

**BIOACTIVITY INVESTIGATION OF ANTI-CANCER
ACTIVITIES OF *ELEPHANTORRHIZA ELEPHANTINA*,
DICOMA ANOMALA, *XYSMALOBIUM UNDULATUM* AND
CUSSONIA PANICULATA PLANT EXTRACTS**

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August 2023

DECLARATION OF INDEPENDENT WORK

I, Lesego Proscovia Kgasapane, identity number _____ and student number _____, hereby declare that this research project submitted to the Central University of Technology, Free State, for the Master of Health Science in Biomedical Technology degree, is my own independent work; complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules, and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

SIGNATURE OF STUDENT

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DATE

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ABSTRACT

Cancer is the term used to define a group of diseases associated with uncontrollable proliferation of abnormal cells. More than half of the drugs used currently are from natural products and their derivatives. Medicinal plants have been used as a source of medication for many years. *Cussonia paniculata*, *Dicoma anomala*, *Elephantorrhiza elephantina*, and *Xysmalobium undulatum* are used by traditional healers and herbalists in South Africa and other African countries to treat breast and cervical cancer. The aim of the study was to investigate *E. elephantina* root, *D. anomala* root, *X. undulatum* root, and *C. paniculata* leaf and root extracts for anti-cancer activities. The four medicinal plants were extracted with distilled water (dH₂O), dichloromethane (DCM), methanol (MeOH), and DCM:MeOH (1:1 ratio) solvents using the sequential method of extraction. The MCF7 and Caco2 cell lines were exposed to all extracts with concentrations 10 and 100 µg/mL to determine their toxicity and IC₅₀ values using the Hoechst 33342/Propidium iodide (PI) dual staining method. Thereafter, the effect of cytotoxic extracts on MCF7 and Caco2 cells and their progression through the cell cycle, apoptosis, and mitochondrial membrane potential was investigated using fluorescent staining with annexin V- with a conjugated fluorophore fluorescein isothiocyanate (FITC), annexin V-FITC/PI and Tetramethylrhodamine ethyl ester (TMRE) staining respectively. An anti-inflammation test was performed on the aqueous extract of each plant using Lipopolysaccharide (LPS) induced [nitric oxide](#) production in RAW 264.7 macrophages, and compounds in the *X. undulatum* extracts were identified and characterised using liquid chromatography-mass spectrometry.

Out of the 20 extracts tested, only the *D. anomala* root, *X. undulatum* root, and *C. paniculata* leaf DCM extracts were toxic enough to the two cell lines with IC₅₀ values of 1.8, 26.52, and 7.71 µg/mL for Caco2. For MCF7, the IC₅₀ values were 2.24, 8.99, and 25.74 µg/mL respectively. After 48 hours of treatment with the three extracts, an arrest of early mitosis was evident for the *C. paniculata* and *X. undulatum* extracts on both cell lines; however, for the *D. anomala* extract, the MCF7 cells arrested at the G0/G1 phase, while the Caco2 cells arrested at the early M phase.

Annexin V- FITC staining confirmed apoptosis for the *X. undulatum* extract as a decrease in mitochondrial membrane potential was evident for the Caco2 cells and

relative hyperpolarisation was evident for the MCF cells. The Annexin V-FITC / Plassay showed that there were apoptotic cells after 24 hours of treatment with the *C. paniculata* leaf extract and the *D. anomala* root for the Caco2 cells, but apoptotic cells were evident in the MCF7 cells only after 48 hours.

The aqueous *E. elephantina* extract exhibited anti-inflammatory activity without inducing cytotoxicity against RAW 264.7 macrophages. The *D. anomala* extract also showed a reduction in nitric oxide production at 200 µg/mL; however, this was probably as a result of the associated toxicity at that extract concentration. Due to high activity of *X. undulatum*, its extracts, were selected for identification characterization of present compounds. Chromatographic fingerprinting of the *X. undulatum* medicinal plant resulted in a total of 24 compounds identified from the DCM, MeOH, dH₂O, and DCM:MeOH extracts, and from the 24, five were unknown.

This study indicates that *C. paniculata*, *D. anomala*, *E. elephantina*, and *X. undulatum* medicinal plants could be good candidates for new drug therapies.

TABLE OF CONTENTS

DECLARATION OF INDEPENDENT WORK	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	iii
LIST OF FIGURES	x
LIST OF TABLES.....	xii
LIST OF ABBREVIATIONS	xiii
LIST OF UNITS.....	xiv
LIST OF SYMBOLS	xv

CHAPTER 1: INTRODUCTION AND OVERVIEW OF THE STUDY

1.1 INTRODUCTION	1
1.1.1 Cancer as a problem.....	1
1.1.2 Current cancer treatments and their problems.....	2
1.1.3 Medicinal plants as a source of drugs.....	7
1.2 JUSTIFICATION OF THE STUDY	8
1.3 AIM.....	9
1.4 OBJECTIVES.....	9
1.5 SELECTION OF STUDY PLANTS.....	9
1.6 LAYOUT OF THE STUDY	10
1.7 OVERVIEW OF CHAPTERS	10
1.8 REFERENCES	11

CHAPTER 2: THE SELECTED MEDICINAL PLANTS

2.1 TRADITIONAL MEDICINES AND THEIR USES	19
2.2 <i>CUSSONIA PANICULATA</i>	21
2.2.1 Description	22

2.2.2	Distribution	22
2.2.3	Habitat.....	22
2.2.4	Special aspects.....	22
2.2.5	Traditional uses.....	23
2.3	<i>DICOMA ANOMALA</i>	23
2.3.1	Description	25
2.3.2	Distribution	25
2.3.3	Habitat.....	25
2.3.4	Special aspects.....	25
2.3.5	Traditional uses.....	25
2.4	<i>ELEPHANTORRHIZA ELEPHANTINA</i>	27
2.4.1	Description	27
2.4.2	Distribution	28
2.4.3	Habitat.....	28
2.4.4	Special aspects.....	28
2.4.5	Traditional uses.....	28
2.5	<i>XYSMALOBIUM UNDULATUM</i>	29
2.5.1	Description	30
2.5.2	Distribution	31
2.5.3	Habitat.....	31
2.5.4	Special aspects.....	31
2.5.5	Traditional uses.....	31
2.6	REFERENCES	32

CHAPTER 3: PHYTOCHEMICAL ANALYSIS

3.1	INTRODUCTION	39
3.2	BRIEF LITERATURE OVERVIEW.....	40
3.2.1	Alkaloids.....	40

3.2.2	Flavonoids.....	41
3.2.3	Tannins	41
3.2.4	Terpenoids	42
3.2.5	Saponins	42
3.3	METHODOLOGY	43
3.3.1	Plant material collection and verification	43
3.3.2	Extraction method	43
3.3.3	Qualitative phytochemical testing (as described by Harborne, 1973)	44
3.3.3.1	Test for tannins	44
3.3.3.2	Test for phlobatannins.....	44
3.3.3.3	Test for saponins.....	44
3.3.3.4	Test for flavonoids.....	45
3.3.3.5	Test for steroids	45
3.3.3.6	Test for terpenoids (Salkowski test)	45
3.3.3.7	Test for alkaloids	45
3.3.3.8	Test for phenols	45
3.4	RESULTS AND DISCUSSION.....	45
3.5	CONCLUSION	47
3.6	REFERENCES	48

CHAPTER 4: IN VITRO CYTOTOXICITY/ANTI-PROLIFERATIVE ACTIVITY OF *D. ANOMALA*, *E. ELEPHANTINA*, *C. PANICULATA*, AND *X. UNDULATUM* PLANT EXTRACTS

4.1	INTRODUCTION	53
4.2	BRIEF LITERATURE OVERVIEW	53
4.2.1	Apoptosis	54
4.2.2	The cell cycle	56
4.2.3	Cytotoxicity.....	57
4.2.4	Mitochondrial membrane potential	58

4.2.5	Phosphatidylserine (PS) translocation	58
4.3	METHODOLOGY	59
4.3.1	Cytotoxicity and IC ₅₀ determination	59
4.3.2	Cell cycle analysis	60
4.3.3	Mitochondrial membrane potential analysis	61
4.3.4	PS translocation	61
4.3.5	Statistical analysis	62
4.4	RESULTS AND DISCUSSION	62
4.4.1	Cytotoxicity and IC ₅₀ determination	62
4.4.2	Cell cycle analysis	68
4.4.3	Mitochondrial membrane potential analysis	69
4.4.4	PS translocation	70
4.5	CONCLUSION	73
4.6	REFERENCES	75

CHAPTER 5: ANTI-INFLAMMATORY ACTIVITY

5.1	INTRODUCTION	79
5.2	BRIEF LITERATURE REVIEW	80
5.2.1	Inflammation and cancer	80
5.2.2	Nuclear factor kappa B (NF-κB)	81
5.2.3	Cyclooxygenase-2 (COX-2)	82
5.2.4	Nitric oxide (NO)	83
5.3	METHODOLOGY	84
5.4	RESULTS AND DISCUSSION	85
5.5	CONCLUSION	87
5.6	REFERENCES	89

CHAPTER 6: IDENTIFICATION AND CHARACTERISATION OF *X. UNDULATUM* PLANT EXTRACTS

6.1	INTRODUCTION	93
6.2	BRIEF LITERATURE OVERVIEW	93
6.3	METHODS	95
6.3.1	Extraction	95
6.3.2	Liquid chromatography-mass spectrometry (LC-MS) analysis	95
6.4	RESULTS AND DISCUSSION.....	97
6.5	CONCLUSION	101
6.6	REFERENCES	102

CHAPTER 7: GENERAL CONCLUSION AND RECOMMENDATIONS

7.1	INTRODUCTION	Error! Bookmark not defined.
7.2	CONCLUSION	Error! Bookmark not defined.
7.3	RECOMMENDATIONS.....	107

LIST OF FIGURES

Figure 1.1:	Layout of the study	10
Figure 2.1:	(A) <i>C. paniculata</i> leaves and stem; (B) close-up of <i>C. paniculata</i> leaves	21
Figure 2.2:	(A): <i>D. anomala</i> flowers and leaves; (B): <i>D. anomala</i> dried root	24
Figure 2.3:	(A) <i>E. elephantina</i> stem and leaves; (B) <i>E. elephantina</i> dried roots	27
Figure 2.4:	(A) <i>X. undulatum</i> leaves, stem, and flowers; (B) <i>X. undulatum</i> dried root	30
Figure 3.1:	Morphine's chemical structure	40
Figure 3.2:	Flavanol structure	41
Figure 3.3:	Structure of a tannin molecule	42
Figure 3.4:	Isoprene structure	42
Figure 3.5:	Structure of a saponin	43
Figure 4.1:	Intrinsic apoptotic pathway	55
Figure 4.2:	Extrinsic apoptotic pathway	56
Figure 4.3:	Schematic representation of an eukaryotic cell cycle with the positions of checkpoints	57
Figure 4.4:	Schematic representation of the Annexin V assay for apoptosis detection	59
Figure 4.5:	Cytotoxicity of <i>C. paniculata</i> root and leaf extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure	63
Figure 4.6:	Cytotoxicity of <i>E. elephantina</i> root extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure	64
Figure 4.7:	Cytotoxicity of <i>X. undulatum</i> root extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure	65
Figure 4.8:	Cytotoxicity of <i>D. anomala</i> root extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure	66
Figure 4.9:	Cell cycle analysis of the Caco2 (A) and MCF7 (B) cell lines	68
Figure 4.10:	PS translocation on Caco2 (A) and MCF7 (B) cells after 24 hours of treatment with selected extracts and subsequent staining with Annexin V-FITC and PI	71

Figure 4.11: PS translocation on Caco2 (A) and MCF7 (B) cells after 48 hours of treatment with Annexin V-FITC and PI staining	72
Figure 5.1: An example of a pro-carcinogenic scenario that occurs during chronic inflammation	81
Figure 5.2: NF- κ B function in cancer and its molecular targets	82
Figure 5.3: Schematic presentation of how COX-2 contributes to cancer	83
Figure 5.4: NO's contribution to tumour progression	84
Figure 5.5: The effect of aqueous extracts on NO production (A) and cell viability (B) in RAW 264.7 macrophages.....	86
Figure 6.1: LC-MS chromatogram of <i>X. undulatum</i> root	97

LIST OF TABLES

Table 1.1:	Treatment for non-metastasised breast cancer	4
Table 1.2:	Drugs used for metastatic breast cancer and their side effects.....	5
Table 1.3:	Drugs discovered from medicinal plants.....	8
Table 2.1:	Scientific and common names of <i>C. paniculata</i>	21
Table 2.2:	Scientific and common names of <i>D. anomala</i>	24
Table 2.3:	Traditional uses of <i>D. anomala</i>	26
Table 2.4:	Scientific and common names of <i>E. elephantina</i>	27
Table 2.5:	Scientific and common names of <i>X. undulatum</i>	30
Table 3.1:	Phytochemical screening results of the four medicinal plants tested	46
Table 4.1:	Summary of IC ₅₀ values determined from GraphPad Prism version 6 software	67
Table 4.2:	Changes in mitochondrial membrane potential after treatment with melphalan and plant extracts for 24 hours	69
Table 6.1:	Summary of the most abundant compounds present in <i>X. undulatum</i> plant extracts	97
Table 6.2:	Quantities of identified compounds per extract (highest peak intensity).....	99

LIST OF ABBREVIATIONS

AG	Aminoguanidine
Caco	Colon carcinoma
Caco2	Colorectal adenocarcinoma cell line
COX	Cyclooxygenases
COX-2	Cyclooxygenase-2
DAPI	4',6-diamino-2-phenylindole dihydrochloride
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ERBB2	Erythroblastic oncogene B2
Fas	FS-7-associated surface antigen
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
G0 phase	Quiescent phase
G1 phase	Gap phase 1 of the cell cycle
G2 phase	Gap phase 2 of the cell cycle
GLOBOCAN	Global Cancer Observatory
HIV	Human immunodeficiency virus
HPLC-NMR	High performance liquid chromatography-nuclear magnetic resonance
HPV	Human papillomavirus
IC ₅₀	Half maximum inhibitory concentration
iNOS2	Inducible nitric oxide synthase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
M phase	Mitotic stage of the cell cycle
MCF	Michigan Cancer Foundation
MCF7	Human breast cancer cell line
MSE	Microwave solvent extraction
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mz	Mass-to-charge ratio
NF54	Strains of plasmodium falciparum
NF-κB	Nuclear factor kappa B
NOS	Nitric oxide synthase

PBS	Phosphate buffered saline
PI	Propidium iodide
PS	Phosphatidylserine
RAW 264.7	Mouse macrophage cells
RPMI	Roswell Park Memorial Institute
Rt	Retention time
S phase	Synthesis of DNA during the cell cycle
SANBI	South African National Biodiversity Institute
SD	Standard deviation
TB	Tuberculosis
TMRE	Tetramethylrhodamine ethyl ester
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
TRITC	Tetramethylrhodamine-5-(and 6)-isothiocyanate
UPLC	Ultra-performance liquid chromatograph
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

LIST OF UNITS

°C	Degree(s) Celsius
µg/mL	Microgram(s) per millilitre
µL	Microlitre(s)
cm	Centimetre(s)
g	Gram(s)
g/mol	Grams per mole
L/h	Litre(s) per hour
m	Metre(s)
m/z	Mass per charge
mg	Milligram(s)
mg/L	Milligram(s) per litre
mg/mL	Milligram(s) per milliliter
mL	Millilitre(s)
mm	Millimetre(s)

mV	Millivolt(s)
ng/mL	Nanogram(s) per millilitre
nm	Nanometre(s)
nM	Nanomolar(s)
V	Volt(s)
μM	Micromolar(s)

LIST OF SYMBOLS

Ca^{2+}	Calcium ion
CO_2	Carbon dioxide
dH_2O	Distilled water
FeCl_3	Ferric chloride
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
MeOH	Methanol
Mg^{2+}	Magnesium ion
NO	Nitric oxide
O^{2-}	Oxide ion
ONOO^-	Peroxynitrite

CHAPTER 1:

INTRODUCTION AND OVERVIEW OF THE STUDY

1.1 INTRODUCTION

1.1.1 Cancer as a problem

Cancer is a term that describes a group of diseases that can be associated with uncontrollable and abnormal proliferation of body cells that can occur in any part of the body (National Institutes of Health, 2020). Cancer is a genetic and therefore hereditary disease. There are various external cancer-causing agents called carcinogens, which can be classified as biological, physical, and chemical carcinogens. Biological carcinogens include bacterial, parasitic, and viral infections, while physical carcinogens include radiation and ultraviolet (UV) exposure. Chemical carcinogens are found in tobacco, asbestos, aflatoxin or arsenic; to name a few (Blackadar, 2016).

Over the past 20 years, cases of people diagnosed with cancer have increased rapidly across the world (Phillips & Currow, 2010). It is one of the leading causes of death globally and accounted for almost 10 million deaths in 2020 (World Health Organization [WHO], 2022). According to the Global Cancer Observatory (GLOBOCAN, 2018), 14.1 million new cases and 8.2 million deaths were caused by cancer in 2011, with lung cancer at 1.82 million, breast cancer at 1.67 million, and colorectal cancer at 1.36 million cases worldwide. Moreover, in Africa alone there were 847 000 new cancer cases, which underscores the magnitude of the cancer crisis in African countries (Ferlay et al., 2015).

Made et al. (2017) predicted an increase in cancer mortality rates in developing countries due to related incidences or cases including ageing and population growth. These incidences also influence some risk factors associated with economic transitions, which are communicable diseases, unhealthy diets, reproductive behaviours, and other lifestyle tendencies such as smoking and alcohol consumption (Wu et al., 2015).

In 2018, GLOBOCAN recorded 18.1 million cases and 9.8 million cancer deaths. The Union for International Cancer Control emphasised that Asia and Africa had higher

percentages of cancer deaths than their respective incidences since 2012, with Asia at 57.3% and Africa at 7.5%, which is due to high cases of fatality rates in those regions. This was also evident in 2020 cancer reports, where mortality rates were 58.3% in Asia and 7.2% in Africa, whereas these incidences were lower with 49.3% and 5.7% respectively (GLOBOCAN, 2018). In 2020, there were approximately 19 million newly reported cancer cases worldwide, with breast cancer the highest at 11.7%, followed by lung cancer at 11.4%, and colorectal cancer at 10% (WHO, 2022).

The statistics above confirm that cancer is a continuous problem as the numbers of deaths and new cases continue to increase. Breast and cervical cancer affect mostly women, and while men are also affected by breast cancer, cases are rare at 1% of all breast cancer cases (Giordano, 2018). Age, predisposed genetic cancer genes, environmental sources such as exposure to radiation, and family history are some of the risk factors associated with breast cancer in men (Giordano et al., 2004). With regard to cervical cancer in women, the only link is human papillomavirus (HPV) infection. In men it is linked to other infections or diseases such as penile cancers or genital warts as opposed to cervical cancer in women (Doheny, 2022). Although breast cancer is reported to be the major contributor to the increasing number of new cases, it is not the leading cause of cancer deaths as it placed fifth in cancer mortality ranks (WHO: International Agency for Research on Cancer, 2018; WHO, 2022).

1.1.2 Current cancer treatments and their problems

One of the reasons behind the rapid growth in cancer cases, especially breast and cervical cancer, is due to the lack of a cure for the disease (Venkatas & Singh, 2020; Ekici & Jawzal, 2020). Furthermore, the invasive methods or techniques currently used to treat these types of cancer have adverse side effects such as damage to the reproductive system, removal of the breasts, hair loss, vomiting, and others. At times, many cancer survivors end up with long-term disabilities and need constant care and support. This could be due to significant experiences of physical and psychological morbidity, and the affected individuals (both men and women) are burdened, often psychologically as well, by the inability to reproduce as a result of cancer (Redd et al., 2001).

A number of treatment options for both breast and cervical cancer are currently available and they all depend on how much the cancer has grown or if the cancer cells have metastasised. Breast cancer is divided into four different tumour subtypes, namely Luminal A, Luminal B, triple-negative, and human epidermal growth receptor 2. These tumours are treated differently depending on the following: absence or presence of molecular markers for oestrogen, progesterone receptor expression, and erythroblastic oncogene B2 (ERBB2) gene amplification (Waks & Winer, 2019).

Table 1.1 summarises currently available treatment methods for non-metastasised breast cancer and the side effects associated with each type of treatment and drug. Surgery is another type of treatment therapy for non-metastatic breast cancer that is a standard approach used to minimise the continuous follow-ups related to local therapy. The two approaches usually used are total mastectomy or excision together with radiation (Swain et al., 2015). The above-mentioned treatment types are the standard treatment approaches used because it is believed that patients have better overall survival and zero chances of relapse after the surgery. However, contradictory to this, many patients who underwent surgery tend to have complications that include collagen vascular diseases such as scleroderma, positive pathologic margins after lumpectomy, and sometimes disease that, except in very few cases, cannot be treated by removing just a small portion of breast tissue with satisfactory cosmetic results (Waks & Winer, 2019).

Table 1.2 displays drugs used for the treatment of metastatic breast cancer along with their known side effects that survivors and patients still on treatment experience. It also displays the dangers of synthetic or chemically produced pharmaceutical drugs and the quality of life experienced by cancer patients after treatment.

Table 1.1: Treatment for non-metastasised breast cancer

Ingredient	Type of therapy	Common effect	Uncommon but occurring effect	Reference
Tamoxifen	Endocrine therapy	Hot flashes	Uterine cancer, thromboembolic disorder (blood clots)	Francis et al. (2018); Pagani et al. (2014)
Aromatase inhibitor (exemestane, letrozole, or anastrozole)	Endocrine therapy	Hot flashes, joint stiffness	Osteoporosis	Davies et al. (2013); Sanz and Del Valle (2016)
Cyclophosphamide/docetaxel	Cytotoxic chemotherapy	Oedema, myalgias, asthenia, myelosuppression	Febrile neutropenia	Blum et al. (2017); Portier et al. (2006)
Adriamycin/cyclophosphamide	Cytotoxic chemotherapy	Asthenia, myelosuppression, nausea	Cardiac death, leukaemia, febrile neutropenia	Early Breast Cancer Trialists' Collaborative Group (2012); Blum et al. (2017); Portier et al. (2006)
Adriamycin/cyclophosphamide/paclitaxel	Cytotoxic chemotherapy	Asthenia, myelosuppression, nausea, sensory neuropathy	Cardiac death, leukaemia, febrile neutropenia	Early Breast Cancer Trialists' Collaborative Group (2012)
Adriamycin/cyclophosphamide/paclitaxel/trastuzumab	Cytotoxic chemotherapy and ERBB2-directed therapy	Asthenia, myelosuppression, nausea, sensory neuropathy	Congestive heart failure	Porter et al. (2011)
Docetaxel/carboplatin/trastuzumab	Cytotoxic chemotherapy and ERBB2-directed therapy	Asthenia, sensory neuropathy, myelosuppression	Febrile neutropenia, congestive heart failure	Porter et al. (2011)

Table 1.2: Drugs used for metastatic breast cancer and their side effects

Name of the drug	Side effects	References
Trastuzumab emtansine	Fatigue, nausea, elevation of transaminase, thrombocytopenia	Verma et al. (2012)
Olaparib	Fatigue, nausea, thrombocytopenia, myelosuppression	Robson et al. (2017)
Talazoparib	Fatigue, nausea, thrombocytopenia, myelosuppression	Litton et al. (2018)
Palbociclib	Myelosuppression, thrombocytopenia, fatigue	Turner et al. (2015)
Ribociclib	Fatigue, elevation of transaminase, thrombocytopenia, myelosuppression	Quandt et al. (2011)
Abemaciclib	Fatigue, nausea, elevation of transaminase, thrombocytopenia, diarrhoea, myelosuppression	Goetz et al. (2017)

Colorectal cancer is a type of cancer that affects the rectum or colon (large intestine). Although the specific cause of colorectal cancer is often unknown, there are a number of risk factors that may contribute to the prevalence or risk of being affected by this cancer. These risk factors includes; high consumption of red or processed meat diet, obesity, smoking, heavy alcohol consumption, age, family history of colorectal cancer, personal history of colorectal polyps or inflammatory bowel disease, specific genetic condition and lack of physical activities (Centre for Disease control and prevention (CDC), 2020).

Although often many people will display no symptoms during the early stages of the disease, some of the common symptoms include, blood in stool, sudden weight loss, diarrhoea, decreased iron levels and constipation (Sawicki et al., 2021).

World Health Organization (2023) reported that colorectal cancer is the second leading cause of death relating to cancer and the third most common cancer worldwide. In 2018, 1.80 million new cases of colorectal cancer were diagnosed and 862,000 patients died from this disease (Ahmed, 2020; Kanth & Inadomi, 2021). Moreover there were over 1.9 million new cases of and over 930 000 deaths by colorectal cancer in 2020, and it is further predicted that the annual burden of colorectal cancer is expected to increase to 3.2 million new cases (a 63%increase) and 1.6 million deaths (73% increase) by 2040 (WHO, 2023). The above predictions are made based on ageing, population increase and human development, furthermore, increase in exposure to environmental risk factors brought on by the shift in diet and life style towards westernisation is also thought to primarily be responsible for the increase in

the prevalence of colorectal cancer, therefore this is also causing a shift towards low-income and middle income countries as they are becoming more westernized (Keum & Giovannucci, 2019; Xi & Xu, 2021).

A review by Biller & Schrag (2021) reported that in the USA, among the people who are diagnosed with metastatic colorectal cancer, about 70% to 75% survive beyond 1 year, 30% to 35% beyond 3 years, and fewer than 20% beyond 5 years from diagnosis.

There are common and less common subtypes of colorectal cancer, less common types includes; carcinoid tumors, lymphomas, sarcomas and gastrointestinal stromal tumors, whereas common subtypes are rectum, proximal colon and distal colon. Since colorectal cancer is a heterogeneous disease, this makes the prognosis and therapeutic responses different depending on the affiliation of the cancer subtype and also the heterogeneity of the risk factors (Li & Lai, 2009; Keum & Giovannucci, 2019; Xi & Xu, 2021).

Molecular heterogeneity suggests that distal colon cancer is usually associated with a specific chromosomal instability, whereas proximal colon cancer is associated with nucleotide instability pathway, distal colon cancer is however related to specific chromosomal instability (Murphy et al., 2019). Different environmental risks factors also contribute to different types of cancers, on the review by Xi & Xu (2021) they found that lack of physical activity is mostly associated with proximal colon cancer but not distal or rectal cancer. Current Smoking was associated with both proximal and rectal cancer. High alcohol consumption was associated with distal colon and rectal cancers. In conclusion the above mentioned risk factors may also associate with prevalent diabetes and late stage menopause to increase the chance and vulnerability to contracting colorectal cancer (Murphy et al., 2019; Botteri et al., 2020).

Current and efficient methods of prevention of colorectal cancer are; physical activity which will help control body weight gain and is also beneficial for inflammatory and lipid pathways which are likely to influence tumours in the colon. Good diet is also another method of prevention since it is associated with high intake fibre, fruits and vegetables and also avoiding high consumption of red and processed meat. Avoiding alcohol consumption and smoking which could cause some genetic alterations. Moreover the primary prevention strategy implemented in most countries is screening

which increases early detection and helps eliminate precancerous lesions (Hagggar & Boushey, 2009; Algra & Rothwell, 2012; Buskermolen et al., 2019).

In general, cancer is treated the same way – using chemotherapy, surgery, and radiotherapy. The ingredients used and their side effects were listed in Tables 1.1 and 1.2. These adverse effects and contraindications to current cancer treatment clearly indicate that there is a need for other alternatives to treat breast and cervical cancer. Plants are rich in phytochemicals, which have unique medicinal properties that can be of importance, are beneficial to humans, and could lead to the discovery of novel anti-cancer drugs.

1.1.3 Medicinal plants as a source of drugs

Plants have been recognised by humans since the dawn of time, and they have been utilised in several ways throughout history. Plants have played a very important role in the supply of food, medication, clothing, and shelter; among many others. Ancient people, in their search for food, were also able to identify plants that have medicinal purposes. As a result, medicinal plants as a source of ailment relief can be traced back over five millennia to early civilisation (Kalaivani et al., 2012; Shakya, 2016). Even though plants have been used for thousands of years, they continue to present humans with new therapies due to their vast medicinal properties. Natural products and their derivatives account for more than half of all drugs authorised by the American Food and Drug Administration (Chavan et al., 2018). Moreover, in developing countries, between 70% and 90% of the population still use traditional medicines and this is because of the most potent and promising element, namely secondary metabolites, in these plants (Chin et al., 2006; Robinson & Zhang, 2011). For these reasons, natural products have been extensively investigated in the search for novel drug discoveries (Chen et al., 2015).

Many drugs have been derived from medicinal plants already; from the treatment of headache, toothache, cancer, malaria, and leukaemia to analgesic and anti-

inflammatory agents, among others. Table 1.3 lists some drugs that were derived from medicinal plants and are already on the market.

Table 1.3: Drugs discovered from medicinal plants

Name of drug	Uses	Plant used
Paclitaxel (Taxol®)	Treatment of breast, ovarian, lung, bladder, prostate, melanoma, and oesophageal cancer	<i>Taxus brevifolia</i> Nutt.
Etoposide	Treatment of tumours and lung cancer	<i>Podophyllum peltatum</i>
Taxol®	Treatment of ovarian, oesophageal, breast, lung cancer, Kaposi's sarcoma, cervical, and pancreatic cancer	<i>T. brevifolia</i>
Vincristine	Anti-tumour, anti-leukemic agent	<i>Catharanthus roseus</i>
Podophyllotoxin	Treatment of genital warts	<i>Podophyllum peltatum</i>
Vinblastine	Treatment of Hodgkin's lymphoma, leukaemia, and testicular cancer	<i>C. roseus</i>
Morphine	Treatment of pain	<i>Papaver somniferum</i>

Phytochemicals, also known as secondary metabolites, are abundant in plants and have unique medicinal capabilities. Secondary metabolites are metabolic intermediates that are not required for the plant's survival but rather play a significant role in the plant's interaction with its surroundings. Furthermore, plants use these chemicals to defend themselves from biotic and abiotic stresses (Pagare et al., 2015). Humans found a way of using this to their advantage, as they use secondary metabolites for a variety of purposes, including medicinal purposes. Medicinal plants provide unrivalled benefits when it comes to innovative medical discoveries such as their unique diversity of chemical structure and biological activity. As a result, modern medicinal plant drug discovery research employs a multi-layered strategy that incorporates botanical, phytochemical, biological, and molecular approaches (Kala et al., 2006). In search of the discovery of novel anti-cancer drugs for breast and cervical cancer, this study investigated the anti-cancer properties of *C. paniculata*, *D. anomala*, *E. elephantina*, and *X. undulatum*.

1.2 JUSTIFICATION OF THE STUDY

Due to harmful side effects, reduced quality of life, and relapse of cancer patients after treatment with current anti-cancer drugs, there is a need for alternative methods of treatment. In addition to these unsatisfactory outcomes of current treatment options, there is a need for affordable cancer therapy procedures that will benefit mostly low-

and middle-income countries, which may help to control and hopefully reduce the rapid increase of cancer cases and mortalities.

C. paniculata, *D. anomala*, *E. elephantina*, and *X. undulatum* are some of the largely unexplored plants with reported medicinal benefits. These plants are reportedly used by traditional healers and herbalists for the treatment of breast and cervical cancer; among many other ailments. However, these claims have not been scientifically validated.

1.3 AIM

The aim of this study was to investigate the anti-cancer activities of *E. elephantina*, *D. anomala*, *X. undulatum*, and *C. paniculata* plant extracts.

1.4 OBJECTIVES

The objectives of the study were to extract plants with solvents of different polarities and perform phytochemical analysis on the selected extracts. The crude plant extracts were further investigated for their in vitro cytotoxic activity against cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To determine the cytotoxic/anti-proliferative mechanism of action, the effects of selected plant extracts on cell cycle progression and apoptosis induction were investigated. Finally, the plant extract that displayed the most satisfactory activity was further characterised by liquid chromatography-mass spectrometry (LC-MS).

1.5 SELECTION OF STUDY PLANTS

A literature review was conducted on medicinal plants that were least explored or underexplored as anti-cancer agents following ethnobotanical reports on traditional medicinal plants used in South Africa for the treatment of cancer. Four plants that have not previously been investigated as anti-cancer agents for breast and cervical cancer were selected and investigated in this research project, namely *C. paniculata* (CP2021CUT), *D. anomala* (DA2021CUT), *E. elephantina* (GP0020), and *X. undulatum* (XU2021CUT). The roots of these plants, and the leaves in the case of *C. paniculata*, were selected because they are the parts of the plants that traditional healers and herbalists use in the remedies or concoctions.

1.6 LAYOUT OF THE STUDY

The process of the study is illustrated in Figure 1.1.

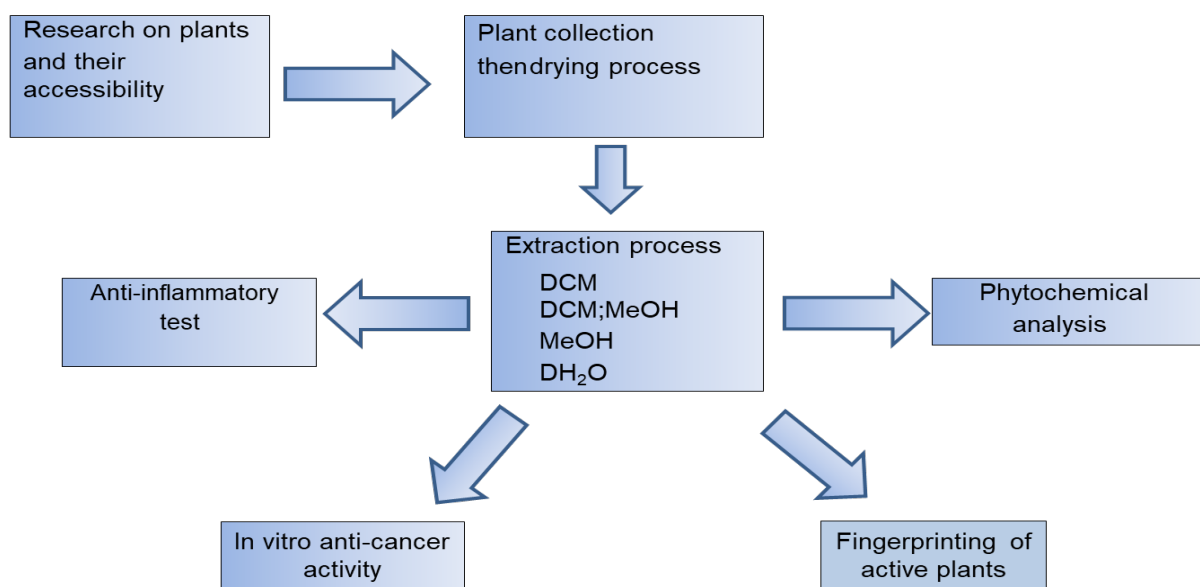


Figure 1.1: Layout of the study

1.7 OVERVIEW OF CHAPTERS

Chapter 1 summarises cancer and cancer as problem. This chapter also explains how medicinal plants can be an alternative source of cancer treatments and mentions existing drugs that were derived from medicinal plants.

Chapter 2 presents an overview of selected medicinal plants. The chapter also justifies the choice of medicinal plants given the history, traditional uses, and the dependency on traditional medicine by traditional healers, herbalists, and underdeveloped communities.

Chapter 3 summarises the phytochemical constituents of the studied plants. Qualitative phytochemical analysis was performed to screen the selected plants for the presence of dominant secondary metabolites in order to estimate the plants' medicinal properties relative to phytochemical constituents, where the secondary metabolites present were indicated by a change in colour when adding a specific reagent to the mixture.

Chapter 4 presents the in vitro cytotoxic activity / anti-proliferative activity of the four medicinal plant extracts of different polarities against the human breast cancer cell line

MCF7 and the colorectal adenocarcinoma cell line Caco2. Extracts that showed good activity against the two cell lines were further investigated to examine the mechanism of cell death induced by the extracts.

Chapter 5 outlines the anti-inflammatory activity of the aqueous extracts, where nitric oxide (NO) production was investigated in response to RAW 264.7 and to confirm the absence of toxicity as a contributory factor. Cell viability was assessed using the MTT assay.

Chapter 6 summarises the fingerprinting of the most active plant, namely *X. undulatum*.

Chapter 7 presents the general conclusion of the study.

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CHAPTER 2:

THE SELECTED MEDICINAL PLANTS

2.1 TRADITIONAL MEDICINES AND THEIR USES

In many communities around the world, medicinal plants have been used and are still being used extensively for the treatment and prevention of many diseases, as well as the promotion of good health in general. The link between humans and their hunt for medicine in nature dates back thousands of years (Stojanoski, 1999). Throughout history, humans have relied on nature for their basic needs such as medicines, flavours, shelter, food, fertilisers, fragrances, clothes, and modes of transportation (Gurib-Fakim, 2006). Humans learned to search for medication in tree bark, seeds, leaves, fruits, and other parts of the plant in their pursuit to fight illness (Petrovska, 2012).

According to the Bible, during different ceremonies following treatment, aromatic plants such as myrtle and incense were used (Genesis 37:25; Matthew 2:11). Moreover, oils of the *Commiphora* species (myrrh), *Cedrus* species (cedar), *Glycyrrhiza glabra* (licorice), *Papaver somniferum* (poppy), and *Cupressus sempervirens* (cypress) are still used today for the cure of diseases that range from colds and coughs to inflammation and parasitic infections, according to the earliest reports carved on clay tablets in cuneiform dating from around 2600 (Dar et al., 2017). Medicinal plants continue to play a significant role in the healthcare system for vast segments of the global population, particularly in developing countries where herbal medicine has a long history of usage. In both developed and developing countries, the discovery and acknowledgement of the medical and economic benefits of these plants are increasing (Dar et al., 2017).

Regardless of how popular and advanced Western medication is around the world, traditional medicine is still used in both developed and developing countries to treat illnesses and diseases, but is mostly popular in developing countries (Dey et al., 2012). In the majority of the developing countries, traditional medicines are the primary source of medication due to the scarcity of healthcare systems and challenges around the healthcare system such as long distances to hospitals, drug shortages, and a long wait to be assisted in hospitals. However, in some cases the use of traditional

medicines is preferred due to historical uses and ethnobotanical knowledge that has been passed from generation to generation (Koduru et al., 2007; Chinsembu, 2016).

Traditional medicine is highly important in South Africa and other African countries as they are often the sole reliable source of basic healthcare. In the eastern Free State in South Africa, the majority of people still rely on agricultural practices and cultural beliefs, which include the use of traditional medicinal herbs (Mothibe & Sibanda, 2019). This results from the poor economic status and limited employment opportunities in that province. Many traditional healers in this province are known to help the communities with the management of many different ailments that include blood pressure, human immunodeficiency virus (HIV), ulcers, different kinds of infections, cancer, tuberculosis (TB), and diarrhoea, among many others, using traditional medicinal plants (Fennell et al., 2004). This has prompted many researchers to conduct research on locally available natural products such as medicinal plants, which are presumed safe and effective, affordable with fewer side effects, and culturally acceptable (Xaba, 2016; Semanya et al., 2018).

According to the literature, when choosing plants to study for their anti-cancer properties, researchers should take into account their ethnopharmacological uses, such as those for inflammatory, infectious, parasitic, and viral illnesses, as these represent disease states that are related to cancer or cancer symptoms (Cordell et al., 1991; Popoca et al., 1998; Steenkamp & Gouws, 2006). Random screening of natural compounds has been a very useful tool or methodology for identifying novel biologically active compounds that have been the source of hundreds of new therapeutic agents (Kalaivani et al., 2012).

For the purposes of this study, the focus was on four medicinal plants, namely *C. paniculata*, *D. anomala*, *E. elephantina*, and *X. undulatum* in search of anti-cancer activities. These plants have been reported for their ability to cure breast and cervical cancer by traditional healers and herbalists; however, these claims have not been scientifically validated.

2.2 CUSSONIA PANICULATA



Figure 2.1: (A) *C. paniculata* leaves and stem; (B) close-up of *C. paniculata* leaves

Source: Researcher (2020)

Table 2.1: Scientific and common names of *C. paniculata*

Family	Scientific name	Common names
Araliaceae	<i>Cussonia paniculata</i>	English: mountain cabbage tree
		Afrikaans: bergkiepersol
		isiXhosa/siSwati: umsenge
		isiZulu: umsengembuzi
		Sesotho: motshetse

Source: The Plant List (2010)

C. paniculata belongs to Araliaceae family, which is a family of mostly angiosperms, with approximately 46 genera. Within these genera, 1 400 species have been identified (The Plant List, 2010; Walker & Levers, 2012). Araliaceae is reported to be rich in different types or classes of secondary metabolites such as cerebrosides, saponins, sterols, triterpenes, diterpenes, and acetylenic lipids, which have diverse biological activities (Ashmawy et al., 2020). Among the genera in this family are the ginseng (*Panax*) and ivy (*Hedera*). The ginseng genus consists mostly of trees or shrubs and only a few herbs and climber plants, whereas the ivy genus consists of

house plants and ornamental plants grown for display purposes (Encyclopaedia Britannica, 2015).

2.2.1 Description

C. paniculata is a slow-growing pachycaul succulent tree with a thick or swollen stem. The roots of the plant are also swollen and thick and can grow up to 5 m high. The tree has large and digitate leaves that are cabbage blue in colour with a thick wax layer. They have seven to nine, but sometimes up to 13, leaflets that can grow up to 30 cm in length. Newly emerging leaves are green in colour. They bear small green stalked flowers in spikes that form a big, branched inflorescence at the end of the branches, which occurs from January to April. The branches become fleshy purple and maroon fruits start to appear during May to June (Reyneke & Kok, 1987; South African National Biodiversity Institute [SANBI], 2002).

There are two subspecies of *C. paniculata*, namely *C. paniculata* subsp. *paniculata* and *C. paniculata* subsp. *sinuata*. The only differences between the two subspecies are their leaves and size. *C. paniculata* subsp. *paniculata* is a smaller tree that has leaflets with no lobes, whereas *C. paniculata* subsp. *sinuata* is a larger tree that has deeply lobed leaves and it is the more common one of the two (Reyneke & Kok, 1987; SANBI, 2002).

2.2.2 Distribution

In South Africa, *C. paniculata* is found in the Eastern Cape, KwaZulu-Natal, Free State, and Gauteng provinces. It is also found in Lesotho and southeastern Botswana (SANBI, 2002; Walker & Levers, 1988).

2.2.3 Habitat

C. paniculata is usually found in mountains, rocky places, and high-altitude grasslands of up to 2 100 m.

2.2.4 Special aspects

It is a medicinal plant that can tolerate frost and survive drought.

2.2.5 Traditional uses

The leaves of *C. paniculata* are used as a food source for goats, the wood of the tree is used in wagons for brake blocks, and the roots can be used as a source of water and food for humans, where they are eaten raw, and the tree also provides good shade (SANBI, 2002).

Moreover, there are many traditional uses of this plant and different parts of it are used to treat different illnesses. Tetyana et al. (2002) reported that *C. paniculata* leaves are traditionally used to treat early nervous and mental diseases in the form of a decoction and in the form of an enema for the treatment of colic and dysmenorrhea. Other traditional uses of the plant include performance of magic tricks, treatment of malaria, as an analgesic, and for inflammation and wounds (De Villiers et al., 2010). Tribal communities such as the Xhosas use the raw fruits of the tree as a dietary supplement and the roots of the tree are peeled and eaten raw as food or a source of water (SANBI, 2002). Kose et al. (2015) reported that traditional healers in Lesotho use the leaves of *C. paniculata* to treat cervical cancer, breast cancer, and wounds.

2.2.6 Scientific investigations

C. paniculata was found to have anti-inflammatory and analgesic activities, aqueous extracts were also found to be toxic towards rats, showing 80% mortality at 400mg/kg (Adedapo et al., 2008). The plant was also found to have anti-microbial, moderate anti-malarial activity and cytotoxicity towards leukaemia cells at IC_{50} greater than 50 μ g/mL (De Villiers et al., 2010).

2.3 *DICOMA ANOMALA*

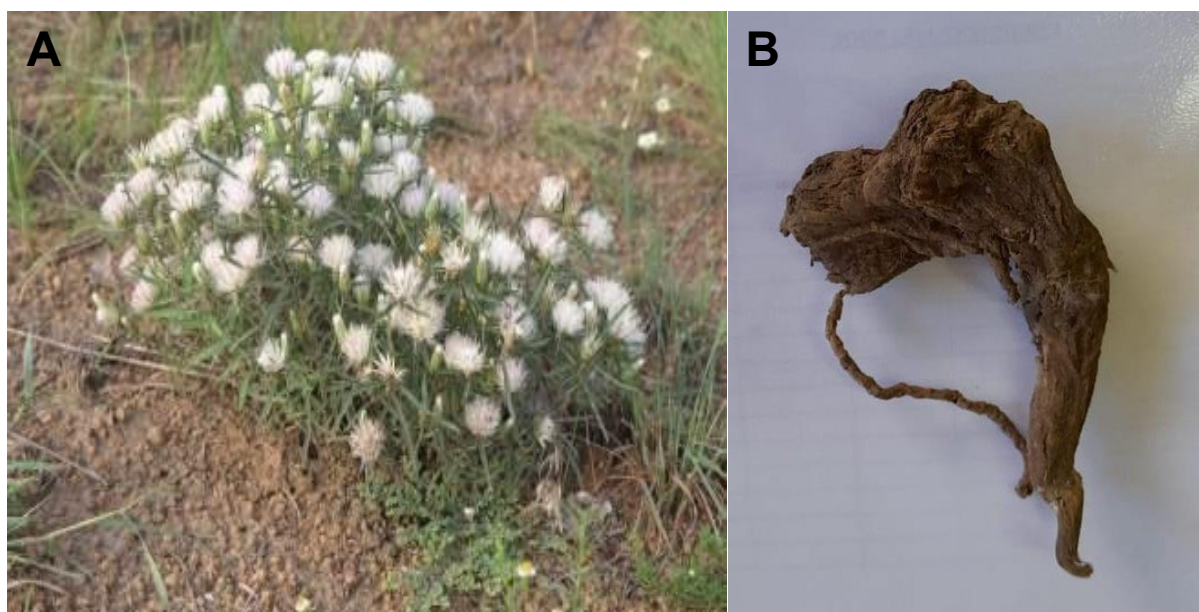


Figure 2.2: (A): *D. anomala* flowers and leaves; (B): *D. anomala* dried root

Source: Researcher (2020)

Table 2.2: Scientific and common names of *D. anomala*

Family	Scientific name	Common names
Asteraceae	<i>Dicoma anomala</i>	English: fever bush, stomach bush
		Afrikaans: maagbitterwortel, kalwerbossie, koorsbossie, gryshout, maagbossie
		isiXhosa/siSwati: inyongana
		isiZulu: isihlabamakhondlwane, umuna
		Sesotho: hloenya, mohlasetse

Source: SANBI (2007)

D. anomala belongs to the Asteraceae family, which is also known as the daisy family, aster family, or composite family of flowering plants. It is one of the largest plant families with approximately 1 620 genera and 23 600 species of herbs, shrubs, and trees that are found all over the world (Encyclopaedia Britannica, 2015). For the longest time, members of this family have been used as food and in the form of medicine. Despite their diversity, most members in this family have a similar chemical composition; for example, all species are a good source of inulin, which is a natural polysaccharide with very strong prebiotic properties. They also have strong anti-oxidant, anti-inflammatory, and anti-microbial activities, as well as diuretic and wound-healing properties (Rolnik & Olas, 2021). Polyphenols, phenolic acids, flavonoids, acetylenes, saponins, sterols, and triterpenes are among the phytochemical

compounds found to be responsible for the pharmacological effects reported for the Asteraceae family (Koc et al., 2015).

2.3.1 Description

D. anomala is characterised by its stiff, sharp-pointed bracts, enormous flower heads, and mauve-white florets that are surrounded by numerous white bristles. *D. anomala* is a perennial shrub with an underground tuber that grows prostrate, decumbent, or upright. A woody rootstock produces a few to many straight stems. The stems are covered with hairs and very thin, alternating, stalkless, linear, or narrowly lanceolate leaves with a glabrous upper surface and a white-hairy under-surface. With stiff, thin, sharply pointed bracts and slender white, mauve, purple, or pink tubular florets, the flower heads are terminal, single, or in tiny clusters. The pappus is made up of several barbellate bristles. Flowering takes place from November to July, with the months of February and March being the peak flowering time (Germishuizen & Meyer, 2003; SANBI, 2007).

2.3.2 Distribution

D. anomala is found throughout sub-Saharan Africa, resulting in a wide range of morphological variations. In South Africa, it is found in the Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North West, and Northern Cape provinces (Balogun & Ashafa, 2017).

2.3.3 Habitat

It grows in summer rainfall zones, on doleritic or sandy soils south of the Sahara, in rocky grasslands, slopes or flat grassland, and in savannah, at elevations ranging from 165 to 2 075 m. It is, however, not found in winter rainfall areas (SANBI, 2007).

2.3.4 Special aspects

D. anomala is a medicinal plant that can withstand a limited supply of water.

2.3.5 Traditional uses

The root decoction of the plant is used for the treatment of gall sickness, while sores and wounds are treated with the powdered form of the plant. This plant is also given

to children orally or in the form of an enema when it is suspected that they are suffering from blood problems. The Afrikaner community in South Africa uses the root decoction for the treatment of diarrhoea, colic, toothache, intestinal worms, dysentery, sterilisation, and haemorrhoids. For fever treatment, the same root decoction is mixed with a herb called melkbos (*Euphorbia mauritanica*) (Germishuizen & Meyer, 2003).

The Xhosa tribe chew the roots of the plant to induce vomiting when they suspect that someone has ingested poisoned food (Hutchings & Van Staden, 1994). The plant is also believed to treat coughs and respiratory problems. Table 2.3 summarises the different traditional uses of *D. anomala* in different countries.

Table 2.3: Traditional uses of *D. anomala*

Habitat	Part(s) Used	Medicinal Use	Country Practiced
Stony grasslands, rocky hillsides, and savanna forests. Altitude from 165 to 2075 meters.	Roots	Abdominal Pain	Zimbabwe
	Roots	Cardiovascular Diseases	Namibia and South Africa
	Tuber	Asthma	South Africa
	Leaves and roots	Breast Cancer	Lesotho
	Roots	Cataracts	Zimbabwe
	Leaves and roots	Diabetes	Lesotho and South Africa
	Root	Renal Problems	Swaziland and South Africa
	Root	Malaria	Zimbabwe
	Root	Pneumonia	South Africa and Zimbabwe
	Root	Syphilis	Zimbabwe
	Root	Cough	Malawi, Namibia, and South Africa
	Root	Hemorrhoids	Namibia
	Root	Intestinal worms	Lesotho, Malawi, and South Africa
	Flower and roots	Wounds and sores	Lesotho, Malawi, and South Africa

Source: Chota et al. (2020)

2.3.6 Scientific investigations

D. anomala was reported to have antibacterial activity against *Microsporum canis*, *Candida albicans*, *Staphylococcus aureus* and *Trichophyton mentagrophytes* microorganisms. It is also reported to have anti-cancer activity against MCF7, MDA-MB-231 and MCF-12A cell lines as well as anti-parasitic activity against NF54 *p. falciparum* strain (Balogum & Ashafa, 2017; Maroyi, 2018; Chota et al., 2020; Shafiq et al., 2020).

2.4 ELEPHANTORRHIZA ELEPHANTINA



Figure 2.3: (A) *E. elephantina* stem and leaves; (B) *E. elephantina* dried roots

Source: Researcher (2020)

Table 2.4: Scientific and common names of *E. elephantina*

Family	Scientific name	Common names
Fabaceae	<i>Elephantorrhiza elephantina</i>	English: eland's bean, eland's wattle, elephant's root
		Afrikaans: baswortel, elandsboontjie, leerbossie, looiersboontjie
		Shona: mupangara
		Sesotho/Setswana: mositsane
		isiXhosa/isiZulu: intolwane

Source: SANBI (2007)

E. elephantina is from the genus *Elephantorrhiza*, which means elephant root, in reference to the huge underground stem seen in most members of the genus (Palmer et al., 1972). Many phytochemical compounds have already been identified in this species that are responsible for its diverse medicinal uses. These phytochemical compounds include saponins, phenolic compounds, tannins, sugars, flavonoids, triterpenoids, esters, fatty acids, anthraquinones, polysterols, and anthracyanidins.

2.4.1 Description

E. elephantina is a perennial suffrutex with unbranched, unarmed aerial stems that can grow up to 0.9 m in height, which represent the canopy of a much bigger tree

below the ground that is represented by these stems. The immature branchlets and bark have a dark reddish-brown colour. The leaves are dark green, bipinnately complex, and have two to 17 opposed or sub-opposite pairs of pinnae on each side. The flowers are golden yellow to pale yellowish white and clustered in axillary, single, or grouped racemes close to or projecting from the ground level. The flowering of this plant is independent of spring rain and occurs in September to November. The seeds are dark brown and ellipsoid formed, whereas the fruits are dark or reddish-brown compressed pods (Schmidt et al., 2002; Jansen & Cardon, 2005; SANBI, 2010).

2.4.2 Distribution

This species has a wide distribution, and populations differ greatly in terms of the number of pinnae pairs and the number, size, and form of the leaflets. Furthermore, of all *Elephantorrhiza* species, *E. elephantina* is the most widespread and most commonly encountered in the southern parts of Angola, Botswana, Mozambique, Namibia, Zimbabwe, Eswatini, Lesotho, and South Africa. In South Africa, *E. elephantina* is found in the Eastern Cape, Free State, Limpopo, Gauteng, KwaZulu-Natal, Mpumalanga, North West, and Northern Cape provinces.

2.4.3 Habitat

E. elephantina is frequently found in grassland and open scrub in hot, dry areas at elevations of 1 060 to 1 360 m (Fern, 2018).

2.4.4 Special aspects

It is a medicinal plant that is drought resistant, and honeybees feed on it.

2.4.5 Traditional uses

E. elephantina is a valuable plant resource in Southern Africa, where it offers food and medicine to locals, and the bark of its tuberous rhizome is sometimes crushed and mixed with water to make a paste that is used for tanning and dyeing products (Coates-Palgrave, 2002). In Southern Africa, subterranean rhizomes, sometimes known as roots, are one of the most commonly used parts of *E. elephantina* as they are used as traditional medicine for diarrhoea and dysentery, stomach illnesses, skin diseases and acne, haemorrhoids, and perforated peptic ulcers, as well as for emetics. Together

with *Pentanisia prunelloides*, *E. elephantina* is used as a concoction for the treatment of eczema (Maroyi, 2017). In Lesotho and Zimbabwe, a root decoction is used to treat abdominal pains, and in Mozambique, the root decoction is taken orally for the treatment of anaemia (Ribeiro et al., 2010; Kose et al., 2015). In Botswana, the powdered form of *E. elephantina* rhizome is used as a remedy for sexually transmitted infections, early menstruation in children, erectile dysfunction, to clean the womb after abortion, and to stop bloody diarrhoea in children by wiping the anus with it (Maroyi, 2017).

The Lesotho community also uses the rhizome decoction (Kose et al., 2015) for the treatment of breast cancer, herpes, TB, infertility, and intestinal ailments. The young shoots of *E. elephantina* are eaten by cattle. The seeds have a sweetish flavour followed by a scorching sensation, and are commonly roasted as a coffee replacement in Southern Africa.

2.4.6 Scientific investigations

A number of pharmacological activities have been reported on *E. elephantina*, these includes anti-inflammatory, anti-plasmodial, anti-oxidant, anti-bacterial, anti-fungal as well as anthelmintic activity. A number of secondary metabolites like flavonoids, tannins and steroids among many others were also identified on the rhizome of the plant (Maroyi, 2017; Maphosa et al., 2009; Clarkson et al., 2004; Mpofu et al., 2014; Aaku et al., 1999).

2.5 XYSMALOBIUM UNDULATUM



Figure 2.4: (A) *X. undulatum* leaves, stem, and flowers; (B) *X. undulatum* dried root
Source: Researcher (2020)

Table 2.5: Scientific and common names of *X. undulatum*

Family	Scientific name	Common names
Apocynaceae	<i>Xysmalobium undulatum</i>	English: milk bush, milkwort, uzura, wild cotton, wave-leaved xysmalobium
		Afrikaans: bitterhout, bitterwortel, bitterhoutwortel, melkbos
		isiZulu: iShongwane, iShongwe, iShinga
		Sesotho: leshokoa, poho-tsehla
		isiXhosa: iyeza elimhlophe, nwachaba, iShongwane

Source: SANBI (2009)

2.5.1 Description

X. undulatum is an annual geophytic plant that can grow up to 1 m in height. It is a robust plant that dies in the winter but grows again from its perennial root stock during spring. It has thick and hairy erect branches and fleshy brown carrot-like roots that are white inside with a unique, nauseating odour. When the plant is damaged or cut anywhere, a milky latex comes out. The large leaves of the plant (80 to 270 x 35 to 50 mm) are hairy and opposite sided, almost heart shaped, stalkless, heavily veined, and with slightly thickened borders. The flowers are cream green to yellowish brown, with their tips curved and covered in short and thick white hairs that are used for the

identification of the species and are borne in small circular clusters along the stem from October to December (Bester, 2009; Schulzke et al., 2011).

2.5.2 Distribution

X. undulatum is widely distributed mainly in the eastern parts of Southern Africa, Angola, Botswana, Kenya, Lesotho, Malawi, Mozambique, Namibia, Eswatini, Tanzania, Zambia, Zimbabwe, and all South African provinces (SANBI, 2009).

2.5.3 Habitat

X. undulatum is found in the Grassland Biome on the edge of most roads and often in seasonally wet places, in open or moist grasslands, and wetlands at altitudes of 84 to 2 000 m above sea level (SANBI, 2009).

2.5.4 Special aspects

It is a pioneer medicinal plant that attracts butterflies.

2.5.5 Traditional uses

In South Africa, poho-tsehla is one of the most extensively utilised herbal remedies. It has a long history of both external and internal use for the treatment of many different ailments. Different ethnic groups in South Africa use poho-tsehla in various ways; for example, the Xhosas use it for the treatment of hysteria and it is often combined with the roots of *Pachycarpus schinzianus* to treat digestion problems since they reportedly have similar medicinal properties (Vermaak et al., 2014). Furthermore, the milky latex is applied to festering wounds to inhibit maggot growth. The Tswana people chew a portion of the root as an antidote to food poisoning, while the Zulus grind the stem into water and use it as an emetic in poisoning cases. The Nama people take the root of the plant to relieve flatulence and diarrhoea and they also use a paste of powdered roots and cold water for the treatment of lumpy skin disease in cattle (SANBI, 2009; Pooley, 2003).

Poho-tsehla has many historic uses across the rest of South Africa. The powdered root is used as a decoction to treat intestinal issues such as colic, diarrhoea, dysentery, stomach discomfort, and digestive issues and as an antidote for food poisoning. It is

also used to relieve menstrual and afterbirth cramps due to its anti-spasmodic properties. Because of its sedative effects, the powdered root is used as snuff and helps to alleviate headaches (Watt & Breyer-Brandwijk, 1962; De Wet et al., 2012).

Other traditional uses of poho-tsehra include the treatment of urinary tract infections, syphilis, heart failure, and coughing and it is also used as an emetic, diuretic, and tonic. Kose et al. (2015) reported that traditional healers in Lesotho use poho-tsehra roots to treat breast cancer, cervical cancer, TB, headaches, and phlegm. The young leaves of the plant are also used as a spinach or green vegetable by the Sesotho people. The plant is also believed to be “magical” and is used as a protective charm to divert storms during religious rituals and to make dogs keen hunters (Schulzke et al., 2011; SANBI, 2009).

The information provided shows that plants possess different active compounds with different pharmacological effects that can be used for drug development. Not many plants are thoroughly documented and scientifically investigated for drug development. It is therefore important to screen and document more medicinal plants, as historical use shows that they have the potential to be developed into novel drugs for different ailments that currently do not have a cure, to replace treatment methods that are expensive, or to replace treatments that currently leave patients with adverse side effects.

2.5.6 Scientific Investigations

Xysmalobium undulatum phytochemical composition is heavily investigated and a number of compounds have been isolated. It is also reported to possess anti-diarrhoeal activity, anti-bacterial and anti-cancer activity (Schulzke et al., 2011; Buwa & van Staden, 2006; Gouws et al., 2021; Brendler et al., 2023)

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CHAPTER 3:

PHYTOCHEMICAL ANALYSIS

3.1 INTRODUCTION

Phytochemicals, also known as secondary metabolites, are naturally occurring and biologically active compounds found in plants. The term “secondary metabolites” comes from the fact that plants have secondary biological pathways that allow them to synthesise chemical compounds from primary metabolic processes (Awuchi et al., 2020; Delgoda & Murray, 2017). Cowan (1999) suggested that secondary metabolites make a significant contribution to a plant’s smell, taste, and colour, and that plants produce these secondary metabolites as a response to specific environmental stressors or stimuli such as damage induced by herbivores, pathogenic attacks by bacteria, fungi, or viruses, pollution, UV exposure, or any kind of nutritional deprivation. They are created and employed by plants for protection as they are synthesised as part of the plant’s defence mechanisms. An example of a secondary metabolite is phytoalexins, which are a group of compounds that have anti-bacterial and anti-fungal properties that are produced or synthesised when the plant is attacked by fungi or bacteria (Böttger et al., 2018; Pagare et al., 2015; Xaba, 2016).

Phytochemicals from medicinal plants are becoming very popular due to their numerous medicinal applications. They are essential in the treatment of a variety of ailments that include asthma, arthritis, and cancer; among many others. These phytochemicals from medicinal plants, unlike pharmaceutical compounds, have limited negative side effects and can therefore be regarded as “human friendly” as a result of their ability to treat ailments with minimal or no harm to human beings (Banu & Cathrine, 2015).

Furthermore, phytochemicals differ in their roles, depending on whether they are primary or secondary. Amino acids, chlorophylls, proteins, nucleic acids, and sugars are classified as primary phytochemicals, whereas alkaloids, flavonoids, lignans, steroids, phenols, saponins, and glucosides are regarded as secondary metabolites because of their different functions in plant metabolism (Saxena et al., 2013). More than 4 000 phytochemicals have been identified and categorised based on their

protective function, physical traits, and chemical properties (Harborne, 1999; Koche et al., 2016; Xaba, 2016).

3.2 BRIEF LITERATURE OVERVIEW

Secondary metabolites are classified into the following groups based on their chemical structure and functions: alkaloids, flavonoids, tannins, terpenoids, and saponins.

3.2.1 Alkaloids

Alkaloids are the second biggest group of compounds found in all living things, and they are thought to contain a wide range of medicinal properties. In an acidic environment, their compounds are water-soluble and in a neutral and basic environment, compounds have lipophilic characteristics. Alkaloids are poisonous to herbivores due to their anti-inflammatory, analgesic, anaesthetic, adaptogenic, and toxicity properties (Aniszewski, 2007; Saxena et al., 2013). In their pure form, most alkaloids are colourless, non-volatile, crystalline solids and they tend to have a bitter taste. Previous studies have indicated that two types of alkaloids, namely vinblastine and vincristine, are greatly used in the treatment of many cancers as chemotherapeutic agents (Encyclopaedia Britannica, 2020). The structure of morphine, as an example of an alkaloid, is shown in Figure 3.1.

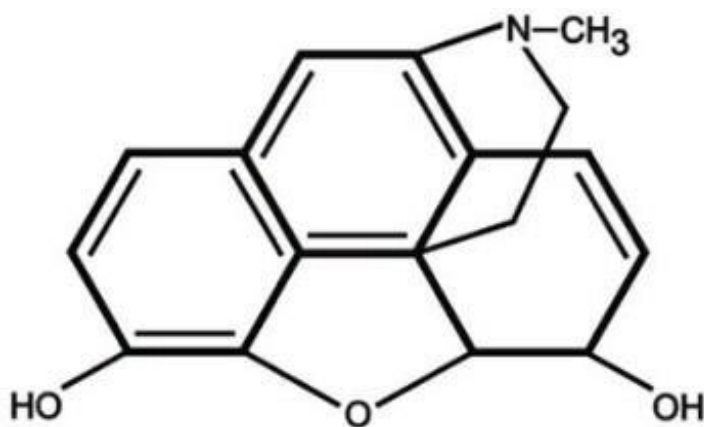


Figure 3.1: Morphine's chemical structure

Source: Braenden et al. (1955)

3.2.2 Flavonoids

Flavonoids are a class of secondary metabolites with a polyphenolic structure widely found in plants used in the human diet. Examples of foods rich in flavonoids include vegetables, wine, spices, fruit, and tea (Panche et al., 2016). They have a wide variety of health-promoting qualities and they are essential compounds in a number of cosmetic, medicinal, and pharmacological applications. They are reported to have anti-carcinogenic, anti-inflammatory, anti-mutagenic, and anti-oxidative activities (Metodiowa et al., 1997; Havsteen, 2002; Saxena et al., 2013). Figure 3.2 illustrates the structure of flavanol, as an example of a flavonoid compound.

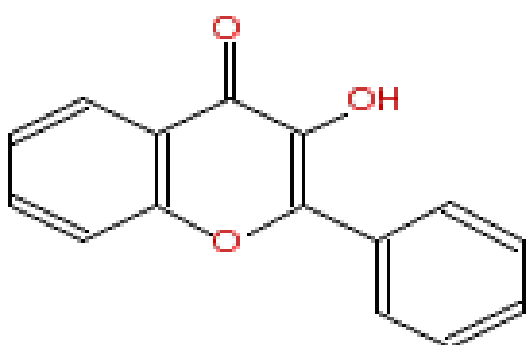


Figure 3.2: Flavanol structure

Source: Barrett et al. (2018)

3.2.3 Tannins

Tannins are a complex class of plant secondary metabolites that are soluble in polar solutions and can precipitate proteins. This is a unique characteristic that distinguishes them from other polyphenolic substances. They are known to have a wide range of polymers and oligomers, which makes them a very complex class of secondary metabolites due to their heterogeneous nature with polyphenolic compounds (Silanikove et al., 2001; Saxena et al., 2013). Tannins are found in a number of plants, especially woody flowering plants, and they possess anti-inflammatory, anti-oxidant, astringent, anti-septic, anti-phagocytic, anti-microbial, anti-neoplastic, and anti-tumour properties (Ntsoelinyane, 2014; Xaba, 2016; Encyclopaedia Britannica, 2020). The structure of a tannin molecule is presented in Figure 3.3.

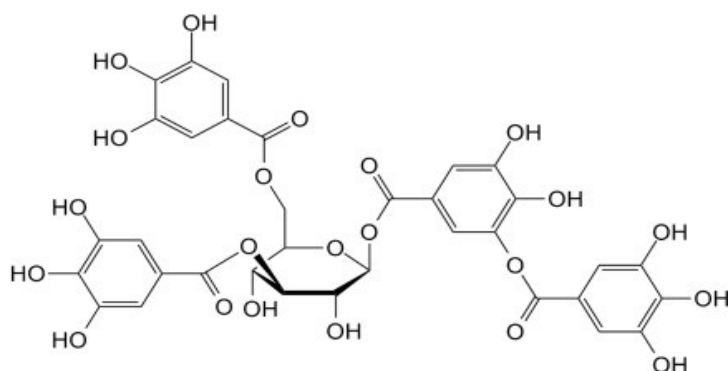


Figure 3.3: Structure of a tannin molecule

Source: Aguilar et al. (2007)

3.2.4 Terpenoids

Terpenoids are a group of natural compounds derived from isoprene units with five carbons (see Figure 3.4). Most of these compounds consist of multicyclic structures with functional groups and basic carbon skeletons that differ from one another. They are also considered the largest group of natural products as they are found in all living organisms (Devi, 2018). Terpenoids play a significant role as plant hormones and they are responsible for chemical defences against insect herbivores and microbial diseases. Previous studies have reported terpenoids to have medicinal properties such as anti-ulcer, anti-malaria, anti-microbial, anti-carcinogenic, and diuretic activity (Xuan & Khanh, 2016).

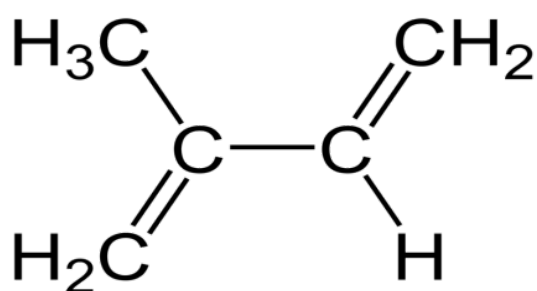


Figure 3.4: Isoprene structure

Source: Almarie (2020)

3.2.5 Saponins

“Saponin” comes from the Latin word *sapo*, which means soap, because of their surfactant abilities that allow them to produce stable foam when shaken in an aqueous solution. They are large molecules with a hydrophobic backbone made up of 30 carbon atoms (triterpenoid) or 27 carbon atoms with a six-ring spirostane or five-ring furostane

skeleton (steroid) backbone and a hydrophilic backbone made up of a few saccharide residues attached via glycosidic bonds to the hydrophobic scaffold (Stanimirova et al., 2011). Saponins have been reported to reduce cholesterol levels in both animals and humans (Moghimipour & Handali, 2015). Waheed et al. (2012) identified and isolated a new steroidal saponin glycoside that can cause cancer cells to undergo cell-selective apoptosis or necrosis. It was also reported that saponins possess the following biological and medicinal activities: anti-diabetic, anti-viral, anti-fungal, anti-cancer, anti-bacterial, anti-inflammation, analgesic, anthelmintic, insecticidal, and molluscicidal action (Xaba, 2016; El Aziz et al., 2019). Figure 3.5 illustrates the structure of saponin.

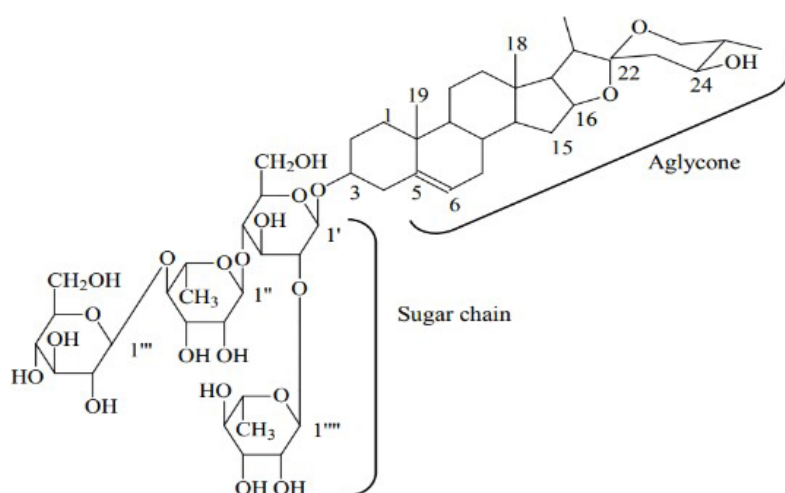


Figure 3.5: Structure of a saponin

Source: Moghimipour and Handali (2015)

3.3 METHODOLOGY

3.3.1 Plant material collection and verification

Plants were collected from Thaba 'Nchu at Chubane farm (29°03'03.0"S 26°53'46.11.0"E). All the collected plants were authenticated by Prof. Zietsman from the National Museum in Bloemfontein.

3.3.2 Extraction method

The plant materials were washed and dried at room temperature (20-25 C. Fifty grams of each plant was ground to a fine powder. The powdered plant materials were added in 500 mL solvents of different polarities, namely dichloromethane (DCM), methanol

(MeOH), DCM:MeOH, and distilled water (dH₂O). The mixtures of the solvent and powdered plant material were shaken for 72 hours on a shaker using a sequential method of extraction, except for the dH₂O extract. After 72 hours, the mixtures were filtered and then concentrated using a rotary evaporator (BUCHI- Recirculating chiller (F-305); Heating bath (B-300 base); Rotavapour (R-300); Vacuum pump (V-300); Interface (I-300 Pro), while the dH₂O extract was dried using a freeze dryer (Alpha 1-2 LDplus Martin Christ). The concentrated extracts were then transferred into vials and stored in a cool room until further analysis.

3.3.3 Qualitative phytochemical testing (as described by Harborne, 1973)

3.3.3.1 Test for tannins

The dried powdered plant samples (0.5 g each) were boiled in 20 mL of dH₂O respectively. A few drops of 0.1% ferric chloride (FeCl₃) solution were then added to half the volume of the filtrate. The appearance of an intense green, brownish-green, or a blue-black colouration indicated the presence of tannins in the plant sample. This was then confirmed by adding a few drops of iodine to a second portion of the filtrate, which yielded a faint bluish colouration.

3.3.3.2 Test for phlobatannins

The dried powdered plant samples (1.0 g) were mixed in 20 mL of dH₂O respectively and filtered. The mixture was thereafter boiled with 1% aqueous hydrochloric acid (HCl). The presence of phlobatannins was observed by the presence of a red precipitate at the base of the test tubes.

3.3.3.3 Test for saponins

The dried powdered plant samples (2.0 g) were boiled in 20 mL of dH₂O in a water bath and filtered. Thereafter, 10 mL of the filtrate was mixed with 5 mL of dH₂O and shaken vigorously for a stable, persistent froth. The frothing was then mixed with three drops of olive oil, shaken vigorously, and observed for the formation of an emulsion. The presence of saponins was indicated by the formation of a heavy emulsion.

3.3.3.4 Test for flavonoids

The dried powdered plant samples (2.0 g) were heated with 10 mL of ethyl acetate over a steam bath for three minutes. The mixture was thereafter filtered and 4 mL of the filtrate was shaken and 1 mL of diluted ammonia solution was added to it. A yellow colouration indicated a positive test for flavonoids.

3.3.3.5 Test for steroids

The dried powdered plant samples (0.5 g) were mixed with 2 mL of acetic anhydride respectively. This was followed by the addition of 2 mL sulphuric acid (H_2SO_4). A colour change from violet to blue or green indicated the presence of steroids.

3.3.3.6 Test for terpenoids (Salkowski test)

Dried powdered plant samples (5 g) were mixed in 2 mL of chloroform and thereafter 3 mL of concentrated H_2SO_4 was carefully added to form a layer. A reddish-brown colouration at the interface indicated the presence of terpenoids.

3.3.3.7 Test for alkaloids

The dried powdered plant samples (0.5 g) were mixed with 8 mL of 1% HCl, warmed, and filtered. Thereafter, 2 mL of the filtrate was treated with a few drops of freshly prepared Dragendorff's reagent. The presence of precipitate showed a positive result.

3.3.3.8 Test for phenolics

The dried powdered plant sample (0.5 g) was mixed with 1 mL of dH_2O . A few drops of neutral 5% FeCl_3 solution was added to the mixture. A dark-green colour indicated the presence of phenols.

3.4 RESULTS AND DISCUSSION

Table 3.1 reports on the phytochemical screening that was performed on *D. anomala*, *E. elephantina*, *C. paniculata*, and *X. undulatum* plant powders. Positive signs (+) indicate the presence of secondary metabolites, which means that there was a colour change or formation of precipitate during the test, whereas negative signs (-) indicate the absence of a particular secondary metabolite; there was thus no colour change or

formation of precipitate and multiple signs (++ or +++) indicates colour intensity, as shown in Figure 3.6.

Table 3.1: Phytochemical screening results of the four medicinal plants tested

Plant extracts	Flavonoids	Tannins	Saponins	Terpenoids	Alkaloids	Steroids	Phenols
<i>D. anomala</i> roots	+	+++	+	+++	-	+++	-
<i>E. elephantina</i> roots	+	+++	+	+	-	+	+++
<i>C. paniculata</i> leaves	-	+++	+++	++	-	-	++
<i>C. paniculata</i> roots	-	++	++	+++	-	-	++
<i>X. undulatum</i> roots	+++	+	-	+++	+++	+	-

Only *C. paniculata* (both the roots and leaves) tested negative for flavonoids and steroids, while all tested plant powders tested positive for tannins and terpenoids. *X. undulatum* tested positive for all phytochemicals, except for saponins, and in the test for phenols, *C. paniculata* (both the leaves and roots) and *E. elephantina* root powder tested positive.

To the researcher's knowledge, not much phytochemical screening has been performed on the roots and leaves of *C. paniculata*. Dovgii et al. (2005) isolated and elucidated the following compounds: 3-O-β-D-glucopyranoside, 3-O-α-L-arabinopyranosides of oleanolic, ursolic, 3-O-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranoside, 3-O-α-L-arabinopyranosyl-28-O-α-L-rhamnopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosides, and 28-O-α-L-rhamnopyranosyl-(1→4)-O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranoside from *C. paniculata* leaf isopropanol extract. *C. spicata* is the most studied species in the *Cussonia* genus; which include anti-oxidation studies (Amoo et al., 2012), in vitro anti-plasmodial evaluation (Kraft et al., 2003), and, among others, phytochemical screenings and structural elucidation by Gunzinger et al. (1986), where they elucidated two saponin structures from the aqueous stem bark extract of the *C. spicata* plant. The compounds isolated were [α-L-arabinofuranosyl-(1→4)β-D-glucuronopyranosyl-(1→3)]-β-hydroxyolean-12-en-28-oic acid, and (α-L-arabinofuranosyl-(1→4)-β-D-galactopyranosyl-(1→2) β-D- glucuronopyranosyl-(1→3)]-3β-hydroxyolean-12-en-28-

oic acid, where they found that these two compounds were responsible for the molluscicidal activity of the extract.

Tlotlo and Olivier's (2022) review of *E. elephantina* reported that the roots or rhizomes of the plant consist of flavonoids, tannins, and phenolic compounds, which supports the phytochemical screening results of this study. Xaba (2016) tested the different *X. undulatum* extracts for the presence of secondary metabolites and found that the aqueous extract contain tannins and alkaloids, while the ethanol extract contained flavonoids and alkaloids, which supports the findings of this study; however, Xaba's (2016) tests for terpenoids and steroids were negative, which contradicts the results of this study. This may be due to the fact that this study used plant powder for the test while Xaba (2016) used solvents of specific or different polarities, which caused the extract to contain specific compounds or phytochemicals relative to the solvent polarity.

The total phenolics and flavonoids contents of different *D. anomala* root extracts were reported by Matsabisa et al. (2020). The DCM:MeOH extract had the highest phenolic content of 54% and the hexane extract had the highest flavonoid content of 46%. Only flavonoids were detected by this study.

In addition to identifying the constituents of plants, phytochemical screening is effective for determining bioactive compounds that can be used in the synthesis of drugs. There are several types of plant-derived anti-cancer compounds, including acetylenic compounds, phenolics, terpenes, and phytosterols, which are known to be less harmful to healthy cells (Chota et al., 2020). In this study, all the tested plants were positive for terpenoids and only *E. elephantina* and *C. paniculata* tested positive for phenols. This suggests that the individual compounds of the plants could be investigated to determine if some of them have selective toxicity towards cancer cells.

3.5 CONCLUSION

Plants naturally produce secondary metabolites to protect themselves, as a response to specific environmental stressors or stimuli, and as defence against micro-organisms. These characteristics can be employed by humans to help fight diseases.

It is essential to screen natural products since it helps to identify novel active compounds and to determine the efficacy and safety of their use. When a plant is difficult to identify, the study of phytochemicals helps to prove its identity as well, where the documentation of this knowledge may be valuable in the future.

Secondary metabolites, such as alkaloids, flavonoids, saponins, steroids, phenols, and terpenoids, were present in most of the tested plant powders. The phytochemical screening results suggest that *E. elephantina*, *D. anomala*, *X. undulatum*, and *C. paniculata* can be good candidates for drug discoveries due to the presence of various phytochemicals that promote good health and possess the ability to fight different ailments.

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CHAPTER 4:

IN VITRO CYTOTOXICITY/ANTI-PROLIFERATIVE ACTIVITY OF D. ANOMALA, E. ELEPHANTINA, C. PANICULATA, AND X. UNDULATUM PLANT EXTRACTS

4.1 INTRODUCTION

The term “cancer” refers to a class of disorders where a group of cells continue to grow and divide uncontrollably (Hejmadi, 2014). Cancer develops from different types of tissues and displays high genetic diversity. In contrast to normal tissue cells, cancer cells are able to evade physiological mechanisms that promote cell death. They may replicate indefinitely, they are resistant to anti-growth signals (which allows them to continue dividing), they support angiogenesis, and they have the ability to spread to nearby tissues (Spies et al., 2013).

Numerous chemotherapeutic drugs are available and in use for the treatment of cancer; however, the problem of very toxic, unfavourable, and unpleasant side effects persists. Given this, there has been an increase in scientific interest in finding new anti-cancer medications from sources of natural products (Nedeljković & Damjanović, 2019). For centuries, one of the mainstay healthcare systems has been herbal medicine; however, scientific research has not yet been able to confirm the majority of ethnobotanical claims. Research into the safety and effectiveness of plants used for treatment is therefore encouraged because they have played a significant role as sources of anti-cancer drugs. This study thus investigated the anti-cancer activity of *D. anomala*, *E. elephantina*, *C. paniculata*, and *X. undulatum* extracts.

4.2 BRIEF LITERATURE OVERVIEW

Traditional medicine is considered a crucial part of public healthcare, as well as for the treatment of cancer because, in most cases, developing countries cannot afford currently used treatment methods or options due to economic reasons. This is mostly evident in African countries (Njaka, 2016).

Africa is one of the continents with the highest levels of biodiversity in the world, yet the development of chemotherapeutic drugs from medicinal plants receives little

attention. It has been reported that terrestrial plants, marine organisms, and micro-organisms are among the natural sources of more than 60% of the anti-cancer agents currently in use (Shinde et al., 2019; Singh et al., 2020). This thus emphasises the need to pursue the search for natural products that can provide anti-tumour agents with novel structures and unique mechanisms of action.

Cancer is defined as a cell's ability to proliferate without the ability to die. Apoptosis is a process of cellular suicide brought on by the activation of a specific intracellular programme, whereas necrosis is a type of cell death that occurs in response to apoptotic inhibition or shock such as infection, trauma, or toxins (Priante et al., 2021). Apoptosis must be controlled since too much apoptosis can cause stroke-induced neuronal damage and neurodegenerative disorders, while too little apoptosis can cause cancer, autoimmune diseases, or chronic inflammatory diseases (Zangemeister-Wittke & Simon, 2001; Sturm, 2008). An advantage of apoptosis is that it prevents an undesired inflammatory reaction while allowing an organism to get rid of unwanted and damaged cells in an orderly manner (Pucci et al., 2000).

4.2.1 Apoptosis

Apoptosis can be initiated by extracellular or intracellular signalling where complex machinery is initiated to begin a chain of events that will eventually destroy nuclear deoxyribonucleic acid (DNA) and break down the cell. It is further sub-classified into two pathways: intrinsic and extrinsic apoptotic pathways (Carneiro & El-Deiry, 2020).

The intrinsic pathway (see Figure 4.1) begins inside the cell. This usually occurs in reaction to cellular signals brought on by DNA damage, separation from the extracellular matrix, a faulty cell cycle, hypoxia, or other types of excessive cell stress. This pathway includes the mitochondrial release of pro-apoptotic proteins, which activate caspases. The intrinsic apoptotic pathway also relies on the activity of pro-apoptotic and anti-apoptotic B-cell lymphoma 2 superfamily proteins, which control the permeability of the mitochondrial membrane (Belkacemi, 2018).

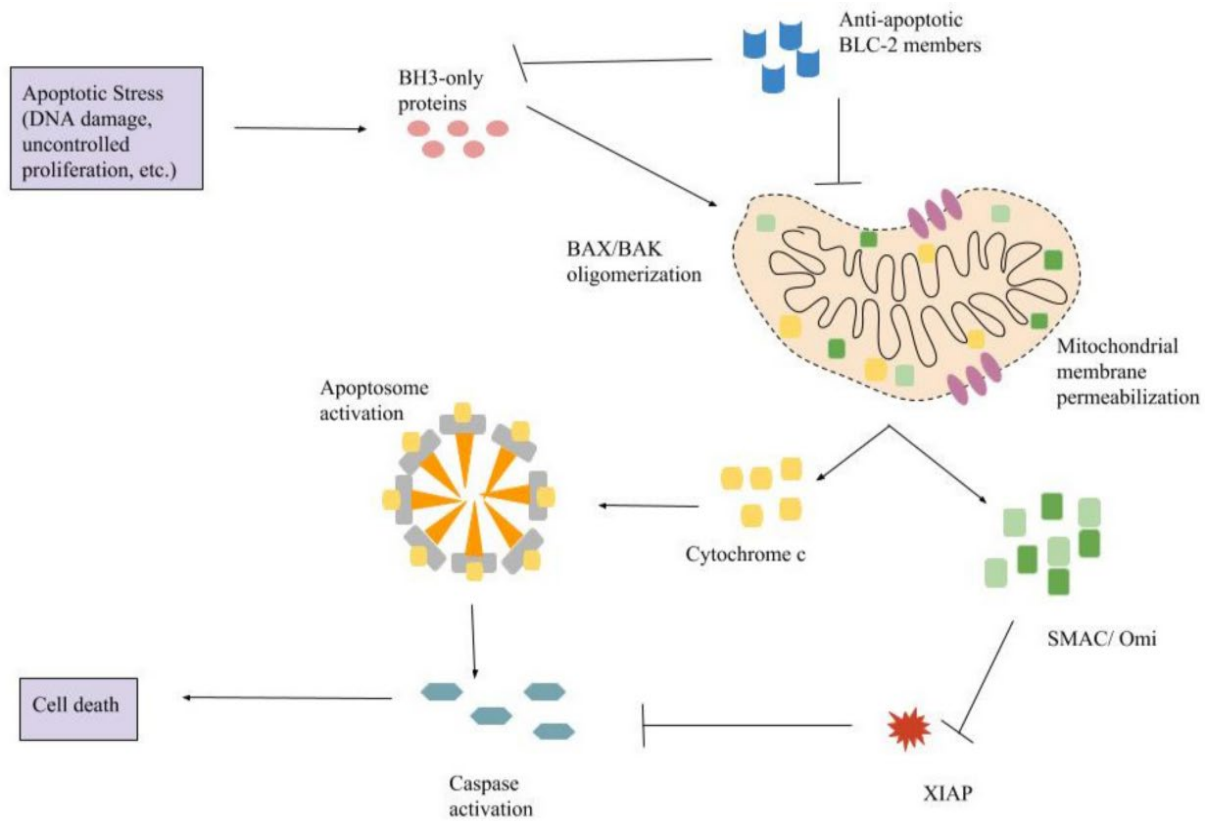


Figure 4.1: Intrinsic apoptotic pathway

Source: Pfeffer and Singh (2018)

The extrinsic apoptotic pathway (see Figure 4.2) is initiated by cell membrane proteins known as death receptors. These death receptors are present on the surface of the outer membrane of the cell and are members of the tumour necrosis factor (TNF) superfamily, including TNF, FS-7-associated surface antigen (Fas), and TNF-related apoptosis-inducing ligand (TRAIL), with their respective ligands (Carneiro & El-Deiry, 2020).

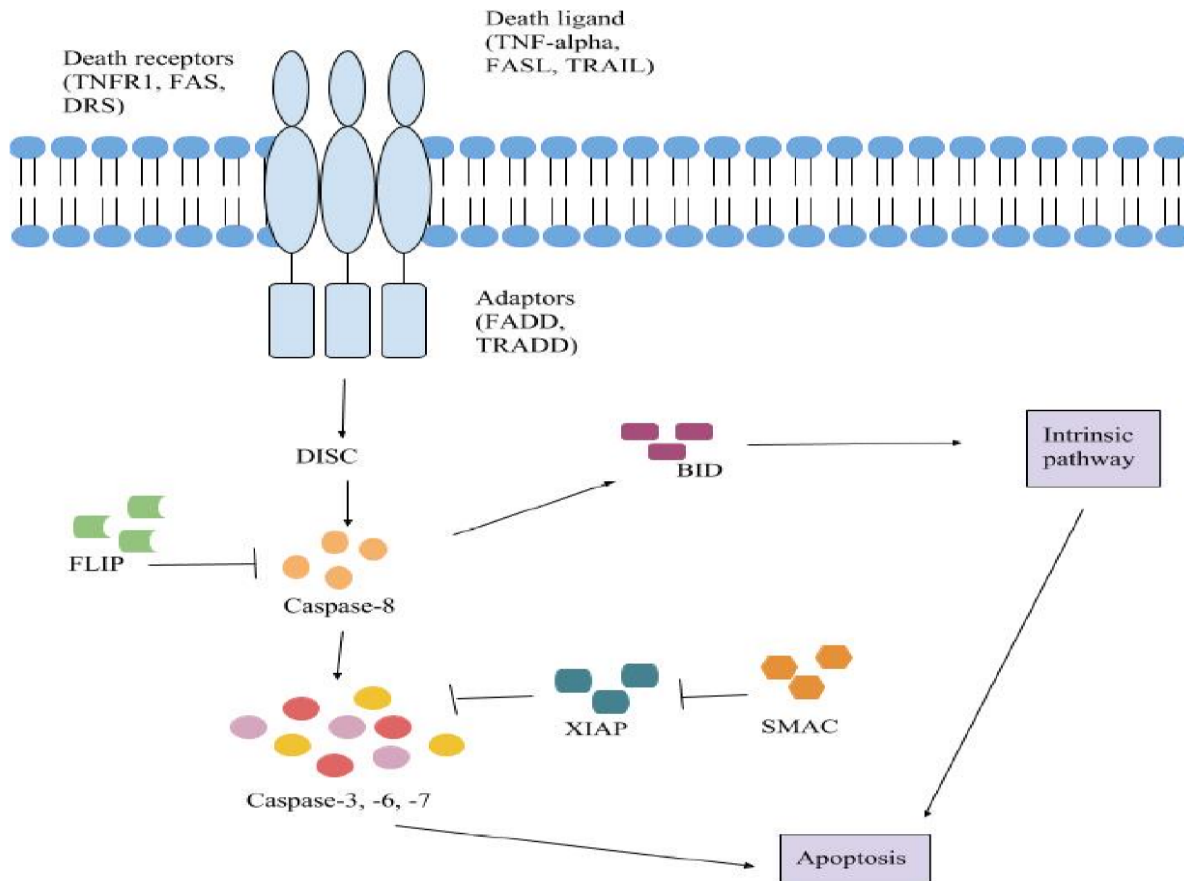


Figure 4.2: Extrinsic apoptotic pathway

Source: Pfeffer and Singh (2018)

4.2.2 The cell cycle

Cell cycle is a conserved mechanism by which cells grow and replicate themselves. The ordinary cell cycle consists of four phases, namely the G1, S, G2, and M phases. Each phase of the cell cycle has checkpoints that act as DNA surveillance systems to stop the accumulation and spread of genetic errors during cell division (see Figure 4.3). These checkpoints can interrupt the process of the cell cycle, or in the case of irreparable DNA damage, they can trigger cell death (Matthews et al., 2022). The link between apoptosis and cell cycle is that they both have significant effects on cellular homeostasis and the emergence and development of cancer.

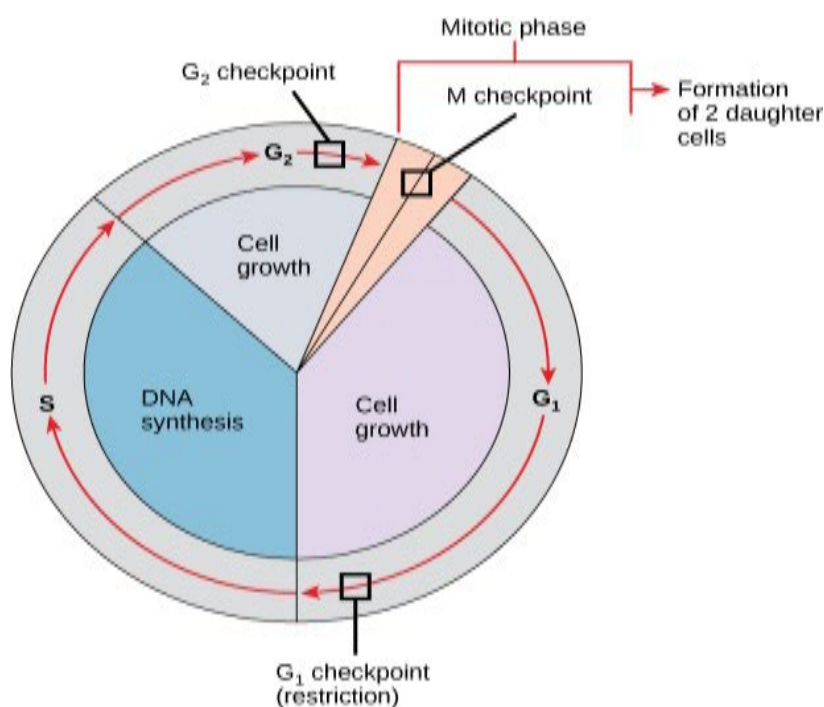


Figure 4.3: Schematic representation of an eukaryotic cell cycle with the positions of checkpoints

Source: Khan Academy (n.d.)

4.2.3 Cytotoxicity

Cytotoxicity is defined as the degree to which a substance can harm a cell (Nelson et al., 2020). The cytotoxicity test is one of the biological evaluation and screening tests that use tissue cells in vitro to observe cell growth, reproduction, and morphological effects through medical devices (Li et al., 2015). Moreover, it is a prominent method for screening pharmaceutical compounds and is widely used in the field of ethnopharmacology for screening plant extracts.

Cytotoxicity assays are used to test the ability of the cell to continue growing while exposed to a test compound or plant extract for a predetermined amount of time. It is usually coupled with cell cycle analysis to determine the state of DNA in response to the treatment of the cell with a specific drug or extract. Cell cycle analysis not only determines the distribution of DNA content, but also helps researchers to identify the targets or pathways that they should focus on during therapeutic strategies for cancer and tumours; hence the screening of anti-cancer activities in this study.

4.2.4 Mitochondrial membrane potential

For many years, it has been known that mitochondria actively contribute to the control of apoptosis. A characteristic feature of apoptosis is the permeability of the mitochondrial membrane. During oxidative phosphorylation, metabolically active cells produce a mitochondrial membrane potential of roughly -180 to -220 mV (Santo-Domingo & Demarex, 2012). The loss of mitochondrial membrane potential and the subsequent release of pro-apoptotic proteins are thought to be signs that the intrinsic mode of apoptosis has begun.

Tetramethylrhodamine ethyl ester (TMRE) is a lipophilic cationic dye that has the ability to cross biological membranes. Due to its positive charge it accumulates in the matrix of mitochondria with a normal membrane potential. When excited at 549 nm, TMRE emits a bright yellow fluorescence at 575 nm (Kalvelytė et al., 2017; Uribe et al., 2017; Swanepoel, 2019). Apoptosis causes a decrease in the mitochondrial membrane potential, which prevents TMRE from accumulating inside the mitochondria and causes the dye to spread out evenly throughout the cytoplasm. This leads to a decrease in the fluorescence intensity, which can be measured by fluorescence microscopy (Creed & McKenzie, 2019).

4.2.5 Phosphatidylserine (PS) translocation

During apoptosis, cells undergo changes to their cellular membranes, and these changes may be used to identify and separate apoptotic cells from the rest of the cells (Majno & Joris, 1995). PS is an amino phospholipid that is mainly found in the cell membrane's inner leaflet. During apoptosis, there is a loss of phospholipid asymmetry that causes the membrane phospholipid PS to be translocated from the inner to the outer leaflet of the plasma membrane (Shin & Takatsu, 2020) (see Figure 4.4).

Annexin V is a calcium-dependent phospholipid-binding protein with a high affinity for binding PS. Annexin V coupled with fluorochromes such as fluorescein isothiocyanate (FITC) may be used to detect apoptosis using flow cytometry or fluorescence microscopy while still maintaining a high affinity for PS. FITC has excitation and emission wavelengths of approximately 495 nm and 519 nm respectively (Swanepoel, 2019). To distinguish between early apoptotic cells, necrotic cells, and dead cells, propidium iodide (PI) is used along with Annexin V. Although PI is a membrane-

impermeable dye, it can penetrate cells with damaged membranes or membranes that have lost their integrity. The Annexin V-FITC/PI assay is thus used to distinguish between viable cells, early apoptotic cells, late apoptotic cells, and necrotic or permeable cells (Catelas et al., 2005; Yong et al., 2015).

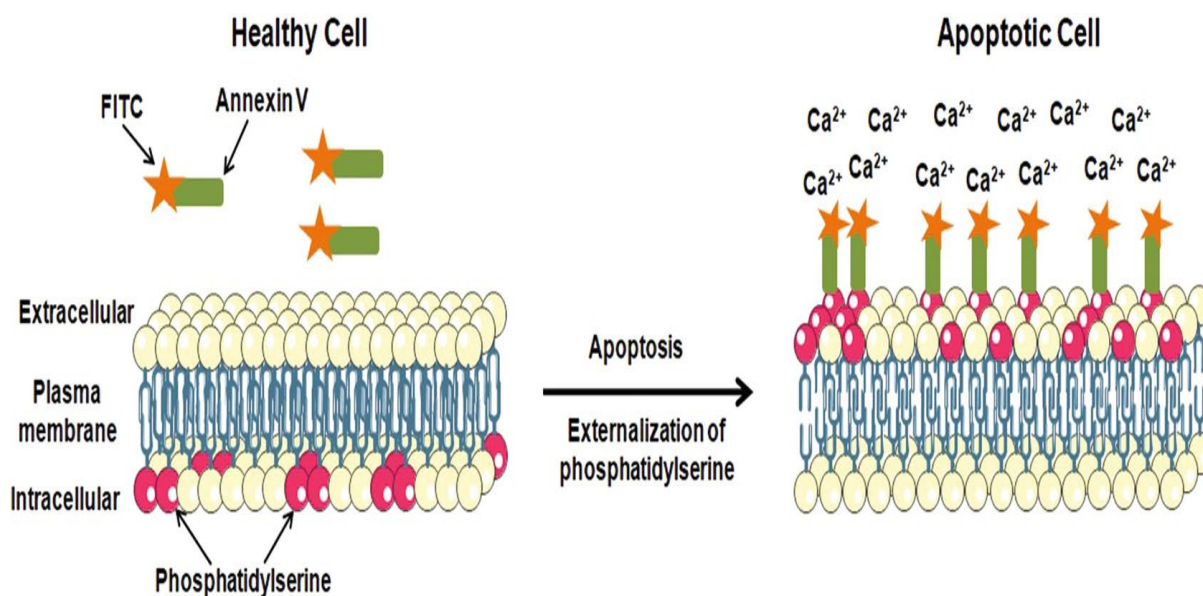


Figure 4.4: Schematic representation of the Annexin V assay for apoptosis detection

Source: Kumar et al. (2021)

This chapter aims to determine the cytotoxicity and mechanism of cell death induced by plant extracts on MCF7 and Caco2 cell lines (see Section 3.3.2). This was achieved by performing cytotoxicity and cell cycle analysis assays, and analysing mitochondrial membrane depolarisation and PS translocation, which are some of the biochemical factors that are characteristic of apoptosis.

4.3 METHODOLOGY

4.3.1 Cytotoxicity and IC₅₀ determination

Two cell lines, MCF7 (human breast cancer, purchased from Highveld Biological, South Africa) and Caco2 (human colorectal carcinoma, purchased from Cellonex, South Africa), were used in this study and five extracts from four plant species were extracted, namely 1) *C. paniculata* roots, 2) *C. paniculata* leaves, 3) *E. elephantina* roots, 4) *X. undulatum* roots, and 5) *D. anomala* roots, with the solvents DCM, DCM:MeOH, and MeOH, as explained in Section 3.3.2.

Dried extracts (50 mg) were reconstituted in 500- μ L dimethyl sulfoxide (DMSO) to give a final concentration of 100 mg/mL. In a humidified 37 °C incubator with 5% carbon dioxide (CO₂), cells were maintained in 10-cm culture dishes in a complete medium consisting of Dulbecco's Modified Eagle's Medium low-glucose cell culture medium supplemented with 10% foetal bovine serum (FBS). Cells were seeded into 96-well culture plates at 4 000 cells per well in 100- μ L aliquots and they were subsequently incubated overnight to attach. The final concentrations of 0.1 to 100 μ g/mL of the plant extract were obtained for treatment by adding an additional 100 μ L of complete medium containing twice the required final concentration of extract to the cells in quadruplicate. The final DMSO concentrations never exceeded 0.25%, and this concentration did not induce any significant change in any of the assays performed. Cells were incubated for 48 hours at 37 °C in a humidified incubator with 5% CO₂ in the atmosphere.

The treatment medium was aspirated and replaced with 100 μ L of phosphate buffered saline (PBS), containing calcium ion (Ca²⁺) and magnesium ion (Mg²⁺), along with Hoechst 33342 at a concentration of 5 μ g/mL. PI was added at a concentration of 10 μ g/mL using 10- μ L aliquots per well of a 110- μ g/mL stock. The cells were then imaged using an ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®). Nine image sites per well were acquired using a 10x Plan Fluor objective. The 4',6-diamino-2-phenylindole dihydrochloride (DAPI) filter was used for Hoechst 33342 and the Texas Red filter for PI. The images were analysed using the MetaXpress® High-Content Image Acquisition & Analysis Software. Untreated cells were used as negative controls and cells treated with 30 μ M melphalan were used as positive controls.

IC₅₀ values were calculated using the average number of live cells per image in each well with GraphPad Prism version 6. Since Hoechst 33342 is a membrane-permeant dye, it binds to the DNA of all cells in the well (including live and dead cells), whereas PI can only permeate the damaged membranes of dead cells. This enables the analysis software to differentiate between live cells (stained with Hoechst 33342 only) and dead cells (stained with Hoechst and PI).

4.3.2 Cell cycle analysis

Cells were seeded for cytotoxicity and IC₅₀ determination. The cells were then exposed to the plant extracts for 48 hours at the calculated IC₅₀. The cells were stained by adding 50-μL aliquots of a mixture of 20-μL Annexin V-FITC and 1-μL Hoechst 33342 in 2-mL PBS with Ca²⁺ and Mg²⁺ after the treatment medium was removed. Following a 15-minute incubation period in the dark at room temperature, images were captured using an ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®) fitted with a 10x Plan Fluor objective, the FITC filter for Annexin V-FITC, and DAPI filter for Hoechst 33342. Nine image sites were captured in each well, which represented roughly 70% of the total well area. The acquired images were analysed using the MetaXpress software and the Cell Cycle Application Module.

4.3.3 Mitochondrial membrane potential analysis

The cells were seeded for cytotoxicity and IC₅₀ determination. Following that, 100-μL staining solution of 25 nM TMRE and 5-μg/mL Hoechst 33342 in PBS with Ca²⁺ and Mg²⁺ were added to each well. The plates were incubated for 30 minutes at room temperature (protected from light). Fluorescence micrographs were captured immediately using an ImageXpress Micro XLS Widefield Microscope (Molecular Devices®) as described for cell cycle analysis, but using a tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) filter for TMRE. The acquired images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Mitochondrial membrane potential was determined using the percentage cells stained with TMRE.

4.3.4 Phosphatidylserine translocation

The cells were seeded for cytotoxicity and IC₅₀ determination. After removing the treatment medium, the cells were stained by adding 50-μL aliquots of a mixture of 20 μL of Annexin V-FITC and 1 μL of Hoechst 33342 in 2 mL of PBS containing Ca²⁺ and Mg²⁺. Thereafter, the cells were incubated at room temperature for 15 minutes in the dark. After adding 1.5 μL of PI to each well, the images were captured as described in Section 4.3.2, but including the Texas Red filter cube for PI. The images were then analysed using the MetaXpress® High-Content Image Acquisition & Analysis Software.

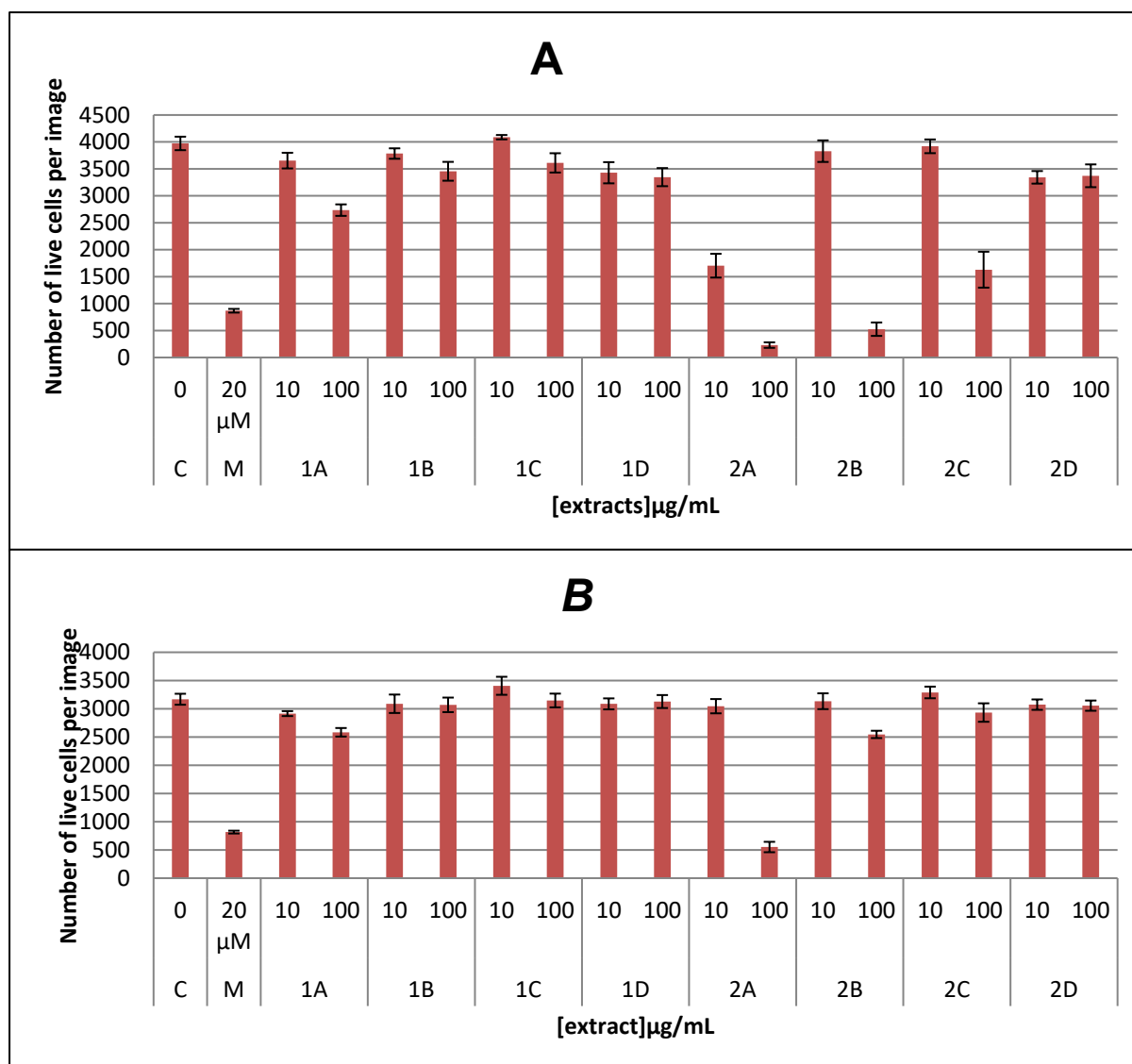
4.3.5 Statistical analysis

All experiments were conducted in quadruplicate. For image analysis, nine images were acquired in each well of a 96-well plate, covering 70% of the total growth area. Statistical analysis was performed using the two-tailed student t-test to compare the results of the treated cells with the controls.

4.4 RESULTS AND DISCUSSION

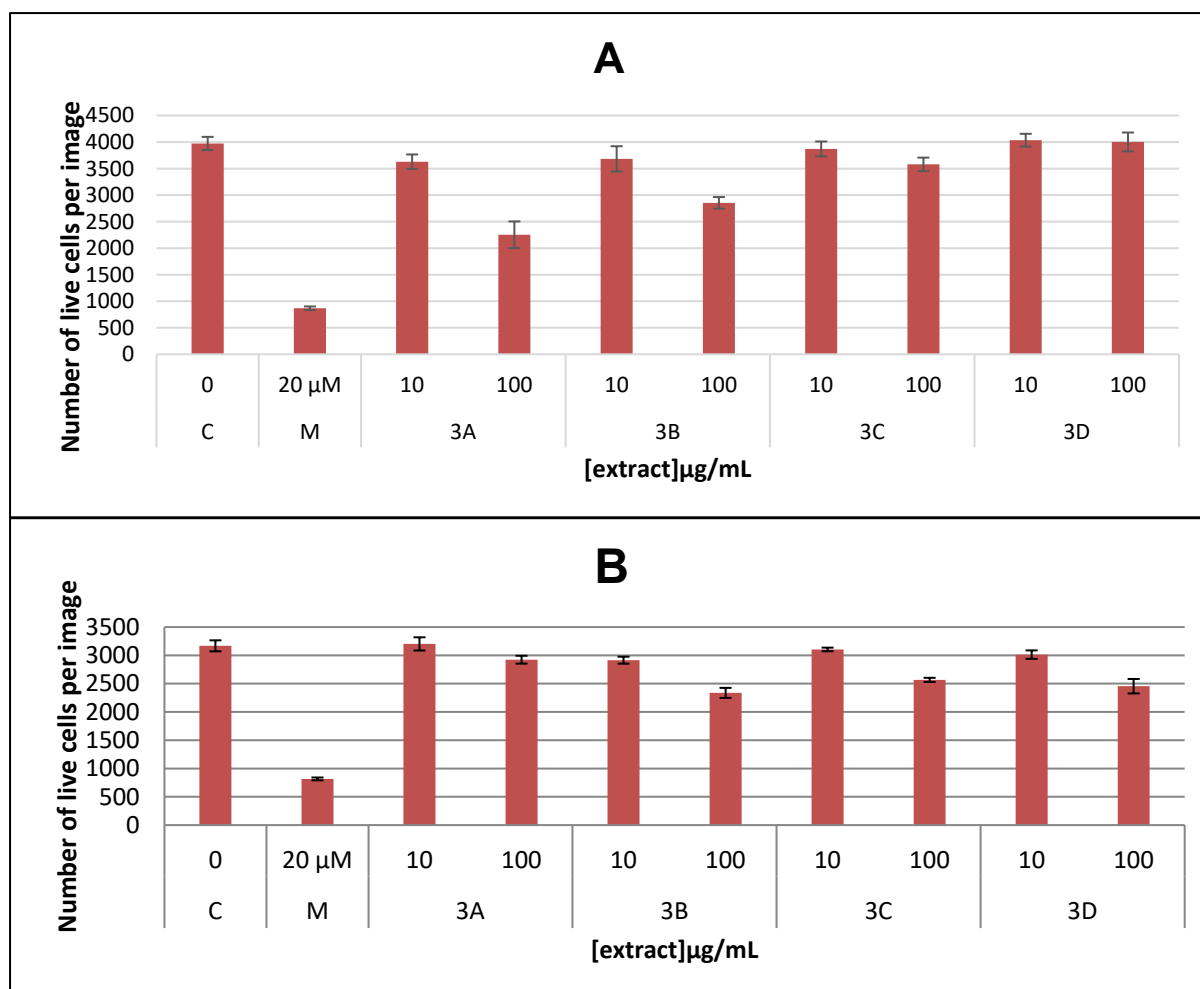
4.4.1 Cytotoxicity and IC₅₀ determination

The Caco2 and MCF7 cells were treated with 10 and 100 µg/mL of each extract mentioned in Section 4.1 to compare their cytotoxic effects. Thereafter, the IC₅₀ values were determined for the extracts that showed the best results. Figures 4.5, 4.6, 4.7, and 4.8 show bar graphs resulting from the treatment of Caco2 cells (A) and MCF7 (B) cells with the 20 extracts at 10 and 100 µg/mL. The DCM extracts (*C. paniculata* leaves, *X. undulatum* roots, and *D. anomala* roots) were found to be the most toxic for both the Caco2 and MCF7 cells. IC₅₀ values were determined for these extracts and were found to be 7.71 µg/mL for the *C. paniculata* leaves, 26.52 µg/mL for the *X. undulatum* roots, 1.80 µg/mL against Caco2 cells for *D. anomala* leaves, 25.74 µg/mL for the *C. paniculata* leaves, 8.99 µg/mL for the *X. undulatum* roots, and 2.24 µg/mL against MCF7 cells for the *D. anomala* roots (see Table 4.1). The rest of the extracts did not exhibit the preferred amount of toxicity, which is to kill more than 50% of the cancer cells at 100 µg/mL. As a result, these extracts were not used for further investigations. Melphalan was used as the positive control.



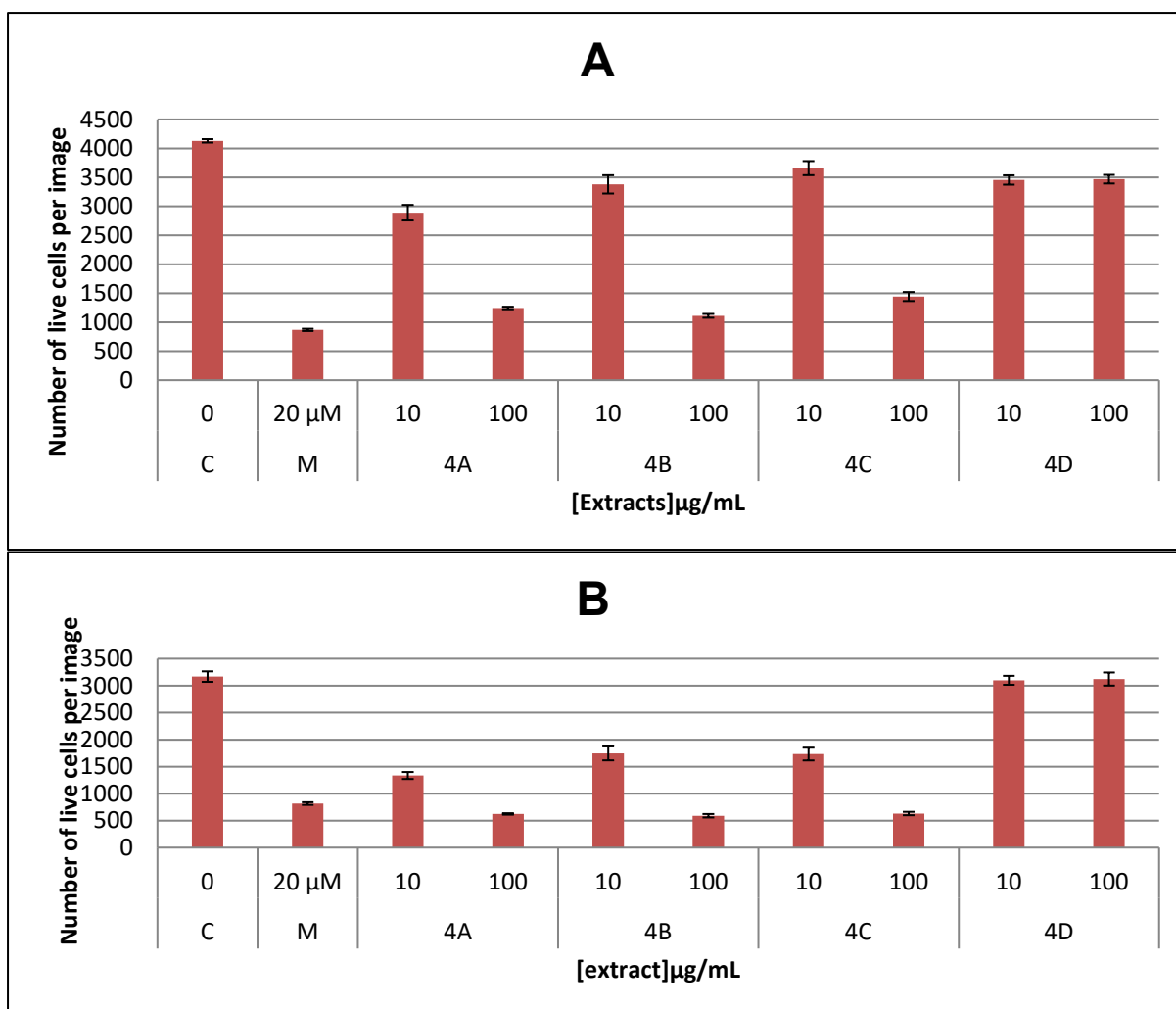
The data points represent the mean $\pm$ standard deviation (SD) of quadruplicate wells; 1 = *C. paniculata* roots, 2 = *C. paniculata* leaves, A = DCM, B = DCM:MeOH, C = MeOH, and D = dH₂O.

Figure 4.5: Cytotoxicity of *C. paniculata* root and leaf extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure



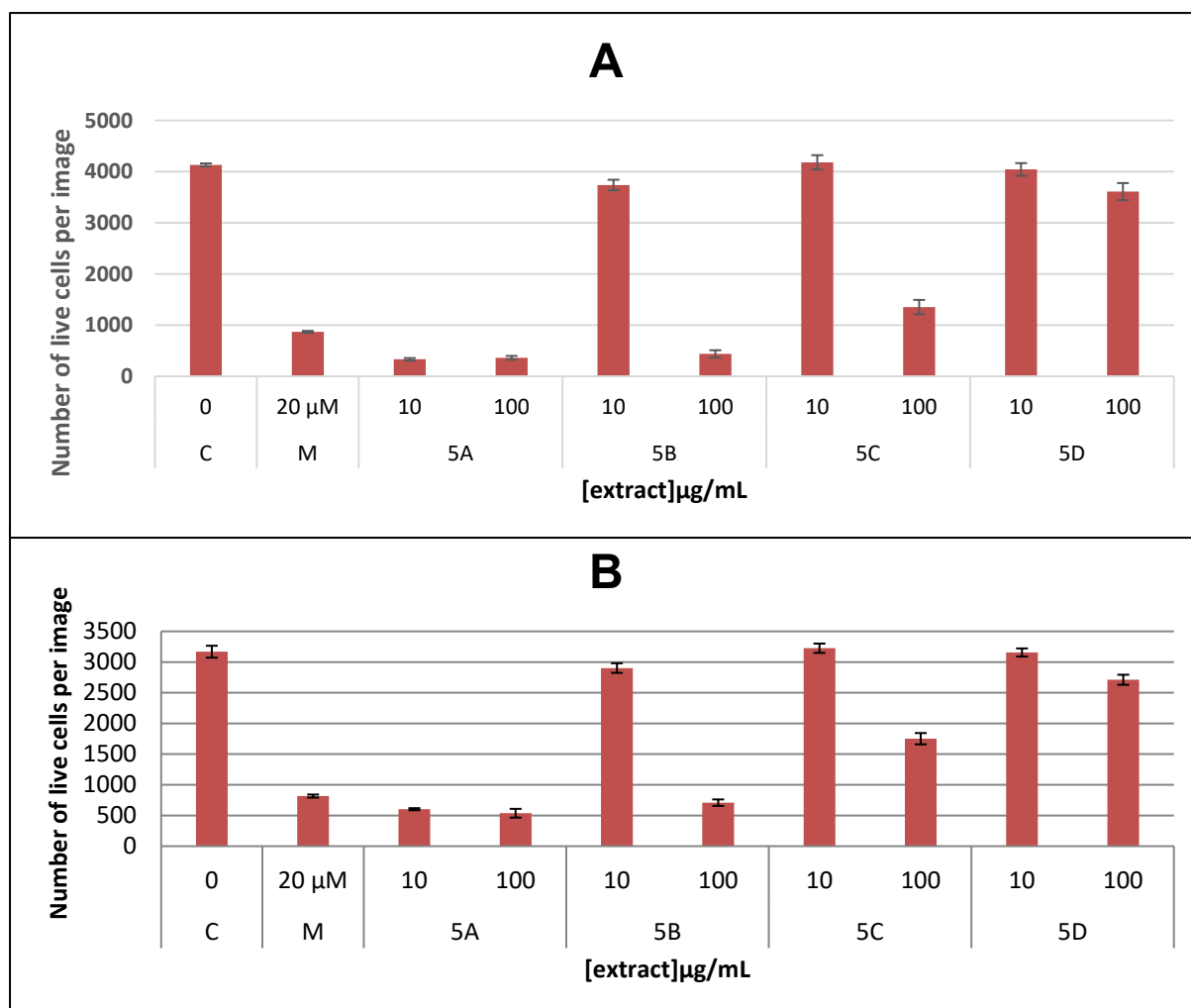
The data points represent the mean $\pm$ SD of quadruplicate wells; A = DCM, B = DCM:MeOH, C= MeOH, and D = dH₂O.

Figure 4.6: Cytotoxicity of *E. elephantina* root extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure



The data points represent the mean $\pm$ SD of quadruplicate wells; A = DCM, B = DCM:MeOH, C = MeOH, and D=dH₂O.

Figure 4.7: Cytotoxicity of *X. undulatum* root extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure



The data points represent the mean $\pm$ SD of quadruplicate wells; A = DCM, B = DCM:MeOH, C = MeOH, and D = dH₂O.

Figure 4.8: Cytotoxicity of *D. anomala* root extracts against Caco2 (A) and MCF7 (B) cells after 48-hour exposure

The effects of silver nanoparticles prepared from *D. anomala* on the MCF7 cancer cell line and the NF54 parasite strain were previously assessed by Tripathy et al. (2020). Their study found that green synthesised silver nanoparticles have anti-proliferative activity against the human hormone-dependent breast cancer cell line MCF7. A dose of 10 μ g/mL was used to determine the nanoparticles' mode of action against the MCF7 cells. Silver nanoparticles were found to damage MCF7 cells at this concentration by generating free radicals, eventually depleting the potential of the mitochondrial membrane, and harming the cells' nuclei. Similarly, *D. anomala* displayed toxicity towards the MCF7 cells in this study.

Many studies have been conducted on *X. undulatum* due to its pharmacological diversity but not much research has been conducted to investigate the activity on the

breast cancer cell line to support the claims made by traditional healers, and the same is evident with *C. paniculata*. A study conducted by Thakur et al. (2019) reported that both *X. undulatum* and *C. paniculata* leaf DCM:MeOH extracts had the potential to significantly reduce the production of β -Amyloid 42 A β 42, which is one of the targets to treat Alzheimer's disease. A study by Smit in 2020 suggested that *X. undulatum* crude aqueous extract has good potential anti-cancer activity, specifically for colon cancer. The results of this study suggest that *X. undulatum* root DCM extract has potential anti-cancer activity and that it was more effective on Caco2 colorectal cancer cells than MCF7 breast cancer cells.

The *E. elephantina* extracts showed no cytotoxic activity towards the MCF7 and Caco2 cell lines. Other studies, however, reported it to have phenolic and flavonoid compounds derived from the root and leaf extracts that possess anti-microbial activity, which suppresses bacterial infections and as a result leads to the healing of infections caused by micro-organisms (Tlotlo & Olivier, 2022).

Table 4.1: Summary of IC₅₀ values determined from GraphPad Prism version 6 software

Cells	<i>C. paniculata</i> leaves (2A) ($\mu\text{g/mL}$)	<i>X. undulatum</i> (4A) ($\mu\text{g/mL}$)	<i>D. anomala</i> (5A) ($\mu\text{g/mL}$)
Caco2	7.71	26.52	1.8
MCF7	25.74	8.99	2.24

According to Gertsch (2009), extracts tested at a high concentration of 200 $\mu\text{g/mL}$ and above are likely to show artificial results from in vitro assays, and even though the data may be reproducible, the effects may be due to non-specific interactions between phytochemicals and the cells. Gertsch (2009) further states that crude extracts should only be considered if IC₅₀ values of less than 50 $\mu\text{g/mL}$ are obtained against cell lines. The IC₅₀ values obtained from this study were less than 50 $\mu\text{g/mL}$ (see Table 4.1). These three extracts were used in further experiments to establish whether their effects were cell cycle dependent and if they induced apoptosis. The ratio of activities of the extracts using the IC₅₀ values towards the two cell lines was found to be 1:1.

4.4.2 Cell cycle analysis

Cell cycle analysis was performed to determine if cell cycle arrest contributed to the decrease in live cell numbers. The Caco2 and MCF7 cells were treated with each of the three selected extracts at their IC₅₀ values for 48 hours. Figure 4.9 shows that both the MCF7 and Caco2 cells arrested at early mitosis for *C. paniculata* and *X. undulatum* extracts, and for the *D. anomala* extract, MCF7 arrested at the G0/G1 phase and Caco2 cells arrested at the early M phase.

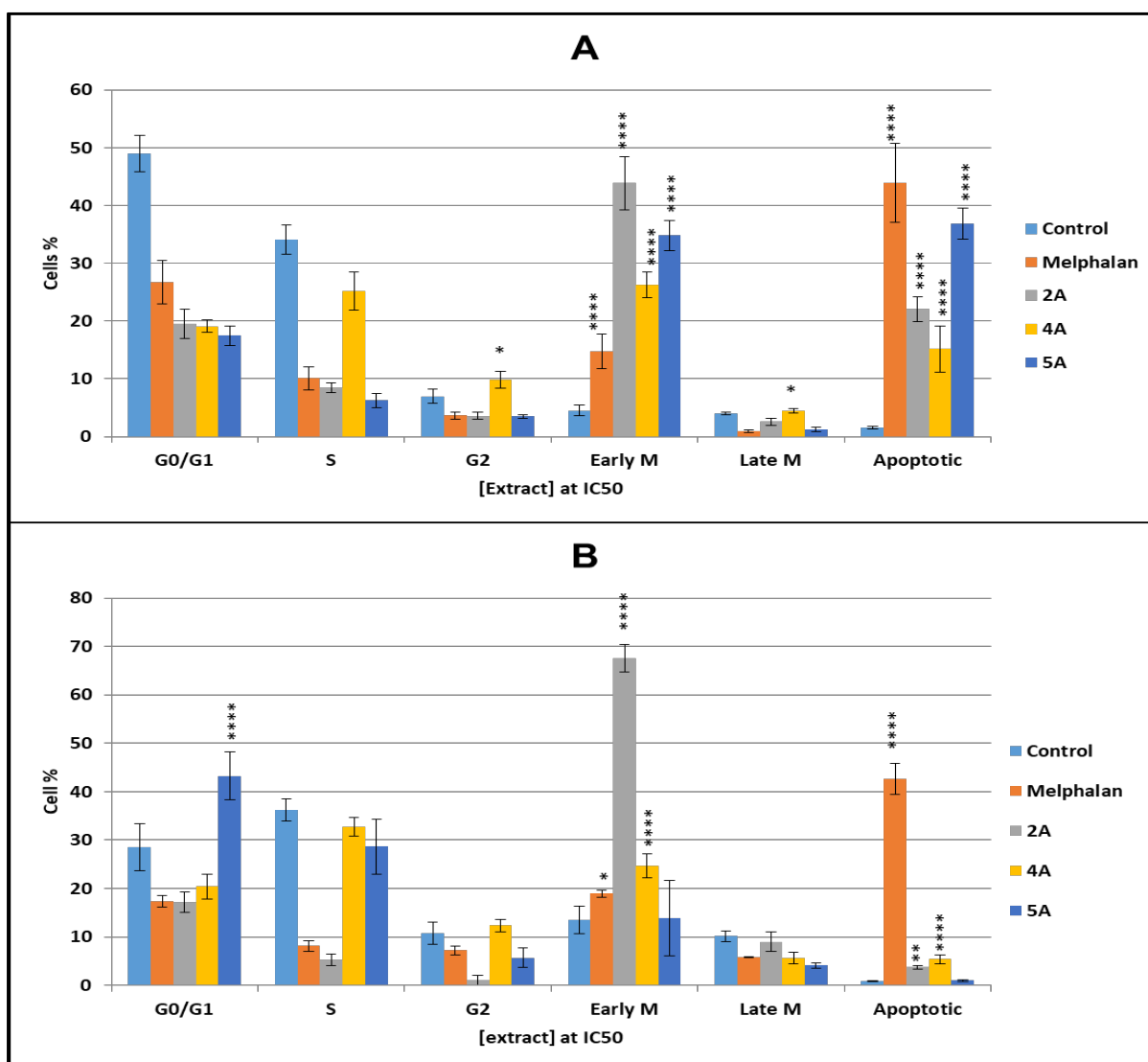


Figure 4.9: Cell cycle analysis of the Caco2 (A) and MCF7 (B) cell lines; control = untreated cells, 2A = *C. paniculata* leaf extract, 4A = *X. undulatum* root extract, and 5A = *D. anomala* root extract. The data points represent the mean $\pm$ SD of quadruplicate wells. The statistical significance compared to the control is indicated as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$, and **** $p < 0.001$.

For both cell lines, the *C. paniculata* leaf extract arrested at the early M phase. Cells that enter mitosis with mutations pass these errors to daughter cells; this checkpoint in the cell cycle therefore prevents a cell with damaged DNA from entering mitosis. For the *X. undulatum* root extract, the arrest was during the S phase for both cell lines; however, there was also a significant delay during the early M phase for both cell lines. During the G1/S phase arrest, cells have a chance to either go through the repair process or to follow the apoptotic pathway (Doan et al., 2019). This may therefore suggest that cells have gone through repair at the S phase but then arrested at the early M phase. A more likely explanation is that the extract contained more than one active compound that induced cell cycle arrest in different phases of the cycle. For the *D. anomala* root extract, cell cycle arrest was at the early M phase for the Caco2 cell line and the G0/G1 phase for the MCF7 cell line. All three extracts therefore acted in a cell cycle-dependent manner to slow down proliferation. The next step was to perform experiments to determine whether this effect induced programmed cell death in the form of apoptosis.

4.4.3 Mitochondrial membrane potential analysis

Depolarisation of the mitochondrial membrane is a sign that the intrinsic mode of apoptosis has begun and that pro-apoptotic proteins have been released from the mitochondria (Pfeffer & Singh, 2018). The *C. paniculata* leaf and *D. anomala* root extracts did not have any significant effects on the mitochondrial membrane potential of the Caco2 or MCF7 cells. The *X. undulatum* root extract induced depolarisation of the mitochondrial membrane potential in the Caco2 cells ($p < 0.005$), and a relative hyperpolarisation in the MCF7 cells ($p < 0.05$) (see Table 4.2).

Table 4.2: Changes in mitochondrial membrane potential after treatment with melphalan and plant extracts for 24 hours

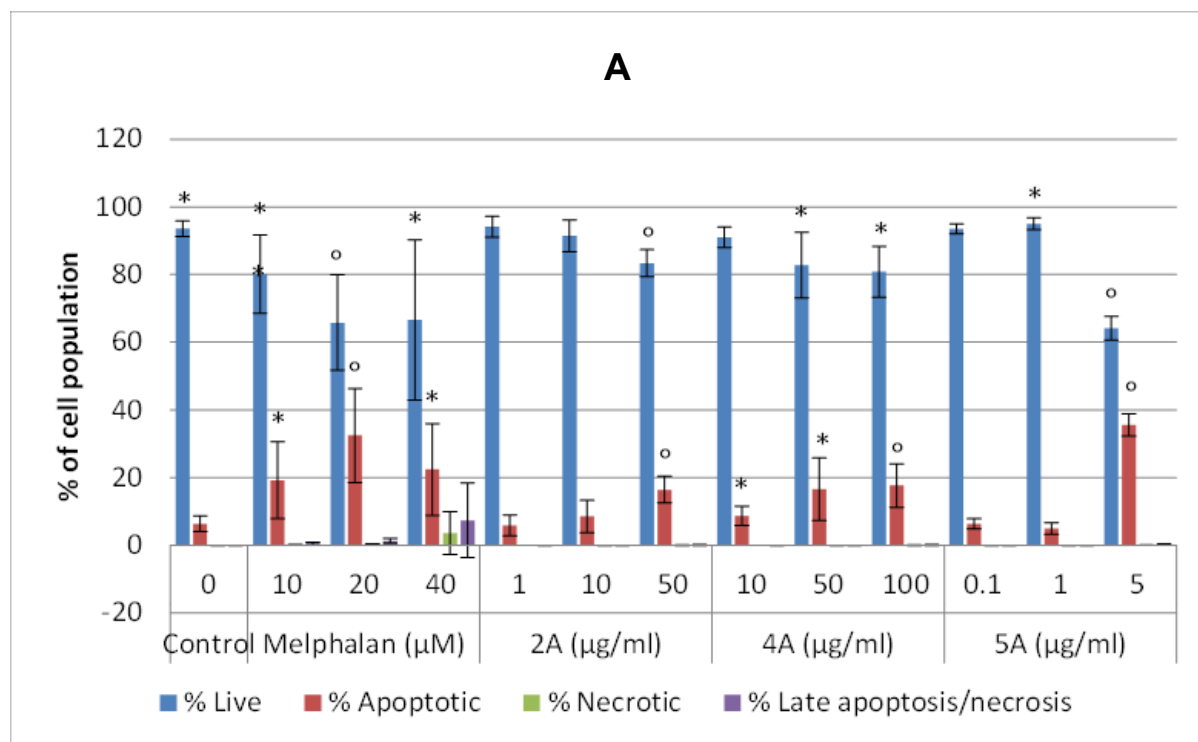
Treatment	Caco2 (% of control cells)	MCF7 (% of control cells)
Control	100 ± 8.11	100 ± 6.61
Melphalan	54.45 ± 37 ****	105.1 ± 5.30
<i>C. paniculata</i>	96.38 ± 9.21	96.05 ± 13.02
<i>X. undulatum</i>	79 ± 3.11***	118.1 ± 8.09 *
<i>D. anomala</i>	96.82 ± 9.74	98.03 ± 13.55

**** $p < 0.001$; *** $p < 0.005$; * $p < 0.05$ compared to control cells.

The mean fluorescence intensity of cells stained with TMRE is expressed as a percentage of control cells ± SD of quadruplicate values.

4.4.4 PS translocation

Due to the loss of membrane asymmetry that occurs when apoptosis is induced, PS translocation is regarded as an early indication of apoptosis (Majno & Joris, 1995). To determine the presence of PS on the outer cell surface, the Annexin V-FITC/PI assay was performed after 24 and 48 hours incubation of the cells. The *C. paniculata* leaf extract and *D. anomala* root extract had a substantial number of apoptotic cells after 24 hours (see Figure 4.10), while the *X. undulatum* root extract showed an increase in apoptotic cells for the Caco2 cell line after 48 hours (see Figure 4.11). The *C. paniculata* leaf and *D. anomala* root extracts showed significant numbers of necrotic cells for the MCF7 cell line after 48 hours.



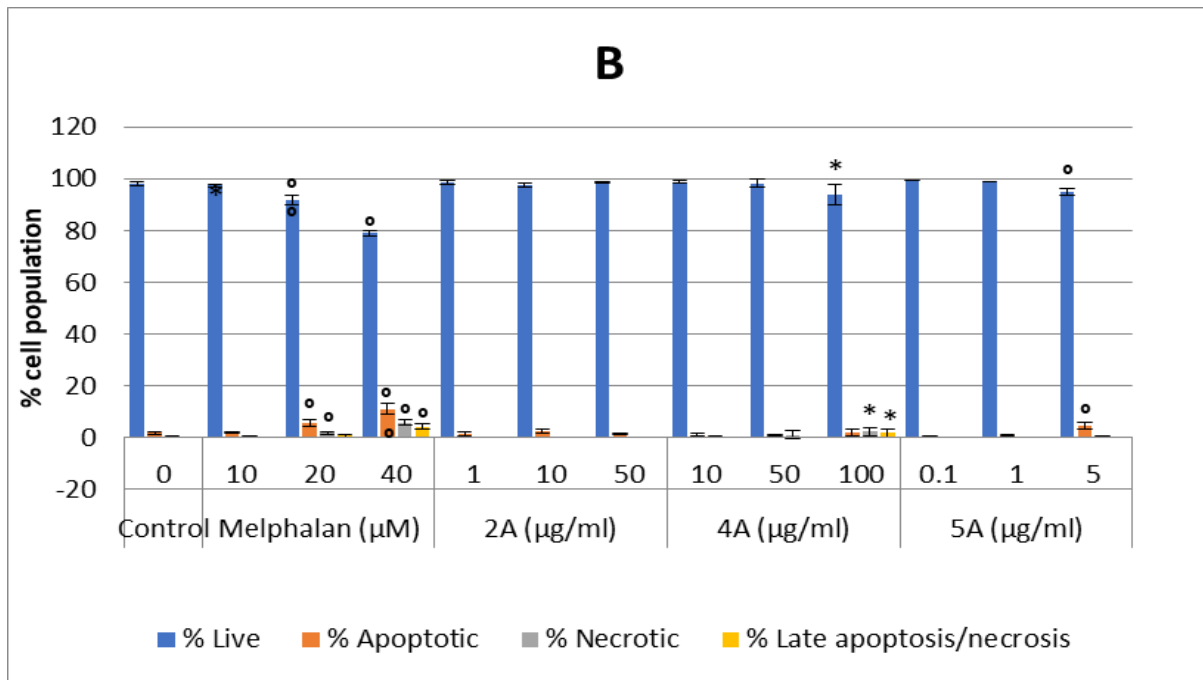


Figure 4.10: PS translocation on Caco2 (A) and MCF7 (B) cells after 24 hours of treatment with selected extracts and subsequent staining with Annexin V-FITC and PI; 2A = *C. paniculata* leaf DCM extract, 4A = *X. undulatum* root DCM extract, and 5A = *D. anomala* root DCM extract; control = untreated cells, positive control = melphalan

The data points represent the mean $\pm$ SD of quadruplicate wells. The statistical significance compared to the control cells is indicated as * $p < 0.05$; ** $p < 0.001$ and *** $p < 0.001$.

Figure 4.10 indicates a significant increase in cells stained positive for Annexin V and negative for PI after 24 hours; thus evidence of apoptotic cells and none for necrotic cells. This indicates the start of apoptosis. The *D. anomala* root and *C. paniculata* leaf extracts showed greater increases in apoptotic cells than the *X. undulatum* root extract. Necrotic cells were only seen under treatment with melphalan at the highest concentration used.

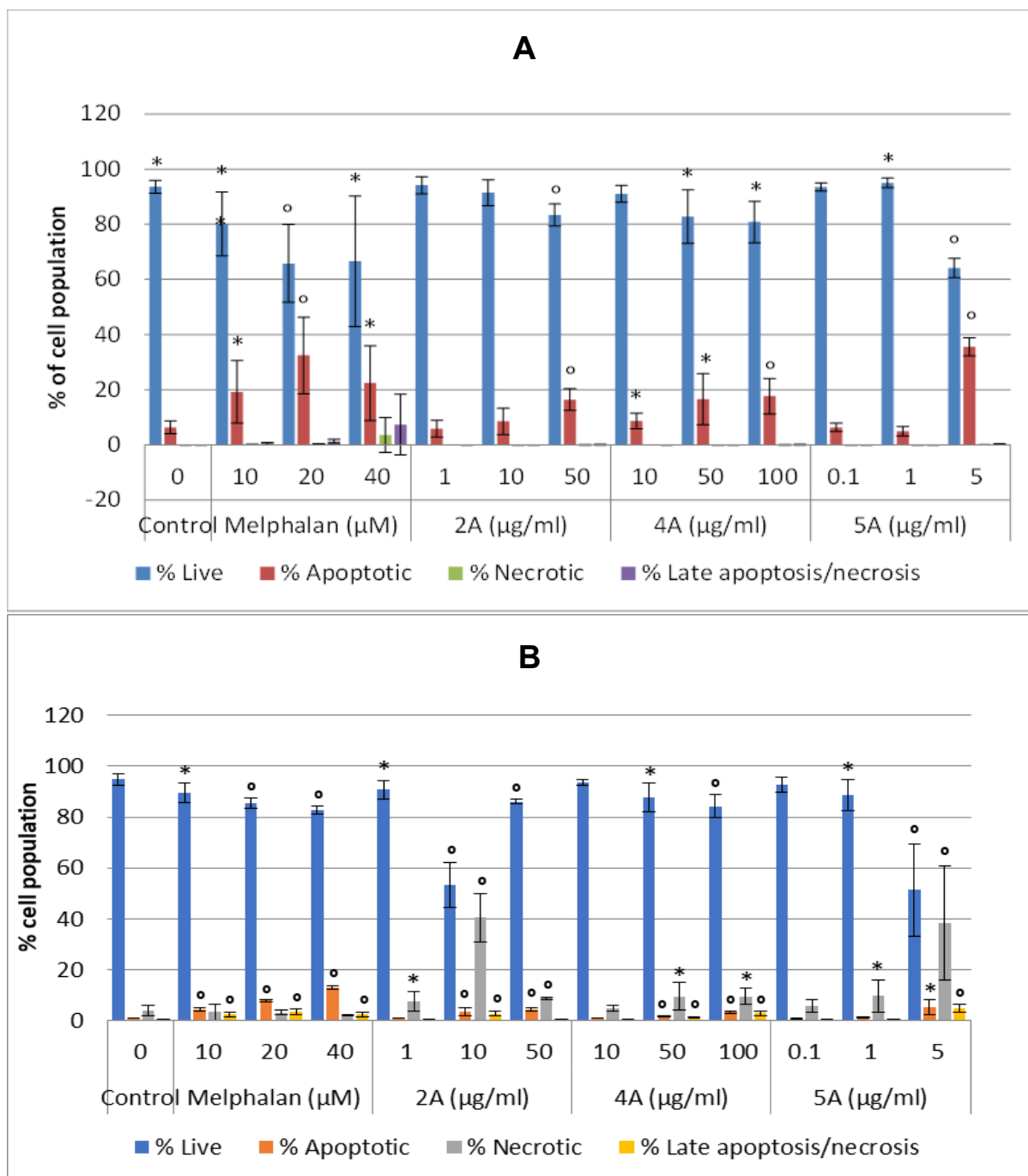


Figure 4.11: PS translocation on Caco2 (A) and MCF7 (B) cells after 48 hours of treatment with Annexin V-FITC and PI staining; 2A = *C. paniculata* leaf DCM extract, 4A = *X. undulatum* root DCM extract, and 5A = *D. anomala* root DCM extract; control = untreated cells and positive control = melphalan The data points represent the mean $\pm$ SD of quadruplicate wells. The statistical significance compared to the control cells is indicated as * $p < 0.05$; ** $p < 0.001$ and *** $p < 0.001$

An increase in PI-positive cells was observed after 48 hours in the MCF7 cells, while no increase was seen for the Caco2 cells (see Figure 4.11). This significant increase in necrotic cells was seen for the *C. paniculata* leaf extract and the *D. anomala* extract for the MCF7 cell line, with a comparatively small increase for the *X. undulatum* root extract. This suggests that the cells did not die due to necrosis for the Caco2 cells. Tripathy et al. (2020) reported that aqueous *D. anomala*-synthesised silver nanoparticles had higher percentages of late apoptotic/necrotic cells compared to their control group according to their Annexin V-FITC/PI staining results against the MCF7 cells, which supports the results obtained in this study.

4.5 CONCLUSION

The mainstay and existing standard of care for cancer includes surgery and chemotherapy, but the toxicity associated with these treatments highlights the urgent need for improved therapies. There is currently no scientific confirmation that *C. paniculata*, *D. anomala*, *X. undulatum*, or *E. elephantina* crude extracts have ever been used to treat cancer, except for the claims made by traditional healers and herbalists.

All 20 extracts of different solvents with different polarities were tested on two cancer cell lines, namely Caco2 and MCF7. The results indicated that the DCM extracts of *C. paniculata* leaves, *D. anomala* roots, and *X. undulatum* roots were the most toxic to the two tested cell lines. IC₅₀ values were determined using GraphPad Prism software and, as Gertsch (2009) mentioned, crude extracts should only be considered if they have IC₅₀ values lower than 50 µg/mL against the tested cell line. For this study, the IC₅₀ values were found to be 7.71 µg/mL for *C. paniculata* leaves, 26.52 µg/mL for *X. undulatum* roots, 1.80 µg/mL for *D. anomala* leaves, 25.74 µg/mL for *C. paniculata* leaves, 8.99 µg/mL for *X. undulatum* roots, and 2.24 µg/mL for *D. anomala* roots for Caco2 and MCF7 respectively. Melphalan was the positive control for all experiments conducted.

DNA cell cycle analysis was performed to determine the effects of the three chosen extracts. After the cells were exposed to the extracts, it was observed that for Extract 2A (*C. paniculata* leaves), arrest at the early mitosis phase was seen for both cell lines

(see Figure 4.9). For Extract 4A (*X. undulatum* roots), cell arrest was seen at the S phase for both cell lines, and for Extract 5A (*D. anomala* roots), cell cycle arrest was seen at the G0/G1 and S phases for the Caco2 cells.

Cells that have undergone apoptosis exhibit unique morphological alterations brought on by biochemical changes in the cells. These included the exposure of PS on the plasma membrane's outer surface and the permeability of the mitochondrial membrane. After determining where in the cell cycle the cells arrested, the mode of cell death was determined using the Annexin V-FITC/PI apoptosis assay. This assay uses the fact that Annexin V protein has a high affinity for PS and PS is translocated to the outer surface of the cell membrane during apoptosis. PI is used to show necrotic cells. The extract concentrations used were set at their respective IC₅₀ values, which were determined over 24 and 48 hours of incubation. Although an increase in PI-positive cells for MCF7 was observed after 48 hours, little or no increase in PI-positive cells for Caco2 cells after the same time was observed, which thus confirms that cell death was not due to necrosis.

Due to apoptosis, the mitochondrial membrane loses its integrity. In this study, the participation of the mitochondria in the onset of apoptosis was determined for the two cell lines, and the *X. undulatum* root extract caused mitochondrial membrane depolarisation. The results of this study confirm that the *X. undulatum* root DCM extract has potential anti-cancer activity and that it was more effective on Caco2 cells than the MCF7 breast cancer cells. It had a very low IC₅₀ of 8.98 µg/mL on the Caco2 cells, but a substantially higher IC₅₀ of 26.52 µg/mL on the MCF7 cells. The extract caused a reduction in mitochondrial membrane potential and an increase in PS translocation in the Caco2 cells, but not in MCF7 cells. The *C. paniculata* leaf DCM extract and the *D. anomala* root DCM extract had no effect on the mitochondrial membrane of either the MCF7 or Caco2 cells. They, however, both increased PS translocation in the Caco2 cells after 24 hours, and an increase in necrotic cells was observed after 48 hours for the MCF7 cells only.

The results obtained from the investigation of the *C. paniculata*, *D. anomala*, *X. undulatum*, and *E. elephantina* crude extracts on how they exert their cytotoxic effect on cancer cells were shown in this chapter. It is recommended that these

extracts, with the exception of the *E. elephantina* crude extract, be tested on normal cells to investigate if their cytotoxicity is selective to normal, healthy cells.

4.6 REFERENCES

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CHAPTER 5:

ANTI-INFLAMMATORY ACTIVITY

5.1 INTRODUCTION

Inflammation is defined as a “complex set of interactions among soluble factors and cells that can arise in any tissue in response to traumatic, infectious, post-ischemic, toxic or autoimmune injury” (Nathan, 2002). It is further described as a mechanism through which the body’s immune system responds in order to heal itself after an injury, to defend itself against foreign substances, or as a protective and reparative way the body responds to injury (Kalden, 1987; Nordqvist, 2017). There are two kinds of inflammation, namely acute and chronic inflammation. Acute inflammation is a healthy response that serves to protect and repair the body against harmful stimuli such as injuries or cuts that may have become infected. Symptoms from acute inflammation include swelling, redness, and pain (CBHS Health, 2015). A few examples of diseases or conditions associated with acute inflammation are sore throat from cold or flu, infected ingrown toenails, bronchitis, and sinusitis (Mayer & Bhikha, 2013).

Although acute inflammation is the initial response to injury and pathogens, it can also progress into a chronic condition. The accumulation of immune cells and pro-inflammatory mediators at the site of inflammation is characterised as chronic inflammation (Maurizi et al., 2018). It is a condition linked to dilated blood vessels that will eventually result in organ damage due to the body’s incapacity to handle such a condition. Chronic inflammation is believed to contribute to conditions such as TB, Alzheimer’s disease, cancer, diabetes, and cardiovascular disorders (CBHS Health, 2015).

Rudolf Virchow was the first physician to hypothesise the connection between inflammation and cancer in 1863 after he observed leukocyte infiltrates in tumours. Initially, it was believed that this observation was a sign of an anti-tumour immune response. Leukocyte infiltrates can, however, have both tumour-suppressive and tumour-promoting effects, as is now well known (Elinav et al., 2013). Since many cancer cells have been found to have up-regulated inflammatory pathways, treating inflammation might also directly influence cancer cells rather than just having an

immune system-mediated effect. Inflammation and immunity have recently been proposed as the seventh hallmark of cancer, which supports the observations made by Virchow in terms of both mechanism and epidemiology (Kamp et al., 2011; Thompson et al., 2015).

5.2 BRIEF LITERATURE REVIEW

5.2.1 Inflammation and cancer

Clinical and epidemiological research has demonstrated a strong relationship between chronic infection, inflammation, and cancer (Thun et al., 2004; Karin et al., 2006; Gomes et al., 2014). Alcohol abuse, asbestos exposure, cigarette smoke, or other conditions that may cause an organ to become chronically irritated lead to inflammation at the site and may eventually result in cancer. Chronic inflammation is also brought on by persistent micro-organism infections, which can lead to cancer (Sfanos et al., 2018). As inflammation progresses, numerous growth hormones, cytokines, and chemokines increase the activation and migration of additional inflammatory cells, lymphocytes, and leukocytes (Maiellaro & Taylor, 2007).

For a tumour cell to survive, grow, proliferate, differentiate, mutate, and migrate, it is influenced by the inflammatory cytokine network. The suppression of anti-tumour immunity occurs when chronic inflammation develops as a result of excessive exposure to pro-inflammatory cytokines, which promote the growth of the tumour (Davern et al., 2021).

Although inflammatory processes continue to be a crucial element of the immune response, they can be controlled and suppressed to offer an alternate method of defence against diseases such as cancer. Chemoprevention has been hypothesised to result from the control of specific cellular inflammatory components, such as the production of reactive species with oxidant-producing enzymes such as cyclooxygenase (COX), nitric oxide synthase (NOS), and the transcription factor nuclear factor kappa B (NF- κ B), which are crucial to the carcinogenesis process. These could eventually offer molecular targets to help with the prevention, early identification, and treatment of cancers that are linked to inflammation (Pathak et al., 2005; Saw et al., 2010). Figure 5.1 illustrates a pro-carcinogenic scenario that occurs during chronic inflammation.

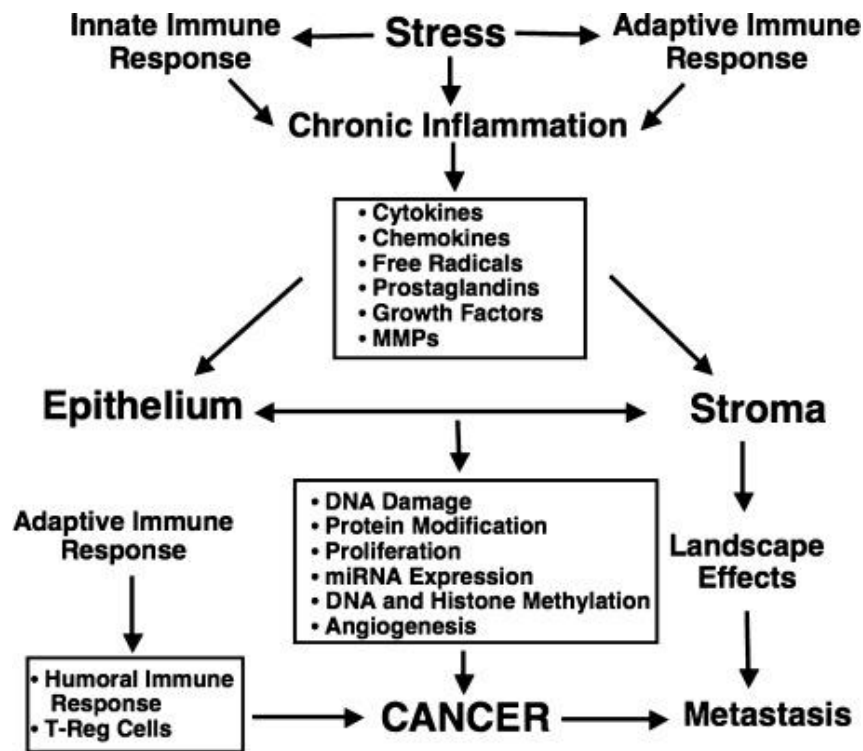


Figure 5.1: An example of a pro-carcinogenic scenario that occurs during chronic inflammation

Source: Perwez Hussain and Harris (2007)

5.2.2 Nuclear factor kappa B (NF-κB)

An important biological relationship between inflammation and cancer is the transcription factor NF-κB (Sansone & Bromberg, 2011; Serasanambati & Chilakapati, 2016). After being discovered approximately two decades ago, mounting evidence has supported its function in mediating a number of pro-tumorigenic pathways. NF-κB regulates a number of genes whose by-products promote angiogenesis, metastasis, and cell cycle progression, as well as the inhibition of apoptosis. Additionally, many NF-κB target genes encode mediators of the innate immune response and inflammation, including cytokines, chemokines, proteases, inducible nitric oxide synthase (iNOS2), and cyclooxygenase-2 (COX-2) (Pathak et al., 2005; Factor, 2015; Gong et al., 2021). These compounds disrupt cellular functions that may cause the development and spread of cancer (see Figure 5.2).

Moreover, NF-κB promotes the expression of a broad variety of target genes, including 1) regulating apoptosis by increasing the expression of the majority of anti-apoptotic proteins that are associated with tumour survival to suppress the death of tumour cells,

2) stimulating tumour cell cycle progression and proliferation through influencing the gene expression of c-Myc, cyclin D1, and COX-2, 3) enhancing the epithelial-to-mesenchymal transition to induce tumour invasion, and 4) creating an inflammatory environment for the growth, invasion, angiogenesis, and metastasis of cancer cells via regulating the gene expression of vascular endothelial growth factors, matrix metalloproteases, and adhesion molecules (Serasanambati & Chilakapati, 2016; Mezzasoma et al., 2016; Caviedes et al., 2017; Li et al., 2020).

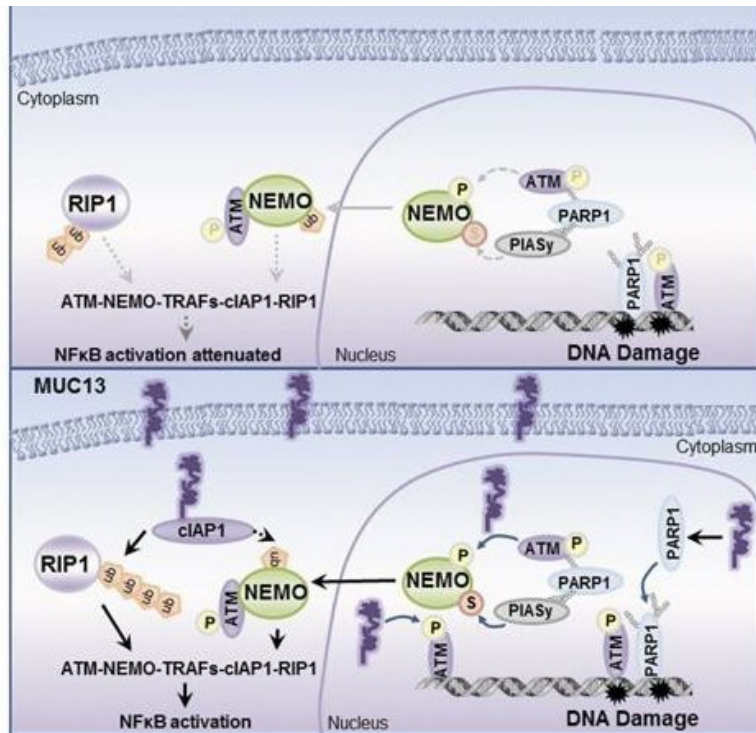


Figure 5.2: NF-κB function in cancer and its molecular targets

Source: Sheng et al. (2017)

5.2.3 Cyclooxygenase-2 (COX-2)

COX-2 is a key player in tumorigenesis as it produces prostaglandins in both cancerous and stromal cells, which prevent apoptosis, increase cell migration, and promote neo-angiogenesis (Tuncer & Banerjee; 2015; Marelli et al., 2017). The expression of COX-2 is activated by cytokines, endotoxins, growth factors, and tumour promoters. It also contributes to local immunological and inflammatory responses through the production of prostaglandins in response to environmental stimuli as it is usually undetectable under resting conditions (see Figure 5.3.) (Wang & DuBois et al., 2018).

Overexpression of COX-2 is seen in a number of cancers, including those of the stomach, liver, oesophagus, pancreas, head and neck, lung, breast, and bladder, and cervical, endometrial, skin, and colorectal cancers when compared to non-malignant tissues (Koki & Masferrer, 2002; Desai et al., 2018).

Singh et al. (2007) state that there is sufficient proof to suggest that COX-2 overexpression contributes to breast cancer tumorigenesis. These claims support the study by Ristimäki et al. (2002), which investigated the expression of COX-2 protein in tissue array specimens from 1 576 invasive breast cancers. The study found that 37.4% of the tumours showed moderate to strong COX-2 protein expression, which was linked to poor long-term disease-free survival.

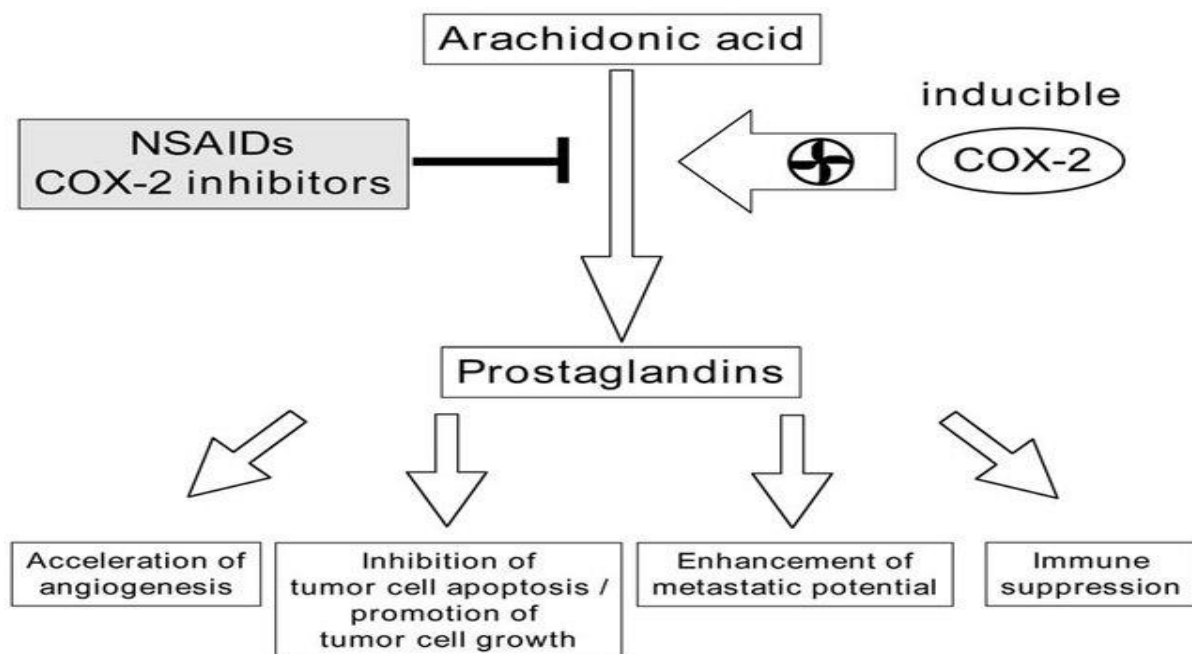


Figure 5.3: Schematic presentation of how COX-2 contributes to cancer

Source: Ohno et al. (2005)

5.2.4 Nitric oxide (NO)

NO is a short-lived molecule synthesised by the enzyme NOS, in a reaction that converts arginine and oxygen into citrulline and NO (Marelli et al., 2017). NO has a variety of roles in cancer therapy, tumour growth, and carcinogenesis, which are all influenced by the cell microenvironment. Numerous studies have found that NO plays a role in the development of genotoxic lesions, as well as the promotion and

progression of tumours by modulating crucial processes such as angiogenesis, tumour cell proliferation, and invasion (Singh & Gupta, 2011) (see Figure 5.4).

Although it participates in normal signalling, NO can also be an apoptotic or cytotoxic molecule when released in high concentrations by inducible nitric-oxide synthase (iNOS) (Bian et al., 2012). A number of chronic inflammatory degenerative disorders may be brought on by an excess of NO, oxide ion (O^{2-}), and peroxynitrite ($ONOO^-$). These molecules may induce oxidative stress and tissue damage. One of the risk factors for cancer has been thought to be macrophage participation in various chronic inflammatory illnesses.

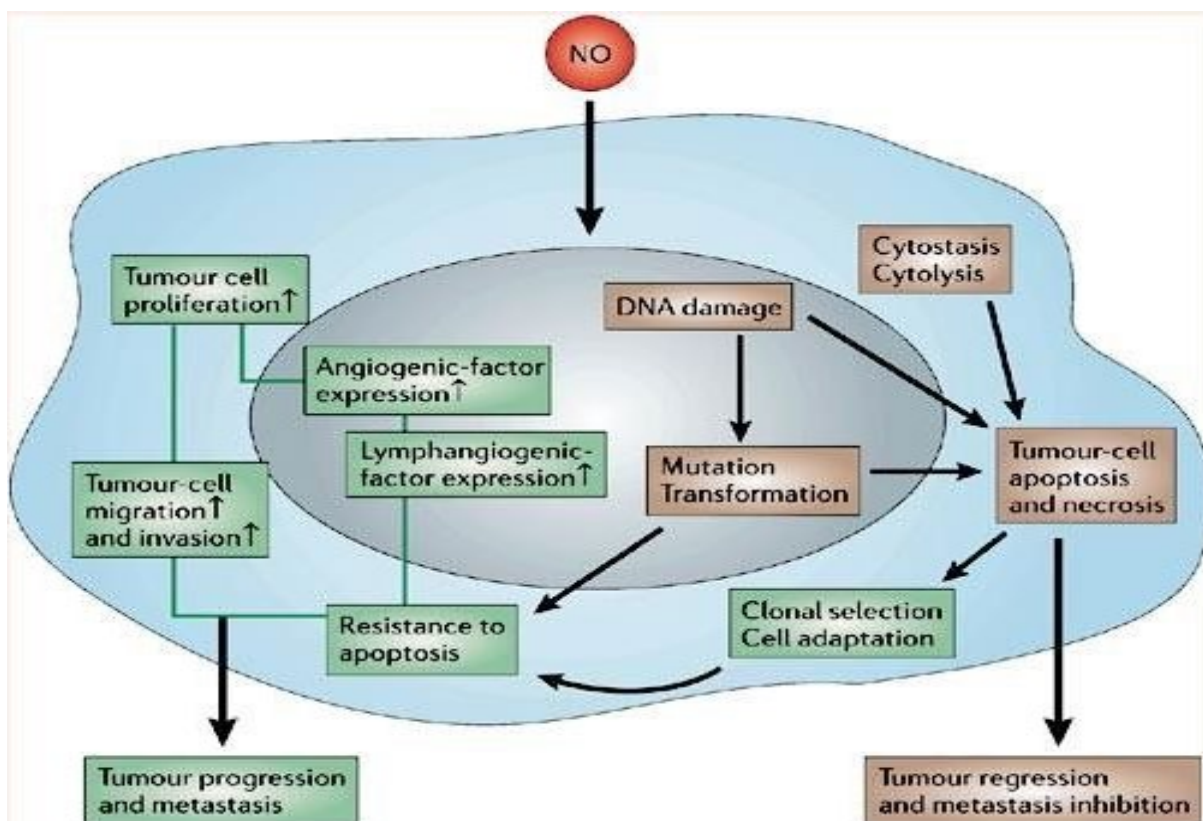


Figure 5.4: NO's contribution to tumour progression

Source: Fukumura et al. (2006)

5.3 METHODOLOGY

RAW 264.7 mouse macrophages were purchased from Cellonex (South Africa). Lipopolysaccharide (LPS), Griess reagent, and aminoguanidine (AG) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Roswell Park Memorial Institute (RPMI)

1640 culture medium and FBS were purchased from GE Healthcare Life Sciences (Logan, Utah, USA).

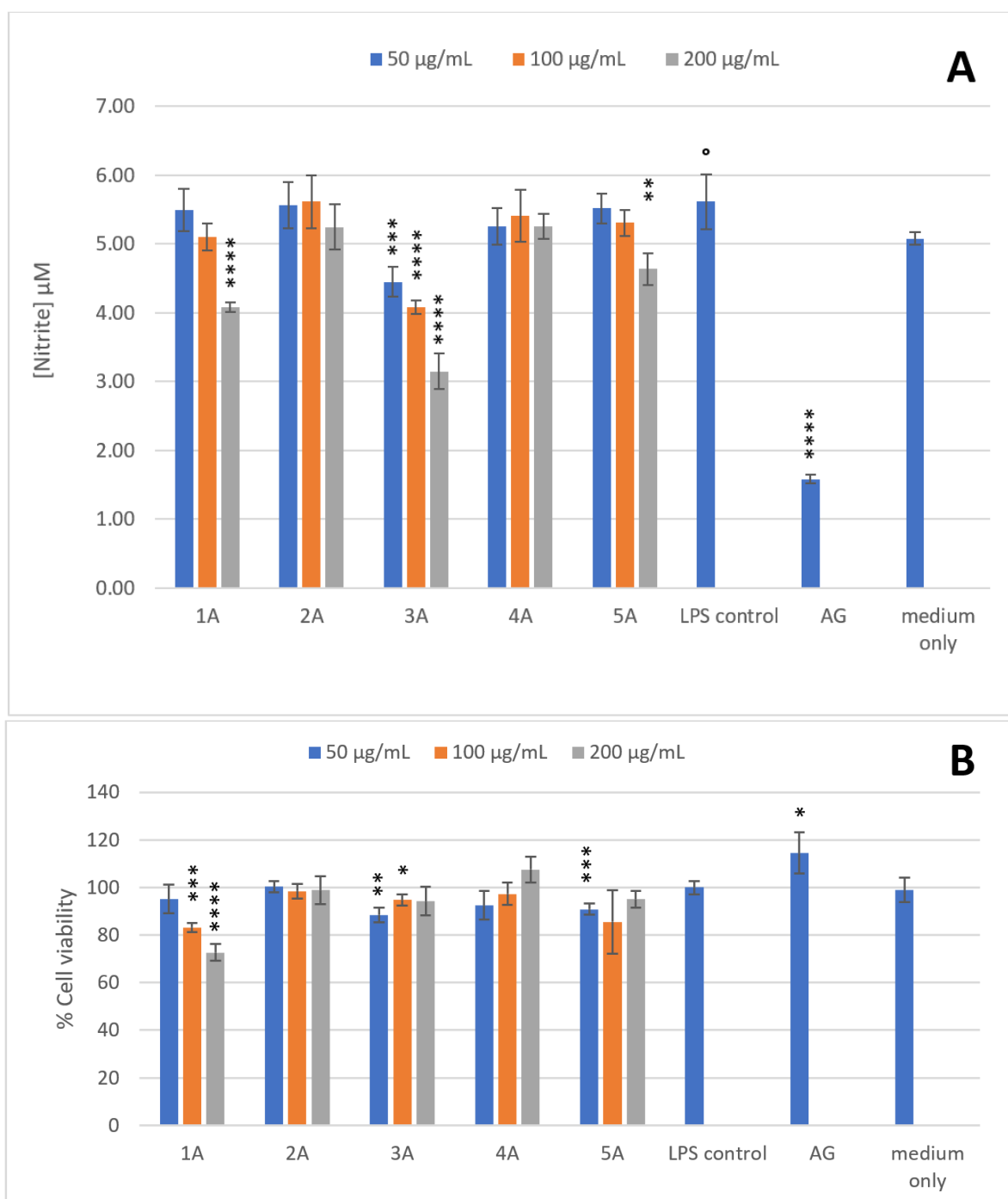
Five aqueous extracts (see extraction process in Section 3.3.2) were solubilised using DMSO to make a stock of 100 mg/mL. Extracts were stored at 4 °C until used. AG (100 µM) was used as a positive control to indicate anti-inflammatory activity.

Cells were seeded in the RPMI 1640 medium supplemented with 10% FBS (complete medium) at 50 000 cells per well. The following day, the spent culture medium was removed and the samples (diluted in RPMI complete medium) were added to give final concentrations of 0, 50, 100, and 200 µg/mL. To assess the anti-inflammatory activity, 50 µL of LPS (final concentration of 500 ng/mL) containing medium was added to all the wells, except the negative control wells, which received 50 µL of RPMI complete medium. AG was used as the positive control at 100 µM. Cells were incubated for a further 24 hours. To quantify NO production, 50 µL of the spent culture medium was transferred to a new 96-well plate and 50 µL of Griess reagent was added. Absorbance was measured at 540 nm. A standard curve using sodium nitrate dissolved in the culture medium was used to determine the nitrite concentration in each sample.

To confirm the absence of toxicity as a contributory factor, cell viability was assessed using MTT. This was done by the removal of the remaining medium and treatments from each well and replacing them with medium containing 0.5 mg/mL MTT and further incubated for 30 minutes at 37 °C. Thereafter, the MTT was removed and 100 µL of DMSO was added to each well to solubilise the formazan crystals. Absorbance was read at 540 nm. Absorbance was measured using a BioTek® PowerWave XS spectrophotometer (Winooski, Vermont, USA).

5.4 RESULTS AND DISCUSSION

The *E. elephantina* aqueous extract (3A) showed the most promising anti-inflammatory activity of the five tested extracts without inducing cytotoxicity against RAW 264.7 macrophages (see Figure 5.5). The *C. paniculata* root aqueous extract (1A) and *D. anomala* root extract (5A) appear to have anti-inflammatory potential at 200 µg/mL; however, these extracts also showed some degree of cytotoxicity at this concentration, which could have resulted in lower NO production.



The data points represent the average $\pm$ SD of quadruplicate values of one experiment. Statistically different compared to medium-only control ($p < 0.05$) or LPS control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$).

Figure 5.5: The effect of aqueous extracts on NO production (A) and cell viability (B) in RAW 264.7 macrophages; 1D = *C. paniculata* roots, 2D = *C. paniculata* leaves, 3D = *E. elephantina* roots, 4D = *X. undulatum* roots, and 5D = *D. anomala* roots

The growth and metastasis of tumours depend on angiogenesis. Previous studies have revealed that both mechanisms have been found to work with NO to mediate angiogenesis directly and indirectly. This was emphasised by Fukumura et al. (2006)

in their review of the role of NO in tumour progression, in which it was stated that high levels of NO activate tumor progression by inducing cell invasion, proliferation, and angiogenesis. *C. paniculata* and *D. anomala* root extracts may have anti-cancer activity by inhibiting NO production, and in so doing, prevent tumor metastasis. Reducing NO production in the tumour environment could therefore be an important target for anti-cancer therapies. Adedapo et al. (2008) showed that the aqueous extract of the stem bark of *C. paniculata* have a significant anti-oedematogenic effect on rat paw oedema induced by carrageenan, which led them to conclude that *C. paniculata* can be effective in acute inflammatory disorders because the carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting as the mediators of acute inflammation. There were no other anti-inflammatory reports on other plants.

5.5 CONCLUSION

The anti-inflammatory assay results showed that only the *E. elephantina* (3A) aqueous extract had an inhibitory effect on the production of NO out of the five tested extracts without being toxic to the RAW 264.7 cells. The *C. paniculata* and *D. anomala* roots inhibited the production of NO at the highest concentration tested (200 µg/mL). To determine the effects of treatment on the viability of the cells, an MTT cytotoxicity assay was performed on the cells after exposure to the extract and LPS, where the results showed that the extracts had no cytotoxic effects, except for *C. paniculata* at 200 µg/mL. AG was used as the positive control at 100 µM.

In conclusion, unresolved inflammation and a pro-tumorigenic microenvironment might result from deregulation in a complex and well-coordinated inflammatory response. Inflammation-related mediators have the ability to cause post-translational modifications, modify gene expression profiles, and point mutations in tumour suppressor genes, all of which can result in genetic and physiological instability and cancer. These changes have been mentioned in chronic inflammatory disorders that are known to increase the risk of developing cancer. However, some of these mediators, such as NO, can act in both pro-tumorigenic and anti-tumorigenic ways, which demonstrates the complexity of their involvement in the human body. Continuing investigations involving NO will likely inspire new cancer treatment methods due to its many biological activities.

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CHAPTER 6:

IDENTIFICATION AND CHARACTERISATION OF *X. UNDULATUM* PLANT EXTRACTS

6.1 INTRODUCTION

Because of the unprecedented availability of chemical variety, products from medicinal plants, whether as pure compounds or as crude extracts, provide limitless prospects for new drug leads. There have been numerous reports of positive biological activity, including analgesic, anti-cancer, anti-bacterial, anti-oxidant, anti-diarrheal, and wound-healing activities of products derived from natural products (Sasidharan et al., 2011). It is therefore essential to identify and characterise the compounds that are responsible for the biological activity in assays, as this will help researchers to identify the targets during therapeutic strategies for cancer and tumours.

The phytochemical analysis report of *X. undulatum* in Chapter 3 (see Table 3.1) showed the presence of flavonoids, tannins, terpenoids, alkaloids, and steroids. Previous studies reported that these secondary metabolites possess anti-carcinogenic, anti-inflammatory, anti-mutagenic, and anti-oxidative activities, as well as astringent, anti-septic, anti-phagocytic, anti-microbial, anti-neoplastic, anti-tumour, anti-ulcer, anti-malaria, diuretic, analgesic, anaesthetic, apoptogenic, and toxicity properties (Metodiowa et al., 1997; Havsteen, 2002; Aniszewski, 2007; Saxena et al., 2013; Ntsoelinyane, 2014; Xaba, 2016; Xuan & Khanh, 2016; Encyclopaedia Britannica, 2020).

Many drugs undergoing clinical trials for a variety of illnesses are of natural origin. Compound identification used to be labour intensive and time consuming and used a great deal of chemicals. Modern approaches to drug development, however, use robotics and full automation to screen hundreds of molecules quickly and utilise a variety of assays with very small quantities of chemicals (Youdim & Saunders, 2010). Several methods, including LC-MS and high performance liquid chromatography-nuclear magnetic resonance (HPLC-NMR) spectroscopy, are used to detect and identify the chemical components of plants (Santos-Buelga et al., 2012).

6.2 BRIEF LITERATURE OVERVIEW

Compounds in plants can be divided into two categories: primary and secondary metabolites. Primary metabolites are essential for the survival of the plant and to provide it with the necessary nutrition (Pagare et al., 2015). Secondary metabolites, also known as bioactive compounds, are those that are primarily active in the treatment and prevention of human and animal diseases. They are typically unique to individual plant species or are species-specific; however, some bioactive substances may be present in many other plant species of a genus, in multiple related genera, or even families (Pagare et al., 2015; Alamgir, 2018).

Due to the plant's medicinal diversity, the phytochemical properties of *X. undulatum* have been thoroughly investigated, which led to more than 18 compounds being isolated and characterised; however, there have only been a few scientific investigations on the biological effects of this plant (Vermaak et al., 2014). Thakur et al. (2021) contributed to existing knowledge on *X. undulatum* with their study on Alzheimer's disease and isolated three active compounds: 1) acetylated glycosylated crotoxigenin, 2) xysmalogenin-3, β -d-glucopyranoside, and 3) crotoxigenin 3-O-glucopyranoside. All three isolated compounds were effective in reducing levels of Alzheimer's disease-associated β -Amyloid peptides A β 42 in vitro. Their structures were further elucidated using NMR and MS.

Many secondary metabolites are found in plants, and phenolic compounds are the most prevalent. Moreover, their distribution in different plants and plant parts is not uniform and due to their structural diversity, phenolic compounds and their stability vary significantly (Santos-Buelga et al., 2012). Phenolic compounds are usually linked to the plant's defence mechanism, and they also play a significant role in other processes, such as accelerating pollination, providing colour for concealment, and offering protection against herbivores (Acamovic & Brooker, 2005; Lin et al., 2016). Plant polyphenols may offer dietary anti-oxidant protection for human health and disease. Phenolic compounds are abundant in plant-based foods and beverages and act as natural anti-oxidants, which are essential for both food and healthcare. According to several studies, phenolic compounds are the dietary anti-oxidants that are most prevalent in typical human diets. Recently, phenolic chemicals have attracted a great deal of attention due to active reports of their speculative role in delaying a number of human ailments (Lin et al., 2016; Motlounge et al., 2020).

A successful drug discovery programme involves several steps; one of which is the quick identification of recognised compounds in natural product extracts (Sashidhara et al., 2007). LC is one of the chromatographic methods that play a crucial role in pharmaceutical businesses and research. LC-based techniques assist in the structural characterisation and quantification of degradation products and contaminants in bulk drug materials and pharmaceutical formulations due to their high resolving power (Beccaria & Cabooter, 2020). Due to its high throughput, selectivity, and sensitivity, the combination of LC and MS has grown in significance in the drug discovery and development process. LC-MS combines the mass analysis of MS with the separation abilities of LC to identify compounds with high molecular specificity and detection sensitivity and produces pure or nearly pure fractions in a chemical mixture (Sashidhara et al., 2007; De Meulder et al., 2016; Stavrianidi, 2020).

Pharmaceuticals are analysed using LC-MS technologies at various stages of their discovery and development. Drug development, product characterisation, metabolism investigations (in vitro / in vivo), and the detection of contaminants and degradation products are some of the stages involved in the discovery of pharmaceutical drugs. Retention periods, molecular weights, and associated fragmentation patterns have significantly increased the use of LC-MS, and they are now recognised as critical analytical elements in the process of developing new drugs (Cobice et al., 2015). The aim of this chapter was therefore to fingerprint *X. undulatum* plant extracts using LC-MS.

6.3 METHODS

6.3.1 Extraction

Two grams of sample from each of the prepared *X. undulatum* extracts, namely dH₂O, MeOH, DCM, and DCM:MeOH, were accurately weighed into a 50-mL centrifuge tube, respectively. Fifteen millilitres of 50% MeOH / 1% formic acid was added and the tubes were tightly capped. Thereafter, the samples were vortexed for one minute, followed by extraction in an ultrasonic bath for one hour. Two millilitres of sample were then withdrawn and centrifuged at 14 000 revolutions per minute for five minutes. The clear supernatant was then transferred into 1.5-mL glass vials for analysis.

6.3.2 Liquid chromatography-mass spectrometry (LC-MS) analysis

A Waters Synapt G2 quadrupole time-of-flight mass spectrometer connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, Massachusetts, USA) was used for high-resolution UPLC-MS analysis. Column eluate first passed through a photodiode array detector before going to the mass spectrometer to allow simultaneous collection of UV and MS spectra. Electrospray ionisation was used in negative mode with a cone voltage of 15 V, desolvation temperature of 275 °C, desolvation gas at 650 L/h, and the rest of the MS settings were optimised for best resolution and sensitivity. Data were acquired by scanning from 150 m/z to 1 500 m/z in resolution mode, as well as in microwave solvent extraction (MSE) mode. In MSE mode, two channels of MS data were acquired: one at a low collision energy (4 V) and the second using collision energy ramp (40 to 100 V) to obtain fragmentation data as well. Leucine enkephalin was used as lock mass (reference mass) for accurate mass determination and the instrument was calibrated with sodium formate. Separation was achieved on a Waters HSS T3, 2.1 × 100 mm, 1.7 µm column. An injection volume of 2 µL was used and the mobile phase consisted of 0.1% formic acid (Solvent A) and acetonitrile containing 0.1% formic acid as Solvent B. The gradient started at 100% Solvent A for one minute and changed to 28% Solvent B over 22 minutes in a linear way. It then went to 40% Solvent B over 50 seconds and a wash step of 1.5 minutes at 100% Solvent B, followed by re-equilibration to the initial conditions for four minutes. The flow rate was 0.3 mL per minute and the column temperature was maintained at 55 °C. The compounds were quantified in a relative manner against a calibration curve established by injecting a range of catechin standards from 0.5 to 100 mg/L catechin. Data were processed using MSDIAL and MSFINDER (RIKEN Center for Sustainable Resource Science, Metabolome Informatics Research Team, Kanagawa, Japan).

6.4 RESULTS AND DISCUSSION

Chromatographic fingerprinting of medicinal plants is used to identify and assess the stability of the plant's chemical constituents as identified by chromatography (Sharma et al., 2009). The aim of this chapter was to identify and characterise the different compounds found in the four plant extracts. Compounds present in *X. undulatum* were shown by peaks generated by the LC-MS instrument used, with molecular masses on the y-axis and retention time on the x-axis (see Figure 6.1).

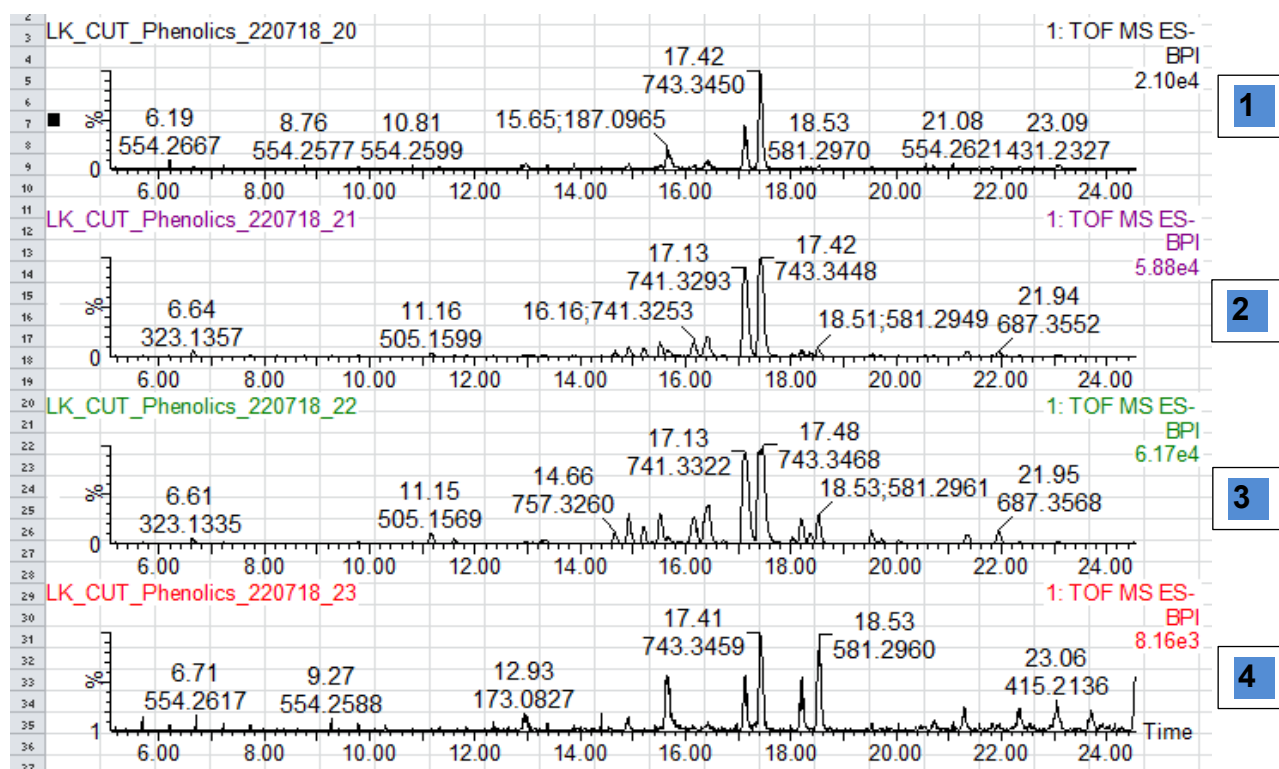


Figure 6.1: LC-MS chromatogram of *X. undulatum* root: 1) dH₂O extract, 2) MeOH extract, 3) DCM:MeOH extract, and 4) DCM extract

Table 6.1: Summary of the most abundant compounds present in *X. undulatum* plant extracts

No.	Average Rt (min.)	Average Mz	Formula	Compound name from PubChem, ChemSpider, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases
1	6,635	323,1363	C ₁₃ H ₂₄ O ₉	Periplobiose
2	11,166	505,1548	C ₂₁ H ₃₀ O ₁₄	Echiside/Allobestonicoside
3	11,6	461,1383	C ₃₀ H ₂₂ O ₅	Blestrin C
4	13,334	759,3467	C ₃₆ H ₅₆ O ₁₇	[(3R,6S)-4,5-diacetyloxy-6-[(3R,6R)-2-ethyl-6-propyl-3-[(2S,5S)-3,4,5-triacetyloxy-6-propyloxan-2-yl]oxyoxan-4-yl]oxy-2-methyloxan-3-yl] acetate

No.	Average Rt (min.)	Average Mz	Formula	Compound name from PubChem, ChemSpider, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases
5	14,645	757,3261	C36H54O17	Anodendrosin G
6	14,93	759,3397	C43H52O12	Methyl 2,4-di-O-benzyl-3-O-[2,3-bis-O-(4-methoxybenzyl)- α -D-galactopyranosyl]-6-deoxy- α -L-mannopyranoside
7	15,199	741,3304	C43H50O11	Allyl 4-O-(2-O,3-O,4-O,6-O-Tetrabenzyl- α -D-Galactopyranosyl)- β -D-Galactopyranoside
8	15,521	743,3484	C43H52O11	Methyl 4-O-(2-O,3-O,4-O,6-O-Tetrabenzyl- β -D-Galactopyranosyl)-2-O,3-O-Dimethyl- α -D-Glucopyranoside
9	16,157	741,3298	C43H50O11	Allyl 3,4-di-O-benzyl-6-O-(3,4-di-O-benzyl- α -D-mannopyranosyl)- α -D-mannopyranoside
10	16,415	743,3445	C43H52O11	Methyl 2,6-di-O-benzyl-4-O-(2,6-di-O-benzyl-3-O-methyl- α -D-mannopyranosyl)-3-O-methyl- α -D-mannopyranoside
11	17,114	695,3267	C35H52O14	Xysmalorin
12	17,418	697,3425	C35H54O14	Uzarin (8Cl)
13	18,037	701,3375	C34H54O15	1-[2,4,5-Trimethoxy-3,6-Bis(Methoxymethoxy)Phenyl]-5-[2,5-Dimethoxy-3,6-Bis(Methoxymethoxy)-4-(2-Methoxymethoxyethyl)Phenyl]Pentane
14	18,199	579,2819	C30H44O11	Antiarigenin 3-O-(6-Deoxy-2-O-Methyl- β -D-Glucopyranoside)
15	18,367	703,3525	C34H56O15	Fumonisin Py2; CHEBI:183918; Q63408599; (2R)-2-[2-[(5R,6R,7S,9S,16R,18S)-6-[(3R)-3,4-Dicarboxybutanoyl]Oxy-16,18-Dihydroxy-5,9-Dimethyl-19-Oxoicosan-7-Yl]Oxy-2-Oxoethyl]Butanedioic Acid
16	18,528	581,2961	C30H46O11	Sorbifolivaltrate C
17	19,539	727,3538	C36H56O15	3'-O- β -D-Glucopyranosyl-2'-O-Isovaleryl-2 β -(2-Desoxy-Atractyligenin)- β -D-Glucopyranoside
18	19,733	903,4005	Unknown	N/A
19	20,052	873,389	C52H58O12	1-O,2-O-Isopropylidene-3-O-[2-(1-O,2-O-Isopropylidene-3-O,4-O-Dibenzyl- β -D-Fructofuranose-6-O-Yl)Phenyl]-4-O,5-O-Dibenzyl- β -D-Fructopyranose
20	21,293	413,1957	Unknown	N/A
21	21,95	687,3568	C34H56O14	Satosporin A
22	22,327	565,3096	Unknown	N/A
23	23,056	415,2142	Unknown	N/A
24	23,716	419,2409	Unknown	N/A

Rt = Retention time; Mz = Mass-to-charge ratio

Table 6.2: Quantities of identified compounds per extract (highest peak intensity)

dH ₂ O (g/mol)	MeOH (g/mol)	DCM:MeOH (g/mol)	DCM extract (g/mol)	Compound name from PubChem, ChemSpider, and KEGG databases
429	2 979	3 016	52	Periplobiose
0	1 880	5 824	91	Echisoside/Allobestonicoside
0	302	2 123	38	Blestrin C
61	611	2 177	27	[(3R,6S)-4,5-diacetyloxy-6- [(3R,6R)-2-ethyl-6-propyl-3- [(2S,5S)-3,4,5-triacetyloxy-6- propyloxan-2-yl]oxyoxan-4- yl]oxy-2-methyloxan-3-yl] acetate
159	1 418	6 877	85	Anodendrosin G
677	4 796	14 409	76	Methyl 2,4-di-O-benzyl-3-O-[2,3- bis-O-(4-methoxybenzyl)-α-D- galactopyranosyl]-6-deoxy-α-L- mannopyranoside
186	4 817	9 426	76	Allyl 4-O-(2-O,3-O,4-O,6-O- Tetrabenzyl-Alpha-D- Galactopyranosyl)-Beta-D- Galactopyranoside
765	7 377	17 678	103	Methyl 4-O-(2-O,3-O,4-O,6-O- Tetrabenzyl-Beta-D- Galactopyranosyl)-2-O,3-O- Dimethyl-Alpha-D- Glucopyranoside
534	7 901	15 860	335	Allyl 3,4-di-O-benzyl-6-O-(3,4-di- O-benzyl-α-D-mannopyranosyl)- α-D-mannopyranoside
1 466	11 356	23 042	481	Methyl 2,6-di-O-benzyl-4-O-(2,6- di-O-benzyl-3-O-methyl-α-D- mannopyranosyl)-3-O-methyl-α- D-mannopyranoside
1 918	20 292	22 087	1 128	Xysmalorin
6 083	18 878	20 121	2 006	Uzarin (8Cl)
131	1 072	3 215	26	1-[2,4,5-Trimethoxy-3,6- Bis(Methoxymethoxy)Phenyl]-5- [2,5-Dimethoxy-3,6- Bis(Methoxymethoxy)-4-(2- Methoxymethoxyethyl)Phenyl] Pentane
345	3 873	14 240	3 751	Antiarigenin 3-O-(6-Deoxy-2-O- Methyl-Beta-D- Glucopyranoside)
258	1 769	5 790	235	Fumonisin Py2; CHEBI:183918; Q63408599; (2R)-2-[2- [(5R,6R,7S,9S,16R,18S)-6- [(3R)-3,4- Dicarboxybutanoyl]Oxy-16,18-

dH ₂ O (g/mol)	MeOH (g/mol)	DCM:MeOH (g/mol)	DCM extract (g/mol)	Compound name from PubChem, ChemSpider, and KEGG databases
				Dihydroxy-5,9-Dimethyl-19-Oxoicosan-7-Yl]Oxy-2-Oxoethyl]Butanedioic Acid
655	4 288	16 408	6 801	Sorbifolivaltrate C
47	1 114	6 333	107	3'-O-Beta-D-Glucopyranosyl-2'-O-Isovaleryl-2beta-(2-Desoxy-Atractyligenin)-Beta-D-Glucopyranoside
29	310	2 167	45	N/A
23	187	1 219	13	1-O,2-O-Isopropylidene-3-O-[2-(1-O,2-O-Isopropylidene-3-O,4-O-Dibenzyl-Beta-D-Fructofuranose-6-O-Yl)Phenyl]-4-O,5-O-Dibenzyl-Beta-D-Fructopyranose
23	40	98	1 718	N/A
191	2 444	7 298	371	Satosporin A
60	70	433	1 353	N/A
20	35	231	2 126	N/A
78	51	161	1 357	N/A

Secondary metabolites are found in different concentrations and combinations in different parts of plants. The type of solvent used, as well as its polarity, can affect the quality, quantity, toxicity, extraction velocity, biosafety, and other biological activities of the extract (Wakeel et al., 2019); the secondary metabolites and compounds identified therefore depend greatly on the solvent and the part of the plant used for extraction.

Compounds present in the *X. undulatum* root extracts are shown by the peaks generated by the LC-MS instrument used, with molecular mass on the y-axis and retention times on the x-axis (see Figure 6.1). A total of 24 compounds were isolated, and out of the 24, five compounds were unknown and two compounds were common in all four extracts. The two common compounds Allyl 4-O-(2-O,3-O,4-O,6-O-Tetrabenzyl-Alpha-D-Galactopyranosyl)-Beta-D-Galactopyranoside and Methyl 4-O-(2-O,3-O,4-O,6-O-Tetrabenzyl-Beta-D-Galactopyranosyl)-2-O,3-O-Dimethyl-Alpha-D-Glucopyranoside had the highest peaks, with molecular mass of 741,33 g/mole and 743,35 g/mole consecutively.

Most of the compounds were found in high quantities in the DCM:MeOH extract. This was expected because the extract contained two solvents. Some of the compounds that have an affinity for DCM will therefore be eluted along with those that have an affinity for MeOH, as well as the compounds that have a polarity that is between DCM and MeOH. The unknown compounds were eluted in high quantities in the DCM extract, with the exception of one with a molecular weight of 903,4005 g/mol. Chapter 4 showed that the DCM extracts were the most toxic towards the MCF7 and Caco2 cells; these compounds therefore need to be isolated and tested individually to determine if they were responsible for the toxicity.

Flavonoids, terpenoids and alkaloids were the three phytochemicals that were found in abundance in chapter 3 (See table 3.1), this may suggest that the two common compounds with molecular mass of 741,33 g/mole and 743,35 g/mole may either be flavonoids, terpenoids or alkaloids. Echinoside and blestin C are the two compounds that were absent from the aqueous extract, which could be because they are not soluble in water. Clark, 2013 reported that phenols are moderately soluble in water, and our results in table 3.1 showed no presence of phenols, this may suggest that Echinoside and blestin C maybe phenolic compounds Kanama et al. (2023) reported uzarin and its isomer allouzarin, xysmalorin and alloxysmalorin compounds as the major compounds found in the roots of *X. undulatum* plant, while two of the compounds, uzarin and xysmalorin, were also identified in this study. Vermaak et al. (2014) also mentioned these two compounds among the 18 they reported.

To the best of the researcher's knowledge, only Thakur et al. (2021) tested the isolated compounds from *X. undulatum* during their study on Alzheimer's disease. The three isolated compounds, 1) acetylated glycosylated crotoxogenin, 2) xysmalogenin-3, β -d-glucopyranoside, and 3) crotoxigenin 3-O-glucopyranoside, were found effective in reducing levels of Alzheimer's disease-associated β -Amyloid peptides A β 42 in vitro.

6.5 CONCLUSION

For the analysis of pharmaceuticals, LC-MS based techniques have demonstrated tremendous promise as they have been essential in the development of new drug candidates, research on drug metabolism, and the identification and characterisation of products and impurities in pharmaceutical products.

A total of 24 compounds were identified from four extracts (DCM, MeOH, DCM:MeOH, and dH₂O) of *X. undulatum* roots. These extracts have pharmacological properties that may be beneficial to human beings (see Section 2.5.1). Their toxicity towards two cancer cell lines was shown in Chapter 4. Only Thakur et al. (2021) have tested individual or isolated compounds from the plant; more biological studies must therefore be performed on previously isolated compounds since the pharmacological effects and benefits of the plant are already proven.

This study identified compounds that other studies did not find. The reason for this may be that the plants are from different locations, which might cause the same species of plant to have different phytochemical constituents, because of exposure to different environmental conditions that could stimulate different chemical responses. The unidentified compounds in the extracts need to be further characterised in order to determine their structure, chemical formulae, and biological activities.

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CHAPTER 7:

GENERAL CONCLUSION AND RECOMMENDATIONS

CONCLUSION Cancer is one of the main public health problems globally. It is a genetic disease but can also be caused by external carcinogens. Cancer cases are increasing due to the lack of a cure. Adverse side effects by current invasive methods of treatment lead to long-term disabilities. Even though the chosen medicinal plants are well known for being used in the treatment and cure of a variety of diseases, as highlighted in Chapter 2, little research has been conducted on their cytotoxicity, anti-cancer effects, and other biological activities. The most reliable source of potential drugs leads is natural products. Plants are one example of natural products that are essential in the fight against different diseases. Phytochemical screening of plant extracts helps to identify novel active compounds and determine their efficacy and safety.

Secondary metabolites such as alkaloids, flavonoids, saponins, steroids, phenols, and terpenoids were present in most of the studied plant powders. The phytochemical screening results suggest that *E. elephantina*, *D. anomala*, *C. paniculata*, *X. undulatum*, and *C. paniculata* are plants that can be good candidates for drug discoveries.

The toxicological characteristics or any possible contaminants of a drug's active ingredients have a direct effect on the safety of the drug product. Different analytical techniques are employed to determine a drug product's quality, quantity, and the materials used in its manufacturing. Chapter 4 investigated the claims made by traditional healers and herbalists that *C. paniculata*, *D. anomala*, *X. undulatum*, and *E. elephantina* are traditionally used to cure cancer. Twenty extracts of different solvents were tested on two cancer cell lines, namely Caco2 and MCF7. The results indicated that the *C. paniculata* leaf, *D. anomala* root, and *X. undulatum* root DCM extracts were the most toxic towards both cell lines, with IC₅₀ values of less than 50 g/mL. DNA cell cycle analysis was performed to determine the effects of the three chosen DCM extracts. Both the MCF7 and Caco2 cells arrested at early mitosis for the *C. paniculata* and *X. undulatum* extracts, and for the *D. anomala* extract, the MCF7 cells arrested at the G0/G1 phase, while the Caco2 cells arrested at the early M phase.

The research further found that the *X. undulatum* root DCM extract had potential anti-cancer activity and was more effective on the Caco2 cells than the MCF7 cells (see Chapter 4). The extracts caused a reduction in mitochondrial membrane potential and an increase in PS translocation in the Caco2 cells, but not in the MCF7 cells. The *C. paniculata* leaf extract and *D. anomala* root extract had no effect on the mitochondrial membrane for either the MCF7 or the Caco2 cells, but increased PS translocation in the Caco2 cells after 24 hours, with an increase in necrotic cells after 48 hours.

RECOMMENDATIONS

It is recommended that these extracts be tested on normal cells to investigate if their cytotoxicity is selective on normal healthy cells. The anti-inflammatory test (see Chapter 5) showed that the *E. elephantina* aqueous extract had an inhibitory effect on the production of NO, while the *C. paniculata* roots and *D. anomala* roots inhibited the production of NO at 200 g/mL. The MTT cytotoxicity assay showed that the extracts had no cytotoxic effects, except for *C. paniculata* at 200 g/mL. This suggests that unresolved inflammation and a pro-tumorigenic microenvironment may result from deregulation in a complex and well-coordinated inflammatory response.

It is a lengthy and costly process to develop a drug. From the first idea to the launch of a commercial product, it is a protracted and complex journey. Throughout this process, an intensive search for a small molecule or biological therapy (target) that resembles a medication is actively undertaken. Once a target is decided on, the pharmaceutical industry uses streamlined early processes, frequently with the assistance of academic centres, to identify molecules that have the necessary properties to produce legal pharmaceuticals. LC-MS-based techniques are essential for the development of new drug candidates, drug metabolism research, and the identification and characterisation of products and impurities in pharmaceutical products. A total of 24 compounds were isolated from the *X. undulatum* roots. It was shown that most compounds were found in higher quantities in the DCM:MeOH extract, which is due to a broader spectrum of polarity. The DCM extract had most of the unknown compounds, and these compounds need to be isolated and tested individually for anti-cancer and other related biological activities.

It is recommended that the extracts be tested on normal cells to determine if some of them have selective toxicity towards cancer cell lines, and individual compounds must also be tested on cancer cell lines and normal cell lines.

Appendices

Appendix A: Additional results

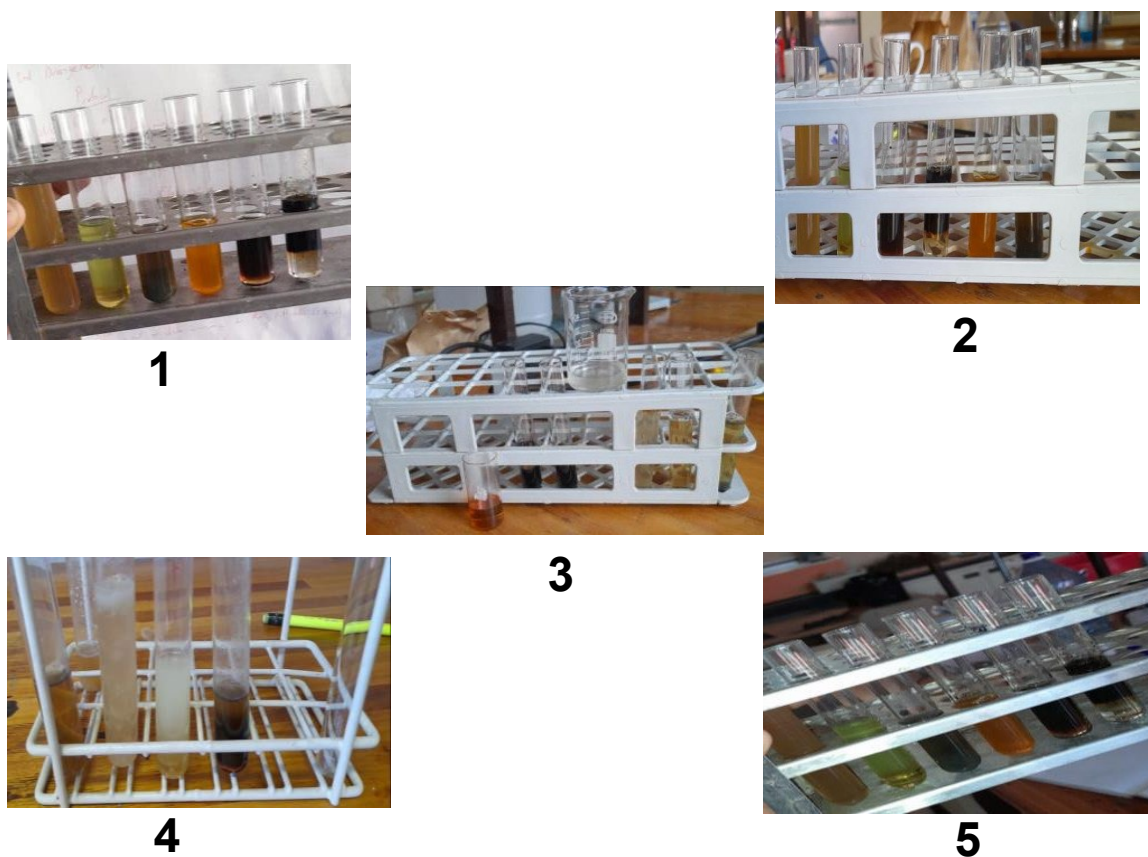


Figure A1: Phytochemical analysis results of the five tested plant powders: 1) *E. elephantina* roots; 2) *D. anomala* roots; 3) *C. paniculata* roots; 4) *X. undulatum*; 5) *C. paniculata* leaves