comparisons HDS test. A P value ≤ 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

4.

4.1 PLANT EXTRACTION

Ruta graveolens and *Malva parviflora* were successfully extracted and the percentage yield of each extraction solvent was calculated. The percentage yield was calculated using the formula in section 3.2. For *M. parviflora*, DCM:MeOH had the highest yield of 25.50%, with DCM having the lowest yield (5.41%). For *R. graveolens*, methanol had the highest percentage yield (19.5516%), with hexane (1.9036%) having the lowest yield. Water extracts were not conducted due to limited plant powder. The percentage yields from each solvent are summarized in Table 2.

Table 2: Percentage yields of *R. graveolens* and *M. parviflora*

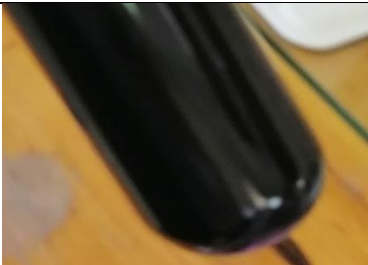
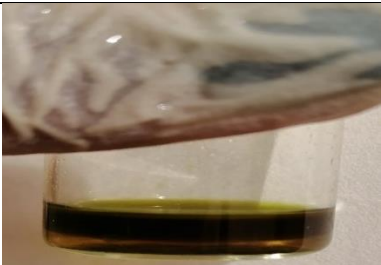
Plant sample	Extraction solvent	Percentage yield
<i>Ruta graveolens</i> L.f	Hexane	1.90
	DCM	16.32
	DCM:MeOH	13.06
	MeOH	19.55
<i>Malva parviflora</i> L	Hexane	21.46
	DCM	5.40
	DCM:MeOH	25.50

	MeOH	12.39

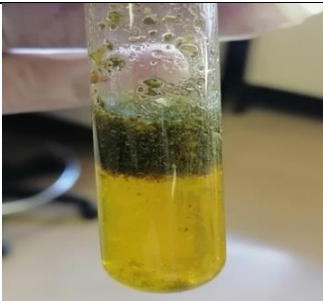
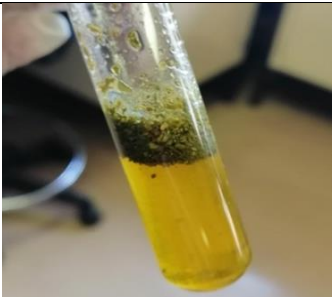
4.2 PHYTOCHEMICAL ANALYSIS

Ruta graveolens L.f and *M. parviflora* L were investigated to determine the absence and presence of phytochemical constituents commonly found in plants. Phytochemical screening was achieved by following the protocols of Kotake (2000), Sofowora (1993), and Harbone (1998), with minor modifications. The reactions from both plants are shown in **Table 3**; and summarized in **Table 4**, below.

Table 3: Visual representations of the phytochemicals detected in *R. graveolens* and *M. parviflora*, “+”, detected, “–“indicates undetected phytochemical.

Phytochemical tested	<i>M. parviflora</i>	<i>R. graveolens</i>
Tannins	 (+)	 (+)
Flavonoids		

	(+)	(-)
Saponnins		
	(+)	(+)
Glycosides		
	(+)	(+)
Phytosterols		
	(+)	(+)

Triterpenoids		
Alkaloids	 (+)	 (+)
Anthraquinones	 (-)	 (-)

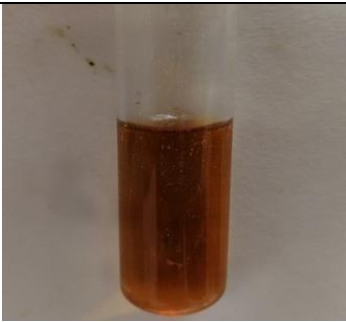
Pentose	 (+)	 (+)
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Table 4: Summary of the phytochemical analysis: + = detected; - = undetected

Phytochemical constituent	<i>R. graveolens</i>	<i>M. parviflora</i>
Phenols and tannins	+	+
Flavonoids	-	+
Saponins	+	+
Glycosides	+	+
Triterpenoids	+	+
Alkaloids	+	+
Anthraquinones	-	-
Pentose	+	+

4.3 CONFIRMATION OF ACQUIRED RESISTANCE OF DU-145/DOX AND DU-145/CDDP CELLS TO DOXORUBICIN AND CISPLATIN

To validate the development of resistance by the established sublines, DU-145/DOX and DU-145/CDDP, to doxorubicin (DOX) and cisplatin (CDDP), an MTT assay and the fold-of-resistance calculation were used. The resistant sublines were established by continual exposure of the cells to doxorubicin and cisplatin. The results (Table 4) show that the resulting IC_{50} of doxorubicin to DU-145/DOX was 54,23-fold higher when compared to the parent DU-145 cell line; and the IC_{50} of cisplatin to DU-145/CDDP cell line was 2,156-fold higher than that of the parent DU145 cell line. To further confirm the resistance phenotype, ABCB1 and ABCC1 gene expression levels were compared to those in DU-145, DU-145/DOX and DU-145/CDDP cells. As shown in Figure 4.4 (a, b and c), MDR-1 had higher expression in DU-145/DOX cells, in both gene and protein levels.

Table 5: Chemo sensitivity assay results. The fold-of-resistance calculation was used to confirm the development of drug resistance by the DU-145/DOX and DU-145/CDDP cells.

CELL LINE	DRUG TREATMENT		RESISTANCE FOLD
	DOXORUBICIN (IC_{50} IN μM)	CISPLATIN (IC_{50} IN μM)	
DU145 ^{WT}	0.2257	1.101	
DU145 ^{DOX}	12.24	0.4804	54,23
DU145 ^{CDDP}	0.06994	2.374	2,156

From the results, it can be concluded that the resistant cell lines acquired resistance against the two chemotherapeutic agents.

4.4 CYTOTOXIC ACTIVITY OF *R. GRAVEOLENS L.f* AND *M. PARVIFLORA L.* EXTRACTS

The *R. graveolens L.f* and *M. parviflora L.* extracts were tested for their cytotoxic activity against the drug-sensitive DU145 and two established drug-resistant (DU145/DOX, DU145/CDDP) prostate cancer cell lines, through the use of an MTT assay. The three cell sublines were exposed to increasing concentrations (1, 10 and 100 µg/ml, a 10-fold dilution); and thereafter, to 0.1, 1, 5, 10, 50, and 100 µg/ml (full dilutions), of the two plant extracts separately. Experiments were completed in triplicate for each extract concentration and growth inhibition of the cells was expressed in the percentage of the untreated cells (negative control), with the results presented as the mean standard deviation (SD). The concentration at which the plant extracts exhibit 50% of the growth inhibition is referred to as the IC₅₀ (minimum growth inhibition). According to the National Cancer Institute (NCI), IC₅₀ values less than 20 µg/ml are considered active; between 20-100 µg/ml are partially active; and those that are more than 100 µg/ml are considered inactive (Ramos-Silva, 2017; Skehan, 1990).

For the present study an extract was considered to be cytotoxic and active at IC₅₀ ≤ 10 µg/ml. The cytotoxic results of each plant are summarized below.

4.4.1 *Ruta graveolens L.f*

Only the DCM extract exerted minimum growth inhibition (IC₅₀) at a concentration of 10 µg/ml (Figure 8(a)). Neither hexane, DCM:MeOH or MeOH exerted any cytotoxic activity on the sensitive prostate cancer DU145 cell line at 10 µg/ml. The DM extract was, therefore, considered active.

(a) No significant growth inhibition was observed on the drug resistant DU-145/DOX cells (Figure 8(a)). The DCM extract only achieved an IC₅₀ at a concentration of 100 µg/ml, but not at 10 µg/ml. No significant growth inhibition was observed on the drug-resistant DU-145/DOX cells (Figure 8(b)). The DCM extract only achieved an IC₅₀ at a concentration of 100 µg/ml, but not at 10 µg/ml. Figure 8 (c), below, shows that no significant growth inhibition was observed on the drug-resistant DU-145/CDDP cells at concentrations below 100 µg/ml. The DCM extract exhibited

some growth inhibition at 10 μ g/ml, but failed to achieve an IC₅₀. An overgrowth was observed at a concentration of 1 μ g/ml hexane, DCM and methanolic extracts.

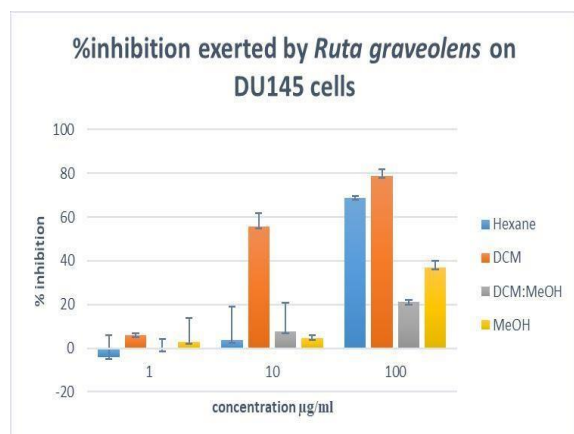


Figure 8(a)

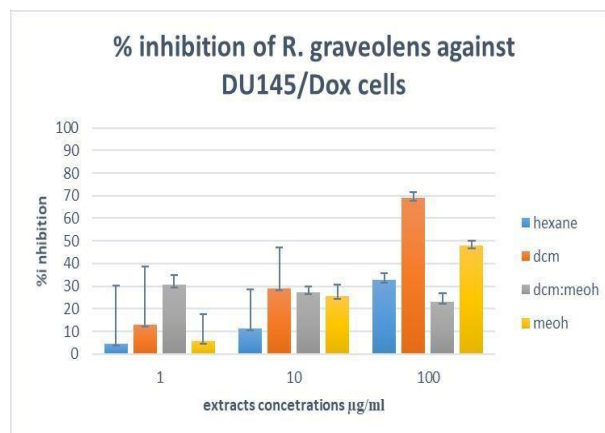


Figure 8(b)

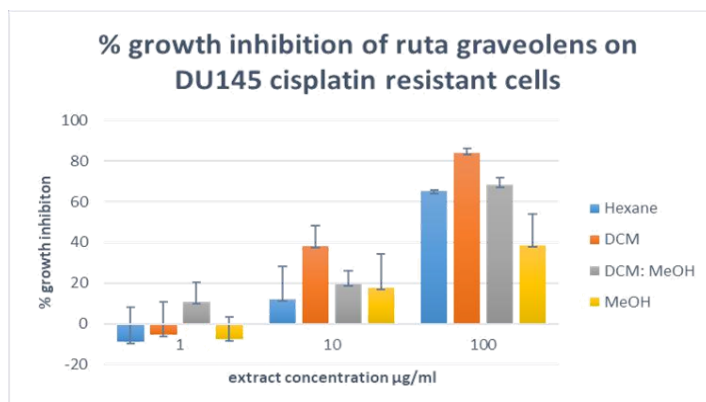


Figure 8(c)

Figure 8 (a, b and c): Cell growth inhibition exerted by *R. graveolens* extracts on drug sensitive DU-45 and drug resistant DU-145/DOX and DU-145/CDDP cells. Cells were treated with different concentrations of plant extracts (1 μ g/ml, 10 μ g/ml and 100 μ g/ml). Each experiment was performed in triplicate for each concentration. Overall, the results (figure 8, a, b and c) indicated that the plant extracts exhibited higher activity against the sensitive DU-145 strains than the resistant cell.

4.4.2 *Malva parviflora* L.

The results obtained following exposure of the cell lines to the *M. parviflora* extracts are shown in Figures 4.4.2, a, b, and c, below.

- (a) Minimum growth inhibition (IC₅₀) for the active extracts was observed at 1µg/ml and 10µg/ml for the DCM:MeOH extract; and at 10µg/ml for the methanolic extract. An overgrowth of cells was observed at a 100µg/ml concentration for the hexane extract (Figure 9(a)). No growth inhibition was observed at any of the tested extract concentrations and overgrowth was observed at the 100µg/ml for the hexane extract (Figure 9 (b)). No significant growth inhibition was observed on the drug-resistant DU145/CDDP cells. Overgrowth was observed at a concentration of 1µg/ml and 10µg/ml for hexane, DCM, dcm:meoh and meoh (Graph 9(c)).

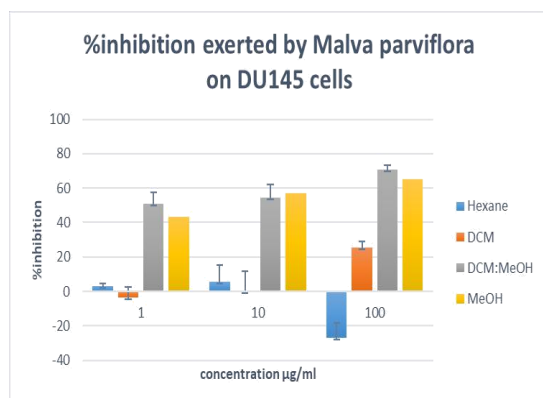


Figure 9(a)

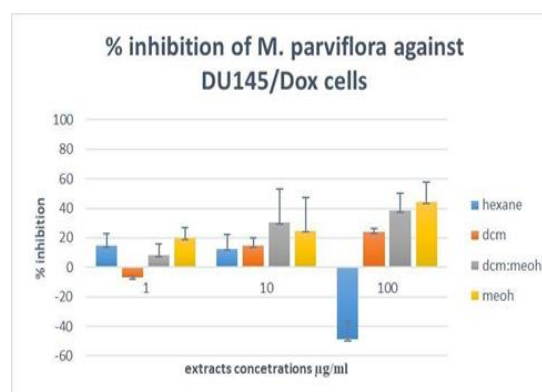


Figure 9(b)

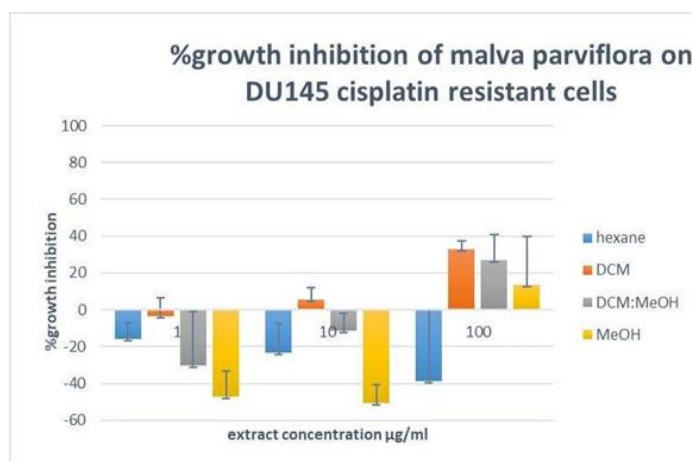


Figure 9(c)

Figure 9 (a, b and c): Cell growth inhibition exerted by *M. parviflora* on drug-sensitive DU-145, DU-145/DOX and DU-145/CDDP cells. Cells were treated with different concentrations of plant extracts (1µg/ml, 10µg/ml and 100µg/ml). Each solvent extract was performed in triplicate for each concentration.

For the **second cytotoxicity assay**, more dilutions of the selected extracts were screened to obtain accurate IC₅₀ values. The DCM extract of *R. graveolens* displayed a concentration-dependent growth inhibitory effect against the DU145 cell line, and achieved an IC₅₀ at a concentration of 5µg/ml. All other extracts were considered inactive against the wild-type parent DU-145 prostate cancer cell line. Interestingly, the methanol extract of *M. parviflora* showed a concentration-dependent growth stimulation effect.

Malva parviflora L. and *Ruta graveolens* L.f., two medicinal herbs, were tested against the drug-resistant (DU-145) and drug-sensitive (wild type) prostate cancer cell lines. The two plant species are employed in traditional medicine to treat a variety of ailments, including fever, gastrointestinal disorders, and inflamed sores; however, nothing is known about how active the plants are against sensitive and resistant prostate cancer cell lines. In order to determine whether medicinal plants might help cancer cells that are resistant to drugs regain their sensitivity to chemotherapy by selectively targeting MDR proteins, the study makes use of in vitro models. From the results, *R. graveolens* DCM extract exhibited 64,66% and 69,82% growth inhibition at a concentration of 50 µg/ml and 100 µg/ml, respectively (Figure 10). The *M. parviflora* DCM+MeOH extract exhibited a dose dependent growth inhibitory effect at concentrations 0,1,5,10,50 and 100 µg/ml even though growth inhibition at 0.1 µg/ml was less than 50% and MeOH extracts exhibited growth inhibitory activity against the DU-145/DOX, but only at concentrations of 50 and 100 µg/ml (Figure 11). Growth inhibitory activity on the DU-145/CDDP was observed at 50 µg/ml and 100 µg/ml (53,59% and 66,89%) for the *R. graveolens* DCM extract (Figure 5); while the *M. parviflora* DCM+MeOH extracts exhibited a >50% growth inhibition at 10 µg/ml concentration (Figure 12). The *M. parviflora* MeOH extract growth inhibition activity was observed at 50 µg/ml and 100 µg/ml concentration, with percentage values of 50,94% and 57, 55% (Figure 12).

The *M. parviflora* plant extracts were the most effective against the drug-resistant cells when compared to the wild type DU-145 cells. Both plants demonstrated anti-

proliferative activity against the tested prostate cancer cell lines. Based on cellular inhibition, this extract exhibits selectivity for drug-resistant cells (Figure 11&12).

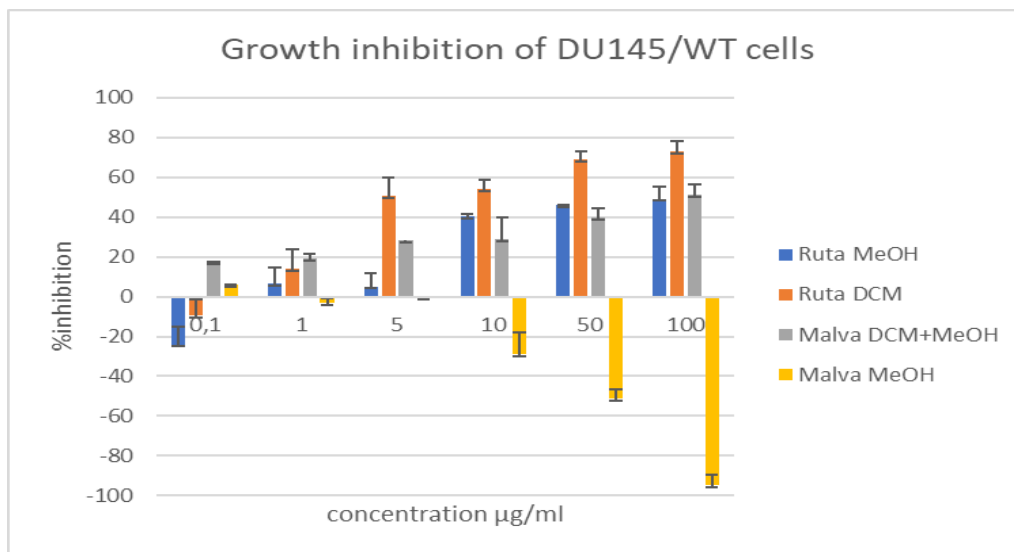


Figure 10: Growth inhibition of DU145/WT prostate cancer cell line by *R. graveolens* and *M. parviflora* plants extracts

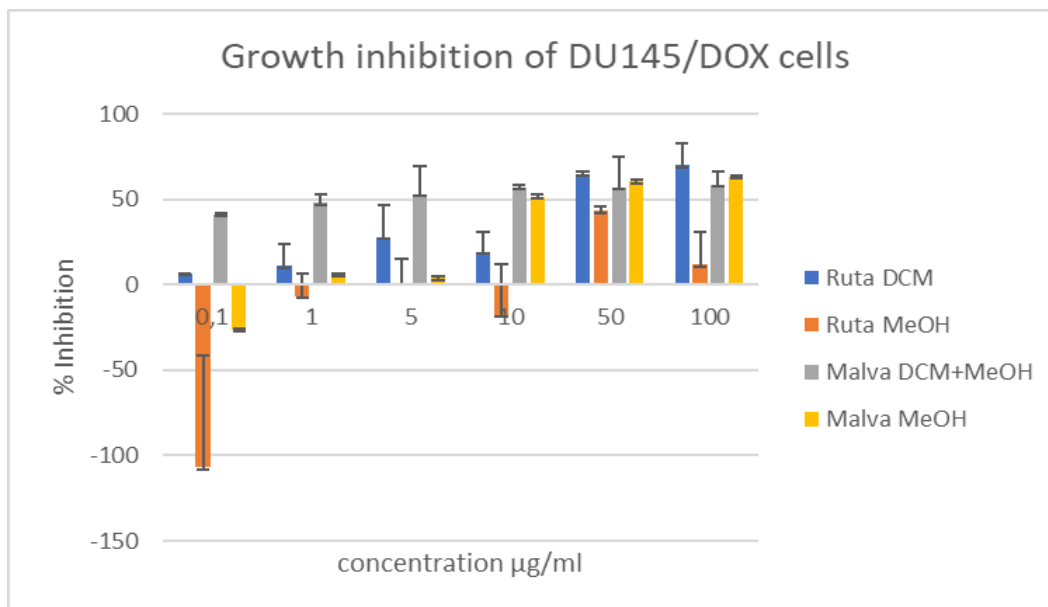


Figure 11: Growth inhibition of DU145/DOX prostate cancer cell line by *R. graveolens* and *M. parviflora*

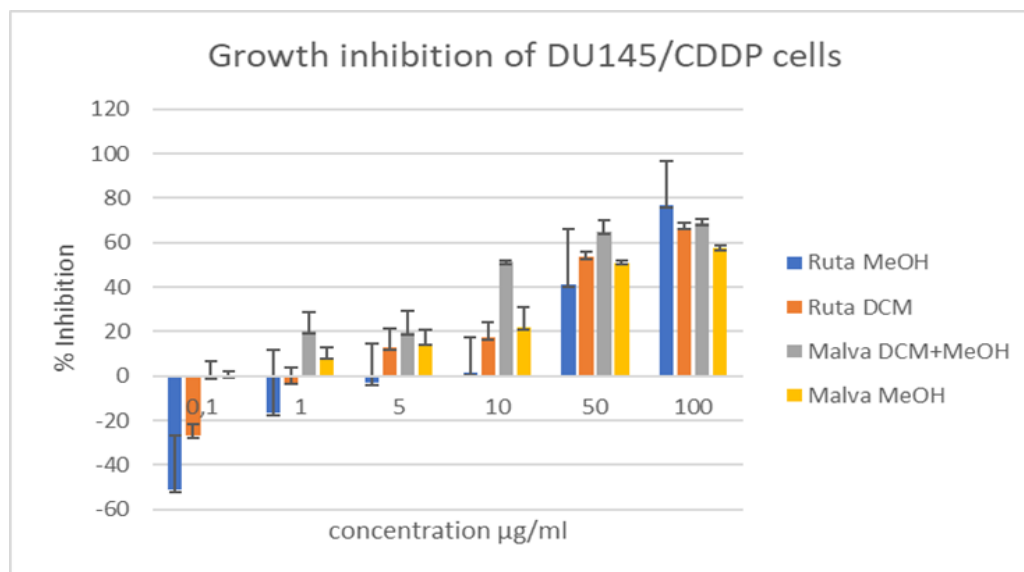


Figure 12: Growth inhibition of DU145/CDDP prostate cancer cell line by *R. graveolens* and *M. parviflora* plant extracts

4.5 RT-QPCR

4.5.1 TOTAL RNA ISOLATION

Good quality total RNA was successfully extracted from cell culture samples (21 of 21, 100%). The RNA quality and quantity data are shown in Table 6 below.

Table 6: Quantification and quality control analysis of RNA from Du-145 cells as determined by absorbance, fluorescence, and integrity analysis using the Tape Station RNA Screen Tape assay, RNA concentration, purity, and RNA integrity number (RIN) data.

Barcode	Sample name	Absorbance (NanoDrop)			Fluorescent Dye (Quantifluor RNA System)	RNA Integrity (TapeStation RNA ScreenTape)
		Concentration (ng/μl)	260/280	260/230	Concentration (ng/μl)	RIN
Vial 1	Du-145WT Control	176.1	1.88	1.02	222.4	9.7
Vial 2	Du-145WT Control	238.5	2.08	1.89	291.3	9.6
Vial 3	Du-145WT Control	259.2	2.08	1.75	341.8	9.5
Vial 4	Du-145Dox-R	242.7	2.01	1.35	347.1	9.7
Vial 5	Du-145Dox-R	549.6	2.02	2.09	462.0	9.5
Vial 6	Du-145Dox-R	395.6	2.06	1.88	385.8	9.4
Vial 7	Du-145-CDDP-R	829.1	2.03	1.63	440.2	9.5
Vial 8	Du-145-CDDP-R	224.3	2.08	1.65	321.0	9.7
Vial 9	Du-145-CDDP-R	367	2.07	2.04	396.7	9.2
Vial 10	Du145Dox-R treated with Malva DCM+MeOH	135.2	1.96	0.33	161.8	9.6
Vial 11	Du145Dox-R treated with Malva DCM+MeOH	49.99	2.02	0.26	66.2	9.2
Vial 12	Du145Dox-R treated with Malva DCM+MeOH	72.54	2.02	0.23	120.8	9.0
Vial 13	Du145Dox-R treated with Malva MeOH	283.8	2.05	0.64	338.1	9.7
Vial 14	Du145Dox-R treated with Malva MeOH	58.0	2.00	0.56	56.0	9.1
Vial 15	Du145Dox-R treated with Malva MeOH	54.23	2.03	0.17	56.1	9.4
Vial 16	Du145CDDP-R treated with Malva DCM+MeOH	286.4	1.67	0.76	314.1	9.1
Vial 17	Du145CDDP-R treated with Malva DCM+MeOH	261.5	2.08	1.34	318.1	9.5
Vial 18	Du145CDDP-R treated with Malva DCM+MeOH	260.3	2.1	1.6	313.8	9.3
Vial 19	Du145CDDP-R treated with Malva MeOH	268.4	2.1	0.49	331.1	9.6
Vial 20	Du145CDDP-R treated with Malva MeOH	146.4	2.1	0.29	205.2	9.7
Vial 21	Du145CDDP-R treated with Malva MeOH	66.58	2.13	0.14	60.7	9.0

4.5.2 qPCR Assay optimization and validation

4.5.2 (a) Optimization of annealing temperature

The optimal T_a of each primer pair was determined empirically by a gradient temperature at 55°C, 57°C, 59°C, 61°C, 63°C, and 65°C.

4.5.2. (b) Optimization of primer concentrations

Different primer concentrations (300 nM, 500 nM, and 800 nM) were tested to determine the optimal primer concentration for each target gene. We found that primer pairs of both ABCB1 and ABCC1 had the lowest C_q values at the primer concentrations of 800 nM and these primer concentrations were chosen as the optimal primer concentration for subsequent reactions. The primer concentrations for the reference genes were used at 500 nM.

4.6 Relative Quantification

Fold-change analysis entails fold changes that are relative to the control group (DU-145) to determine the degree of up-or-down-regulation in the treatment groups.

A.ABCB1

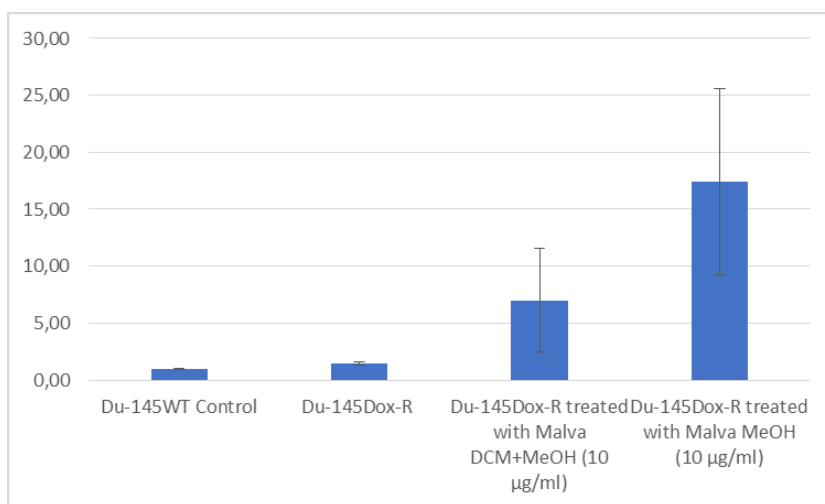


Figure 13 (a): ABCB1 relative gene expression in DU-145 cells following treatment with *M. parviflora*, with normalization to the combination of two reference genes (GAPDH and HPRT1). The bars represent the average of the groups relative to Du-145 differences in gene expression between the indicated groups, with $P < 0.05$ (ANOVA; Tukey HSD's post hoc test). Data are presented as the mean $\pm$ S.D. Statistical significance among groups was established by one way ANOVA using Tukey's multiple group comparison HDS test. A P value ≤ 0.05 was considered statistically significant.

The mRNA expression level of the ABCB1 gene in DU-145; DU-145/DOX and DU-145/ DOX-treated cells was determined using RT- PCR. The DU-145/DOX cells treated with *M. parviflora* DCM+MeOH and *M. parviflora* MeOH cells expressed higher levels of ABCB1 gene in comparison with DU-145 and DU-145/DOX cells.

(B) ABCC1

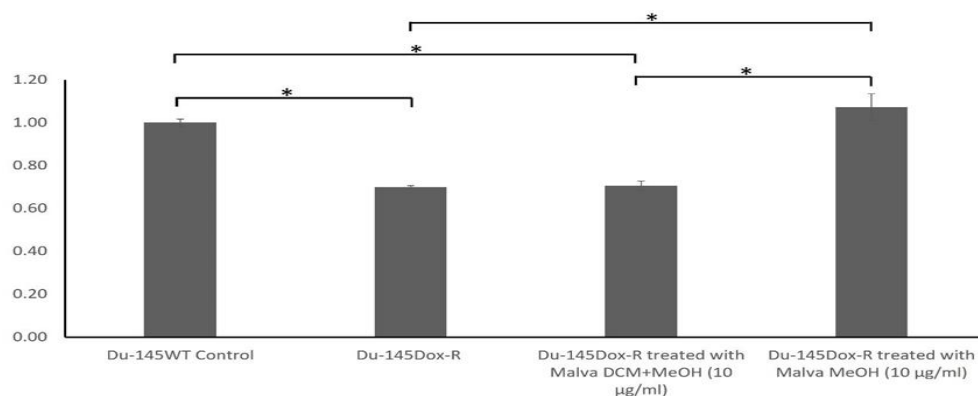


Figure 13(b). The mRNA expression level of the ABCC1 gene in DU-145; DU-145/DOX and DU-145/ DOX cells following treated with *M. parviflora*, was determined using RT- PCR. ABCC1 relative gene expression in DU-145 cells with normalization to the combination of two reference genes (GAPDH and HPRT1). The bars represent the average of the groups relative to Du-145 differences in gene expression between the indicated groups, with $P < 0.05$ (ANOVA; Turkey HSD's post hoc test). Data are presented as the mean $\pm$ S.D. Statistical significance among groups was established by one-way ANOVA using Turkey's multiple group comparison HDS test. A P value ≤ 0.05 was considered statistically significant.

(C) ABCB1

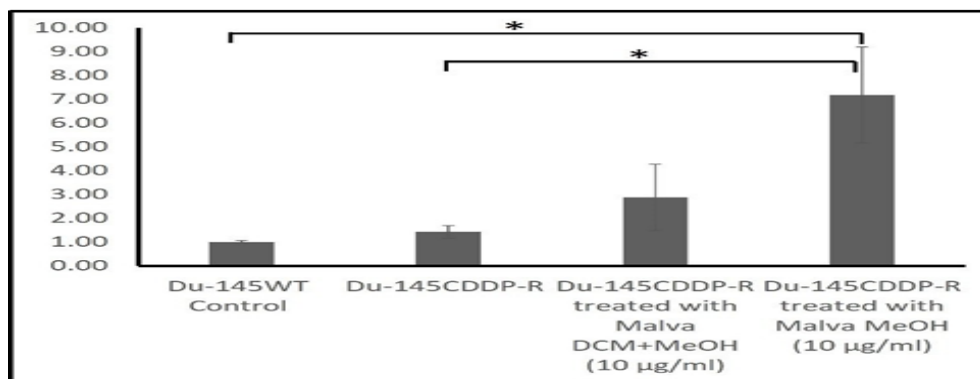


Figure 13(c): The mRNA expression level of the ABCB1 gene in DU-145; DU-145/CDDP and DU-145/ CDDP cells treated with *M. parviflora*, was determined using RT- PCR. The bars represent the average of the groups relative to Du-145 differences in gene expression between the indicated groups, with $P < 0.05$ (ANOVA; Turkey HSD's post hoc test). Data are presented as the mean $\pm$ S.D. Statistical significance among groups was established by one-way ANOVA using Turkey's multiple group comparison HDS test. A P value ≤ 0.05 was considered statistically significant.

The mRNA expression level of the ABCB1 gene in DU-145; DU-145/CDDP and DU-145/ CDDP-treated cells was determined using RT- PCR. The DU-145/CDDP cells treated with *M. parviflora* DCM+MeOH cells expressed higher levels of the ABCB1 gene in comparison with the other cells (Figure13c)

(D) ABCC1

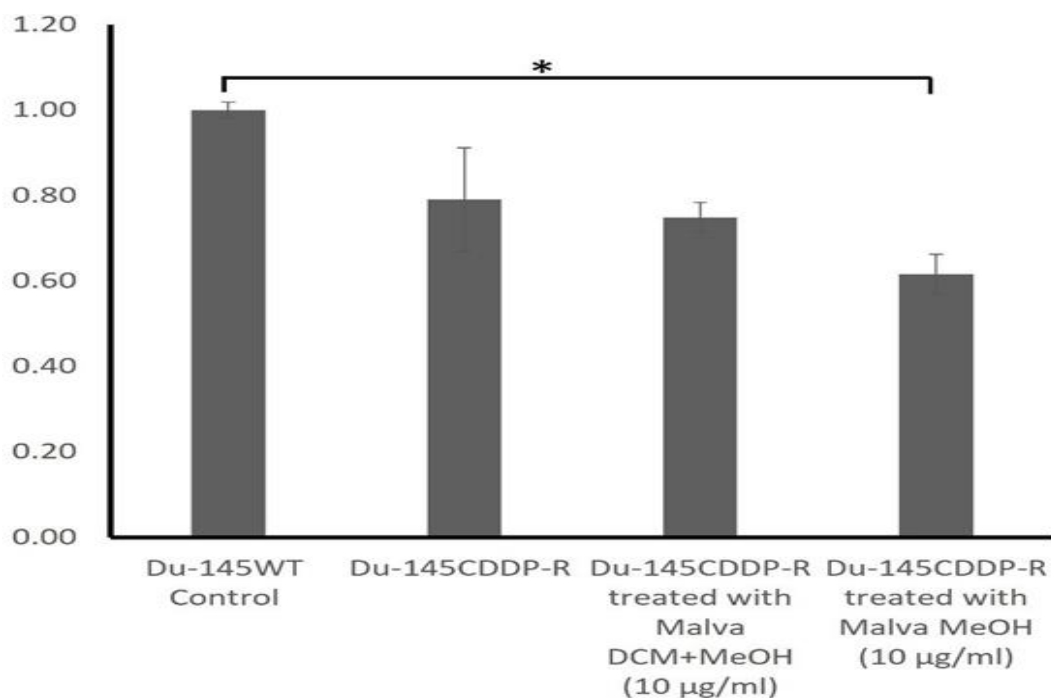


Figure 13(d): The mRNA expression level of ABCC1 gene in DU-145; DU-145/CDDP and DU-145/CDDP- treated cells were determined using RT-PCR. ABCC1 relative gene expression in DU-145 cells with normalization to the combination of two reference genes (GAPDH and HSPCB). The bars represent the average of the groups relative to Du- 145 differences in gene expression between the indicated groups, with $P < 0.05$ (ANOVA; Turkey HSD's post hoc test). Data are presented as the mean $\pm$ S.D. Statistical significance among groups was established by one-way ANOVA using Turkey's multiple group comparison HDS test. A P value ≤ 0.05 was considered statistically significant.

The mRNA expression level of the ABCB1 gene in DU-145; DU-145/CDDP and DU-145/ CDDP-treated cells was determined using RT- PCR. The DU-145/CDDP-R cells treated with *M. parviflora* DCM+MeOH, down-regulation of

the ABCC1 gene was observed in comparison with the other cells (Figure13d)

4.7 DETERMINATION OF MDR PROTEIN FUNCTION IN RESISTANT CELLS

(a) Calcein AM retention in DU-145/DOX cells

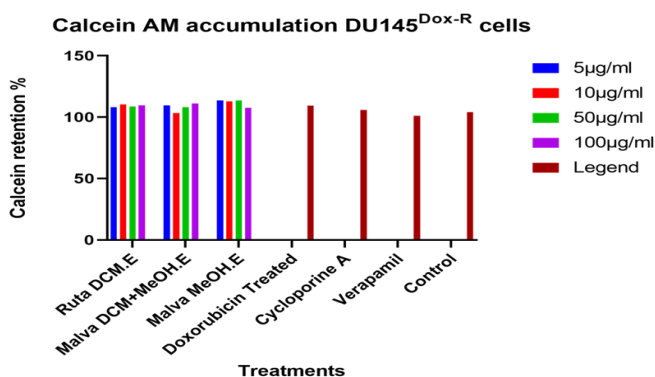


Figure 14(a): Calcein AM retention in DU-145/DOX cells. Each experiment was done in triplicates.

Cells pre-treated with *M. parviflora* showed higher Calcein AM accumulation in comparison with untreated cells (Figure 14a).

(b) Calcein AM accumulation in DU-145/CDDP CELLS

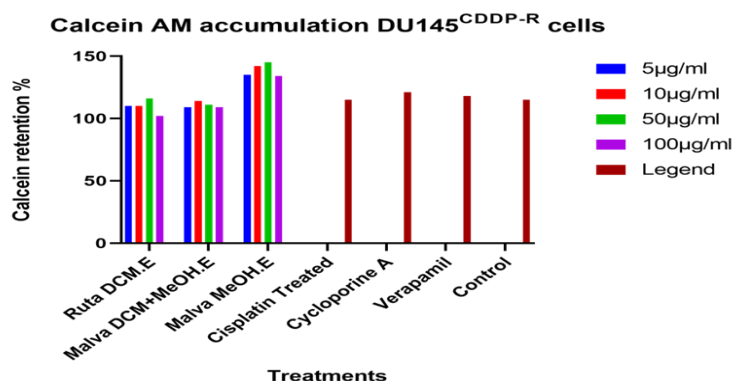


Figure 14(b): Calcein AM retention in DU-145/CDDP cells. Each experiment was done in triplicates.

Cells pre-treated with *M. parviflora* MeOH showed higher Calcein AM retention in comparison with other cell groups (Figure 14b)

4.8 WESTERN BLOT

Using western blot, P-gp and MRP-1 proteins were not found in the cells, Figure 15a&b below. Translational or post-translational mechanisms that increase protein stability and half-life could be the reason we were unable to find these proteins in our investigation. The increase in P-gp protein production in cancer cells was proposed to be explained by chemotherapeutic drug-induced epigenetic changes that affected transcription inside the MDR1 promoter and increased its mRNA stability. An increase in translational rate or a decrease in turnover rate, which would improve protein stability, could lead to an increase in MDR protein level without influencing transcriptional activity.

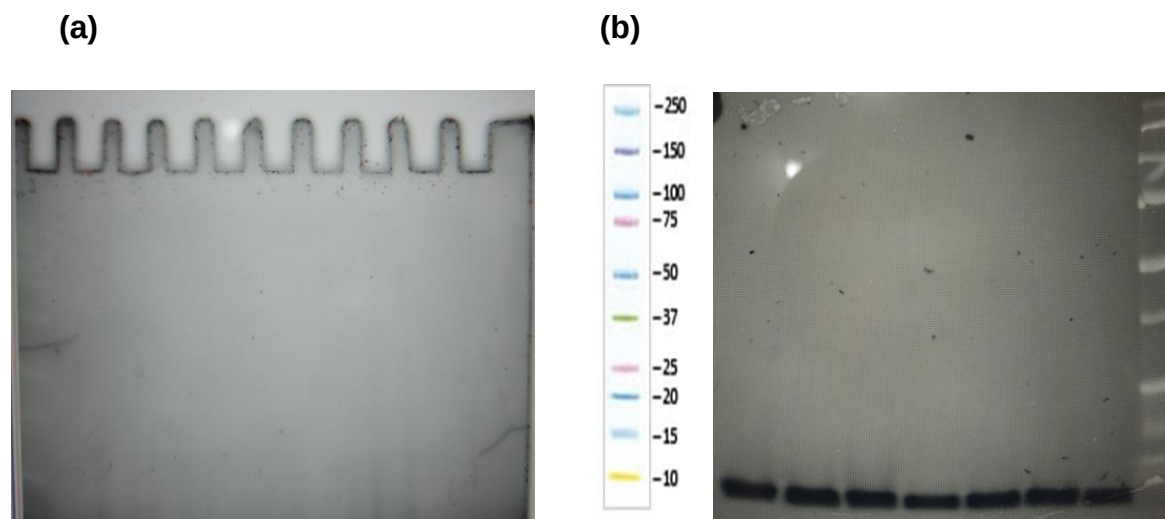


Figure 15 (a) Western blot membrane before staining, (b) western blot membrane after staining with ECL substrates. Protein of interests, P-gp (170kDa) and MRP-1 (190kDa) were not detected after incubating in primary and secondary antibodies.

CHAPTER 5

DISCUSSION

Multidrug resistance remains the main reason for chemotherapy failure in the clinic. Several mechanisms contribute to MDR in tumour cells, but the most common mechanism is the overexpression of ABC transporters. The P-gp, BCRP and MRP1 are the most common ABC transporter cellular membrane proteins that actively pump cytotoxic agents out of the cells. These three proteins may serve as potential targets to overcome MDR (Choi, 2014, Xiao, 2021; Heming, 2022). The expression of MDR proteins has been associated with the degree of tumour progression in a number of cancer types. For example, in patients with acute myeloid leukemia (AML); lung; colon; and breast cancer, a clinical relationship between the expression of P-glycoprotein and chemotherapy response has been observed, showing a poor chemotherapy response in patients expressing high levels of MDR proteins (Sanchez, 2009, Chung, 2016). In prostate cancer, the expression of P-gp and MRP1 has been previously examined with immunohistochemistry, which suggest a relationship between MDR protein expression and MDR phenotype (Sullivan, 2000, Van Brussel, 2001, Heming, 2022). The increased expression of these proteins in tumours without prior treatment is known as intrinsic MDR phenotype which, it has been suggested, is responsible for the poor response to chemotherapy exhibited by some cancer types prior to chemotherapeutic drug exposure (Wang, 2019, Bukowski, 2020). Several P-gp inhibitors, such as verapamil and cyclosporine A, were combined with anticancer agents to circumvent MDR; and even some second- and third-generation inhibitors such as valspodar and tariquidar were tested in clinical trials. However, they were not clinically approved due to their side-effects and pharmacokinetic interactions (Barbuti, 2015, Yu, 2016, Karthika, 2022). Therefore, the identification and development of safe and potent MDR-reversing agents remains as the main objective to circumvent drug resistance in prostate cancer.

The present study aimed to assess the potential anti-proliferative effects of *R. graveolens* and *M. parviflora* against wild type as well as drug resistance reversal potential in drug-resistant prostate cancer cell lines. The DU-145 cell line was selected for the study due to its initial drug sensitivity, time and conditions to develop the resistance phenotype (Sanchez, 2009). The chemotherapeutic agents, doxorubicin and cisplatin, were chosen for the study as they are substrates of P-glycoprotein and multidrug resistance-associated protein-1; and to mimic the conditions and stages prostate cancer patients go through during chemotherapy. To confirm the development of resistance and sensitivity of drugs in the established drug resistant cells, DU-145/DOX and DU-145/CDDP, a chemo-sensitivity assay was performed (Table 5). From the results obtained in the study, the parent DU145 cell line was more sensitive to both doxorubicin and cisplatin, when compared to the drug-resistant cells. The results (Table 5) show that the resulting IC₅₀ of doxorubicin to DU-145/DOX was 54,23-fold higher when compared to the parent DU-145 cell line; and the IC₅₀ of cisplatin to DU-145/CDDP cell line was 2,156-fold higher than that of the parent DU145 cell line. Results obtained from the study were consistent to those obtained from a study conducted by Sanchez (2009), where doxorubicin resistant DU-145 cells were established to test the hypothesis that the over-expression of MDR proteins after drug treatment is related to the increase in drug resistance (Sanchez, 2009). Results obtained from the study were also consistent with those obtained from a study conducted by Zachberg, 2000, where the drug resistant prostate cancer cell line was used to determine the mechanism of drug resistance in prostate cancer .

Phytochemical screening and analysis results obtained from the study (Table 3&4) revealed the presence of important secondary metabolites which have been previously proven to play significant roles in multiple pathways involved in cancer development. Phytochemicals present in the plants include saponins, tannins and flavonoids, which have been reported as displaying antioxidant activity, which exerts a number of biological effects against cancer and other diseases (Rammal, 2018). It has been shown that natural compounds from medicinal plants have anticancer activities, and regulate signal transduction pathways, modulating proteins that control cell cycle

progression and induce apoptotic pathways (Hashem, 2022). They have also been proven to have a therapeutic role in drug resistance reversal in cancer cells. *M. parviflora* has been reported to possess anti-bacterial, anti-inflammatory and anti-irritant activity, but its cancer activity has not yet been fully explored (Singh, 2017). In the current study, *M. parviflora* and *Ruta graveolens* were evaluated for its anti-cancer activity against prostate cancer cell lines. *Ruta graveolens* DCM extract displayed a dose dependent growth inhibitory effect on the DU-145/WT. Similarly, growth stimulatory effects were observed when the DU-145/WT cells were exposed to increasing concentrations of *M. parviflora* (Figure 10). For the DU-145/DOX and DU-145/CDDP cells, the *R. graveolens* DCM extract displayed growth inhibitory effects when exposed to higher concentrations of the extract (Figure 11&12). The *M. parviflora* extracts displayed >50% growth inhibition at a concentration of 10 µg/ml for the resistant cisplatin (DU-145/CDDP) cell line, >50% growth inhibition was observed at a concentration of 10 µg/ml when exposed to the *M. parviflora* DCM+MeOH extract. On the DU-145/CDDP cells, *M. parviflora* DCM+MeOH extract exhibited a dose-dependent growth inhibition. The *M. parviflora* extracts exhibited greater inhibitory effects on the DU-145/DOX cells at a concentration of 10 µg/ml when compared to the *R. graveolens* extracts. Both plants exhibited anti-proliferative activity against the tested prostate cancer cell lines with the *M. parviflora* plants extracts being the most active against the drug resistant cells compared to the wild type DU-145 cells. This extract demonstrates selectivity for the drug resistant cells based on cellular inhibition (Figure 11&12). According to literature, *M. parviflora* methanolic extract inhibited the growth of liver carcinoma, HepG2 cells, *in-vitro* (Abdel-Ghani, 2013). *R. graveolens* has been reported to possess antimicrobial activities. In a study conducted by Benazir, *R. graveolens* displayed cytotoxic activity against liver carcinoma HepG2 cells at a lethal concentration of 62µg/ml, and anti-tumour activity when tested in tumour-bearing animals (Pleethi, 2006, Benazir, 2011). The results obtained suggest that some of the phytochemicals may stimulate the anticancer activity of *M. parviflora* and *R. graveolens*. Since the anticancer activity of the extracts should be mainly attributed to the identified secondary metabolites, these findings warrant further study at the molecular level to develop the

anticancer value of these phytochemicals and to assess the mechanism of action of the phytochemicals. No previous scientific literature has been reported on the plant's anti-proliferative activity against drug-resistant prostate cancer cells.

We also investigated the effects of *R. graveolens* and *M. parviflora* extracts on DOX and CDDP toxicity potentiation and P-gp, MRP-1 expression and function in human, resistant prostate cancer (DU-145/DOX and DU-145/CDDP) cells. P-gp and MRP-1 are the most common expressing ABC transporters associated with MDR in cancer cells. Doxorubicin and cisplatin were used to establish DOX-resistant and CDDP-resistant prostate cancer cells from parent DU-145 cells, confirmed by the increased IC₅₀ observed on the established cells (Table 5) and P-gp mRNA levels (Figure 4.6 a, b, c and d). From the results obtained, the plant extracts had no significant inhibitory effect on the mRNA expression levels of P-gp and MRP-1; instead, the up-regulation of gene expression was observed in the cells (Figure 13 a, b and c). A number of plants and compounds have been associated with increasing *ABCB1* (encoding P-glycoprotein) gene expression, for example St John's wort and rifampicin (Hennessy, 2002; Nabekura, 2018; Elmeliegy, 2020). Up-regulation of the *ABCB1* gene in the DU-145/DOX-R cells treated with the *M. parviflora* MeOH extract was observed relative to the DU-145/WT and DU-145/DOX cells (Figure 13 (a)). No significant difference was observed in the *ABCC1* mRNA expression level in the DU-145/DOX cells for the same extract (Figure 13(b)). Up-regulation of *ABCB1* gene was also observed in the *M. parviflora* treated DU-145/CDDP cells (Figure 13(c)). From the RT-qPCR results, we also observed that *ABCC1* expression was down-regulated when exposed to the *M. parviflora* extracts relative to the control (DU-145/WT) (Figure 13(d)). From the results it can be concluded that the *M. parviflora* has no effect on the down-regulation of the *ABCB1* mRNA expression, instead the tested plant extracts up-regulate the expression of the *ABCB1* mRNA in the drug resistant cells. It can also be concluded that *M. parviflora* plant extracts have significant effect on the *ABCC1* mRNA expression level which therefore warrants the investigation of the drug resistance reversal potential of *M. parviflora* as well as to test the combination effects of *M. parviflora* with chemotherapeutic agents used in the treatment of prostate cancer. Overall it can be

observed that the MDR genes are expressed in prostate cancer cells before and after drug treatment which also explains drug resistance observed in clinical settings before treatment (Seruga, 2011; Xu, 2014; Wang, 2017).

In accordance with other authors, we were able to detect and quantify MRP1 and P-gp mRNAs, but not their proteins, in the DU-145 cells. In the present study, P-gp and MRP-1 proteins were not detected in the cells by western blot (Figure 15), however in a study conducted by Sanchez, 2009 the proteins were detected by immunohistochemistry. A possible explanation for not detecting these proteins in our study, might be translational or post-translational mechanism, which play a pivotal role in increasing protein stability and half-life (Lee, 2023). It was suggested that the increase in P-gp protein expression in cancer cells could be explained by epigenetic changes induced by chemotherapeutic drugs affecting transcription within the MDR1 promoter and increasing its mRNA stability (Lee, 2008, Heming, 2022, Skinner, 2023). An increase in MDR protein level, without change in transcriptional activity, may be due to an increase in translational rate and/or a decrease in turnover rate which results in an increase in protein stability. In addition, we observed that DU-145/DOX cells also developed resistance to cisplatin, like the DU-145/CDDP cells, which also developed resistance to doxorubicin (Table 5). This phenomenon of cross-resistance is known as multidrug resistance. The over-expression of MDR proteins is an adaptive mechanism for cell survival. Therefore, the exposure of cells to chemotherapeutic drugs induces the expression of the MDR proteins, which may also activate other survival pathways (Bukowski, 2020, Vaidya, 2022).

In addition to P-gp and MRP1 expression, a Calcein AM accumulation assay was performed to determine P-gp and MRP1 (MDR proteins) function and activity. Calcein AM is a fluorescent MDR protein substrate and its intracellular accumulation is associated with MDR protein function. After incubating the cells with Calcein AM, we observed that intracellular accumulation (Figure 14a & 14b) in the cells was comparable with that of verapamil and cyclosporine, which are inhibitors of P-gp. This suggests that the plant extracts were effective in inhibiting P-gp and MRP-1 activity. For the DU-

145/DOX results, the positive controls verapamil and cyclosporine A as well as the untreated controls had the same calcein retention (Figure 14a). More calcein retention was observed in the DU-145/CDDP *Malva* MeOH treated cells in comparison with the positive controls and the untreated controls (Figure 14b). From the obtained results it is evident that the *M. parviflora* plant extracts have significant effect on the function of MDR protein in the drug resistant DU-145/CDDP cells (Figure 14b).

CHAPTER 6

6. CONCLUSION AND FUTURE STUDIES

6.1 The extent to which the study objectives were met

The objectives of this study were achieved as follows:

1. Qualitative phytochemical analysis on the pulverized crude plant material indicated the presence of glycosides, saponins, tannins, terpenoids and phytosterols; and absence of flavonoids and anthraquinones in *R. graveolens*. Anthraquinones were undetected in *M. parviflora*.
2. Anticancer assay results revealed that *R. graveolens* L DCM extract inhibited cell growth in the DU-145/WT cells. Cell growth stimulation was observed in DU-145/WT when exposed to increasing extract concentrations of *M. parviflora* MeOH extract.
3. Cytotoxicity results showed that *M. parviflora* DCM+MeOH extract inhibited cell growth in the DU-145/DOX cells.
4. Cytotoxicity results showed that *M. parviflora* DCM+MeOH extract inhibited cell growth in the DU-145/CDDP cells.
5. Cytotoxicity results showed that *M. parviflora* MeOH extract inhibited cell growth in the DU-145/CDDP cells.
6. RT-QPCR results showed down-regulation of the ABCC1 gene in the DU-145/CDDP-R and the DU-145/CDDP-R cells treated with the *M. parviflora* extracts in comparison with the DU-145/WT cells
7. RT-QPCR results showed up-regulation of the ABCB1 gene in the DU-145/DOX-R cells treated with the *M. parviflora* extracts in comparison with the DU-145/WT cells
8. RT-QPCR results showed up-regulation of the ABCB1 gene in the DU-145/CDDP-R and the DU-145/CDDP-R cells treated with the *M. parviflora* extracts in comparison with the DU-145/WT cells

6.2 Concluding remarks

This study has unequivocally confirmed that *Ruta graveolens* and *Malva parviflora* have the potential to provide lead compounds for prostate cancer drug development. This is

proven by the biological activity observed. Further studies to identify the bioactive compounds in the extracts that exhibited anti-proliferative activity are recommended, as well as determining the plants' mechanism of action. In addition, combination studies are necessary to validate and confirm the potential drug resistance reversal activity of *M. parviflora*.

6.3 Study limitations:

A quantitative analysis might have displayed different quantities of the phytochemical components of the plants that are pharmacologically significant, but the study employed a qualitative phytochemical examination.

The MTT assay was used exclusively in the study to measure the cytotoxicity and proliferation of the test substances. Alternative approaches, like the Sulforhodamine B test, which examines the gene expression effect regulation and may be employed to investigate the impacts of mRNA replacement on cell proliferation, could have been utilized.

The study only used western blot to detect proteins. Other sensitive methods such as immunohistochemistry, Elisa ns northern blotting could have been used for the detection of proteins.

6.4 Future studies

The following are suggestions for potential follow-up research that could be conducted after this study:

Biological evaluation of the most active extracts to confirm the mechanisms of action through which the extracts exert their cytotoxic and potential drug resistance reversal effects.

The purification of the most active extracts, to isolate if at all, the compounds responsible for the observed cytotoxic activity.

In vivo studies in mice models to confirm the observed activity in vivo. Biological systems are complex, and involve other processes such as metabolism and bioavailability, which could affect the overall activity of test extracts/isolated compounds, and could influence future studies.

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