THE EFFECTS OF *FLAVERIA TRINERVIA* AND *DICEROCARYUM SENECIOIDES* EXTRACTS ON METASTATIC MDA-MB-231 BREAST CANCER CELLS.

by

Tubake Theona Sebei

RESEARCH DISSERTATION

Submitted in fulfilment of the requirements for the degree of

Master of Science

in

Biochemistry

in the

Faculty of Science and Agriculture

(School of Molecular and Life Sciences)

at the

University of Limpopo.

SUPERVISOR: Dr PKP Chokoe

CO-SUPERVISOR: Prof. VG Mbazima

January 2025

DECLARATION

I, Tubake Theona Sebei, do hereby declare that this research dissertation entitled "The Effects of *Flaveria trinervia* and *Dicerocaryum senecioides* Extracts on Metastatic MDA-MB-231 Breast Cancer Cells", submitted to the University of Limpopo for the degree of Master of Science in Biochemistry has not been previously submitted by me in part or full for the degree or any other degree to any University, and is my own investigation and research.

10/01/2025

Sebei TT (Miss) Date

LIST OF CONFERENCE PRESENTATIONS

Sebei T.T, Mbazima V.G, Chokoe P.K.P. The effects of *Thymus vulgaris* methanolic extract on MDA-MB-231 breast cancer cells. 12th Annual Biomedical Research & Innovation Platform Symposium (BRIP). SAMRC Conference Centre Auditorium & Virtual | MS Teams. 18-19 October 2022.

Sebei T.T, Mbazima V.G, Chokoe P.K.P. Anti-migrative and anti-adhesive effects of *Thymus vulgaris* methanolic extract on MDA-MB-231 breast cancer cells. Pan African Cancer Research Institute's Inaugural International Cancer Meeting. Exploiting Global Networks for the Fight Against Cancer in Africa: Advancing Prevention and Transforming Care. 26 February-1 March 2023.

Sebei T.T, Mbazima V.G, Chokoe P.K.P. investigating the effects of the methanol extracts of *Dicerocaryum senecioides* and *Flaveria trinervia* on migratory and adhesive properties of MDA-MB-231 breast cancer cells. UL-BRIP-UniZulu Research Meeting. 27-30 JUNE 2023.

Sebei T.T, Mbazima V.G, Chokoe P.K.P. Investigating the effects of chloroform and methanol extract of *Dicerocaryum senecioides* and *Flaveria trinervia* on metastatic breast cancer cells. 13th Postgraduate Research Day. Bolivia Lodge, Polokwane. 20-22 September 2023.

Sebei T.T, Mbazima V.G, Chokoe P.K.P. Investigating the effects of chloroform and methanol extract of *Dicerocaryum senecioides* and *Flaveria trinervia* on metastatic breast cancer cells.13th Annual Biomedical Research & Innovation Platform Symposium (BRIP). SAMRC Conference Centre Auditorium & Virtual | MS Teams. 16-17 October 2023.

Sebei T.T, Mbazima V.G, Chokoe P.K.P. Investigating the effects of *Dicerocaryum senecioides* and *Flaveria trinervia* chloroform and methanol extracts on MDA-MB-231 breast cancer cells. UniZulu-BRIP-UL Research Meeting. 26-29 JUNE 2024.

DEDICATION

In loving memory of my dear aunt, Kukie Elizabeth Serite, who courageously fought against colourectal cancer. As I begin on this research journey, it is a heartfelt tribute to her enduring spirit and the impact she had on my life. Each step I take in this research is a testament to the strength she exhibited during her battle and a reflection of the inspiration she continues to instil in me. I remember our shared moments with deep fondness, treasuring the laughter, wisdom, and warmth she brought into my life. The sacrifices she made and the challenges she faced inspired a determination in me to contribute to the understanding and treatment of cancer. This research is not merely an academic pursuit; it is a personal commitment driven by the memory of my aunt. I strive to honor her legacy by investigating into the complexity of cancer, seeking knowledge that may one day contribute to advancements in its diagnosis, treatment, and, ultimately, prevention. Aunt Kukie, though you are no longer physically present, your spirit resonates within me, moving me forward. I hope that, wherever you are, you can witness the progress I've made and feel a sense of pride. Your memory is a guiding light, and I undertake this journey with the hope that it may lead to positive outcomes for others affected by cancer. Thank you for being a source of strength, love, and inspiration. May your soul continue to rest in peace and may this research stand as a meaningful tribute to the remarkable person you were.

ACKNOWLEDGEMENTS

I humbly express my gratitude to the Almighty God for His grace and countless blessings throughout my life.

A heartfelt thank you to my amazing supervisor Dr Chokoe PKP, for her constant support, patience, and constructive suggestions that were crucial for the success of this thesis.

My sincere appreciation to Prof. Mbazima VG, my co-supervisor, for his support as well as invaluable guidance during this research study. It was both an honour and privilege.

I would like to thank the SAMRC-RCDI and National Research Foundation (NRF) for their financial support.

I extend my gratitude to my friends Maenetja KP, Mahlane TT, and Shiloane A, for their constant support and encouragement, always believing that GOD shall see us through. I am also deeply grateful for being a supportive presence during moments of despair, proving a shoulder to lean on.

I am thankful for invaluable contributions of Mohale T, Kgakishe D, Ramasenya G and Makuoa K

To my colleagues and the research team in the Department of Science and Agriculture, your contribution created a positive and supportive working environment. The shared were crucial in overcoming challenges.

To my siblings, beloved grandmother Rakgoale MA, and above all, my mother, Sebei HD, words remain insufficient to convey my appreciation for your boundless love and support. I am beyond blessed, and I am grateful. Thank you, my dearest mother.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
β-catenin	Beta-catenin
CAMs	Cell adhesion molecules
CCK-8	Cell Counting Kit-8
CTCs	Circulating tumour cells
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
E-cadherin	Epithelial-cadherin
ECs	Endothelial cells
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FBS	Foetal bovine serum
FGFs	Fibroblast growth factors
FOXC2	Forkhead box protein C2
GLOBOCAN	Global Cancer Incidence, Mortality and prevalence
MET	Mesenchymal-epithelial transition
miRNA	MicroRNA
MMPs	Matrix metalloproteinases
N-cadherin	Neutral-cadherin
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-κB
PBS	Phosphate buffered saline

ROS	Reactive oxygen species
Smad	Suppressor of mothers against decapentaplegic
TEM	Transendothelial migration
TFs	Transcription factors
TGF-β	Transforming growth factor-β
TNBC	Triple negative breast cancer
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
Zeb	Zinc finger E-box binding homeobox

TABLE OF CONTENTS

ABSTRACT

Medicinal plants have been used for centuries in various traditional healing practices worldwide. Research has revealed some of these plants contain bioactive compounds with potential anticancer and antimetastatic properties. *Dicerocaryum senecioides* and *Flaveria trinervia* have both been reported to have anti-inflammatory and antioxidative effects, which can contribute to inhibiting metastatic processes such as relocation of metastatic cells from a primary organ and subsequent adhesion to a secondary organ. Therefore, the study aimed to investigate potential anti-metastatic effects of *Dicerocaryum senecioides* and *Flaveria trinervia* chloroform and methanol extracts on MDA-MB-231 breast cancer cells. The effect of the extracts on the viability of MDA-MB-231 and HEK–293 human embryonic kidney cells was assessed using the cell counting kit-8 kit. To determine the mode of cell death induced by the extracts, annexin-V and dead cell assay was employed. The effect of the extracts on reactive oxygen species formation and epithelial to mesenchymal transition-related morphological changes were evaluated with the Muse® Oxidative Stress Kit and light microscope, respectively. The transwell and cell adhesion assay were used to investigate the anti-migratory effects of the extracts on MDA-MB-231 cells as well as the ability of the cells to attach to a cell culture plate, respectively. The effects of the extracts on the enzymatic activity of the matrix metalloproteinases-2 and -9 was assessed using gelatin-zymography. Additionally, western blotting was used to evaluate the effect of the extracts on the expression of vimentin. The findings revealed there was no significant effect on MDA-MB-231 and HEK-293 cell viability at extract concentrations below 200 µg/ml, and that cell death occurred through apoptosis at those concentrations. Reactive oxygen species formation and epithelial to mesenchymal transition were suppressed in the MDA-MB-231 cells treated with extracts. Moreover, the extracts suppressed cell invasion by inhibiting the activity of MMP-9 & -2, expression of vimentin as well as subsequent migration, and adhesion. The chloroform extracts of both plant showed better anti-migratory, anti-invasive and anti-adhesive potential than the methanol extract. Thus, this study highlighted *Dicerocaryum senecioides* and *Flaveria trinervia* as potential sources of compounds of intermediate polarity with anti-metastatic activity.

CHAPTER ONE

1.1 Introduction

Breast cancer is one of the most common cancers and the leading cause of death in women worldwide (Bray *et al*., 2004; Carol *et al*., 2015; Zaidi and Dib, 2019). It was also reported to be the leading diagnosed cancer with 2.26 million cases, accounting for nearly 685 000 deaths in 2020 (WHO, 2021). Studies by Guan (2015), Dillekas *et al* (2019), and Wang *et al* (2021) reported that 90% of cancer-related deaths are caused by metastasis, which is the dissemination of tumour cells from a primary tumour to other parts of the body. Current breast cancer treatment methods are limited to stages 1, 2, and 3 of the disease and have severe side effects on the human body (Agrawal, 2014). In stage 4 or metastasised breast cancer, the tumour cells are resistant to the present treatment methods resulting in the high mortality rate associated with cancer metastasis (Massague and Obenauf, 2016; Madell, 2021).

Metastasis is a multi-step process, in which tumour cells detach from the primary tumour and relocate to other parts of the body (Hejmadi, 2013; Riggio *et al*., 2021). The first stage of metastasis involves the detachment of cancer cells from their primary tumour site (Guan, 2015). This stage involves loss of cell-cell adhesion and interactions with the extracellular matrix (ECM) facilitated by the induction of epithelial-to-mesenchymal transition (EMT) (Melzer *et al*., 2017; Janiszewska *et al*., 2020). Epithelial-to-mesenchymal transition is a complex, multi-functional and tightly regulated developmental process in which epithelial cells develop mesenchymal, fibroblast-like properties, reduced intercellular adhesion, and increased motility (McConkey *et al*., 2009). The EMT induction leads to the activation of EMT-associated transcription factors that promote downregulation of E-cadherin causing tumour cells to lose cell-cell adhesion and reduce cell-cell junctions (McConkey *et al*., 2009). Furthermore, mesenchymal makers such as N-cadherin, vimentin, and fibronectin become activated and lead to a greater mesenchymal-like phenotype, with improved migration and increased cell-to-stem cell interaction (Melzer *et al*., 2017).

Some plants used as traditional medicines have shown to have anticancer properties and can be used as starting points for the development of new cancer drugs that can inhibit one or more processes involved in metastasis (Shoeb, 2006; Jain *et al.,* 2016;

Hassan, 2019). However, more research on these plants is needed. Plants have been proven to be potential sources of biological active phytochemicals (Malathi *et al*., 2012). *Flaveria trinervia* is a bifurcated branched herb with oblong, auricle, opposite leaves, and clustered yellow tops (Umadevi *et al*., 2006; Rolnik and Olas, 2021). *F. trinervia* belongs to the *Asteraceae* family, which have a long history in traditional medicine due to their wide range of hepatoprotective, antioxidant, antimicrobial, and anti-inflammatory activities (Achika *et al*., 2014). Furthermore, a study by Umadevi *et al*. (2006) showed that *F. trinervia* leaf extract can be a potential source of phytochemicals with antimetastatic activity due to its wound healing activities, which may be attributed to the antioxidant activity of the flavonoid present in the leaf extract. *Dicerocaryum senecioides* is a prostrate perennial plant widely used both as a nutritional source and a traditional remedy in Southern Africa (Mampuru *et al*., 2012; Rambwawasvika *et al*., 2017). Numerous studies have shown that *D. senecioides* extracts have anti-proliferate, anti-inflammantory, and antioxidant activity (Mphahlele, 2008; Madiga *et al*., 2009; Mampuru *et al*., 2012; Rambwawasvika *et al*., 2019). These properties make *D. senecioides* a potential source of phytochemicals with antimetastatic activity.

1.2 Literature review

1.2.1 Metastasis

Metastasis is a multi-step process alluding to the spread of primary tumour cells to surrounding tissues and organs leading to the formation of secondary or metastatic tumours at the new site (Szczurek *et al*., 2020). Numerous studies suggest that metastatic tumours are responsible for 90% of deaths in cancer patients (Seyfried and Huysentruyt, 2013; Song *et al*., 2017; Szczurek *et al*., 2020). Metastasis, illustrated in **Figure 1.1**, features several steps consisting of: (1) cell detachment from the primary tumour and induction of epithelial-mesenchymal transition (EMT); (2) invasion of the basement membrane and cell migration; (3) intravasation into the surrounding vasculature system; (4) survival and transportation through circulation; (5) extravasation from the vasculature to secondary tissue; and finally, (6) metastatic colonisation and mesenchymal-epithelial transition (MET) of the cells (Hapach *et al*., 2019; Hernandez-Caballero, 2013; Melzer *et al*., 2017). Despite decades of research on metastatic progression, the precise mechanisms of each step remain unclear (Martin *et al*., 2013; Melzer *et al*., 2017; Riggio *et al.,* 2021).

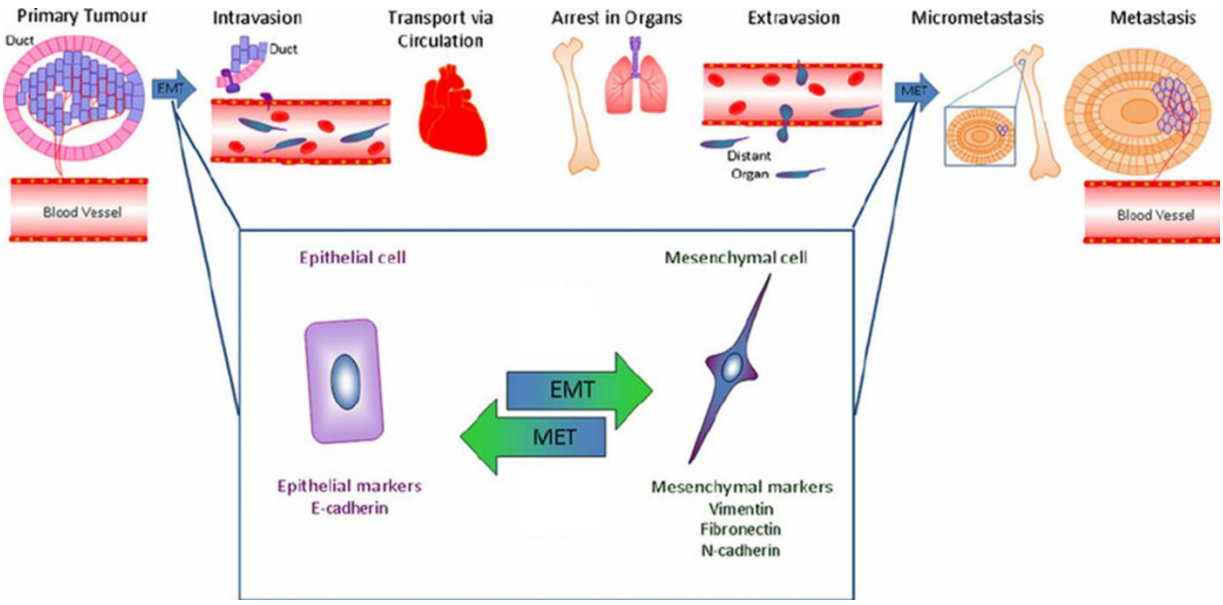

Figure 1.1: The metastasis cascade (Drabsch and Dijke, 2011).

1.2.1.1 Cell detachment from the primary tumour and possible induction of epithelial-mesenchymal transition (EMT)

A tumour is defined as a mass of cancer cells connected to one another by cell-to-cell adhesion molecules such as cadherins, integrins, selectins and Ig superfamily, that allow interactions between several proteins on the cell surface (Janiszewska *et al,* 2020). Moreover, the cells also bind to a mesh of protein known as an extracellular matrix (ECM) (Hejmadi, 2013; Poltavets *et al*, 2018). Therefore, for cancer cells to metastasise, they must first break away from the ECM and their neighbouring cells (Deng *et al*., 2021).

Epithelial cadherin (E-cadherin) is a protein involved in cell-cell adhesion and its suppression during epithelial-mesenchymal transition (EMT) is crucial for tumour cell detachment (Babaei *et al*., 2021; Na *et al*., 2020; Wendt *et al*., 2011; Yu *et al*., 2015). Normally, epithelial cells undergo anoikis (induction of apoptosis or cell death) by detaching from the extracellular matrix and their neighbouring cells (Babaei *et al*., 2021). However, detached tumour cells exhibit certain resistance to anoikis, which is achieved by suppression of E-cadherin and overexpression of Neural cadherin (N-cadherin) through the EMT process (Babaei *et al*., 2021; Melzer *et al*., 2017). EMT allows the tumour cells to transition from an epithelial phenotype to a mesenchymal stem-like phenotype (Ribatti *et al*., 2020).

Epithelial-mesenchymal transition (EMT) is a reversible cellular biological process whereby epithelial cells lose cell-cell junctions, apical-basal and epithelial markers to acquire a spindle-cell shape, cell motility and mesenchymal markers (Lai *et al*., 2020). EMT occurs during embryonic development, wound healing, tissue fibrosis, and cancer progression or metastasis in response to signals received from their microenvironment (Babaei *et al*., 2021). The signals activate key regulators such as nuclear factor-κB (NF-κB) and growth factors as well as other EMT modulators such as ECM components and developmental signalling pathways (Guan *et al*., 2020; Vella *et al*., 2020; Dobnath *et al*., 2022).

These signalling pathways will jointly aim to change gene expression through modulating several transcription factors (TFs) or master regulators of EMT such as TWIST family proteins, Zinc finger E-box binding (Zeb) homeobox family proteins, and Snail Family proteins, as shown in **Table 1.1** (Foroni *et al*., 2012; Taddei *et al*., 2014). Ultimately, these TFs will promote the suppression of epithelial markers such as E-cadherin (Micalizzi *et al*., 2010). Suppression of epithelial markers will lead to the upregulation expression of mesenchymal markers such as N-cadherin, matrix metalloproteinases (MMPs) and fibronectin (Deshmukh *et al*., 2021). A decrease in the expression of E-cadherin will then lead to the loss of cell-cell adhesion within the primary tumour, thus triggering the transition from an epithelial phenotype to mesenchymal cancer cells (Lusby *et al*., 2022). Tumour cells with a mesenchymal phenotype are characterised by increased cell motility, invasiveness, migration, resistance to anoikis/apoptosis and expression of ECM components (Dave *et al*., 2012).

Table 1.1: Epithelial-mesenchymal transition-related markers.

Upregulated	Down-regulated	Activated	Cell functional acquisition
N-cadherin	E-cadherin	β-catenin	Increased migration
Vimentin	Desmoplakin	Smad-2/3	Increased invasion
Fibronectin	Cytokeratin	NF-κB	Increased scattering
Snail1 (Snail)	Occludin	Snail1 (Snail)	Stem-cell phenotype
Snail2 (Slug)	Claudin	Snail2 (Slug)	Drug resistance
Twist	miRNA200 family	Twist	
Goosecoid FOXC2 Zeb1, Zeb2 MMP-2, MMP-3, MMP-9			

β-catenin: beta catenin, E-cadherin: epithelial cadherin, FOXC2: forkhead box protein C2, miRNA: micro RNA, MMP: matrix metalloproteinase, N-cadherin: neural cadherin, NF-κB: nuclear factor – kappa B, Smad: suppressor of mothers against decapentaglegic, Zeb: zinc finger E-box binding homeobox.

1.2.1.2 Invasion of the basement membrane and cell migration

Invasion of the basement membrane (local invasion) involves the infringement of tumour cells from the primary tumour mass into nearby tissues (Shenoy and Lu, 2016). This cell invasion and migration is enabled by the formation of actin-rich membrane protrusions acquired by the tumour cells during EMT termed invadopodia (Santiago-Medina *et al*., 2016). Invadopodia secrete proteases that facilitate breaching of the basement membrane, as well as the remodelling, and degradation of the ECM (Masi *et al*., 2020; Shenoy and Lu, 2016). **Figure 1.2** depicts two main modes of invasion employed by the detached tumour cells, namely, single cell (a and b) and collective (c) invasion (Lintz *et al*., 2017; Melzer *et al*., 2017).

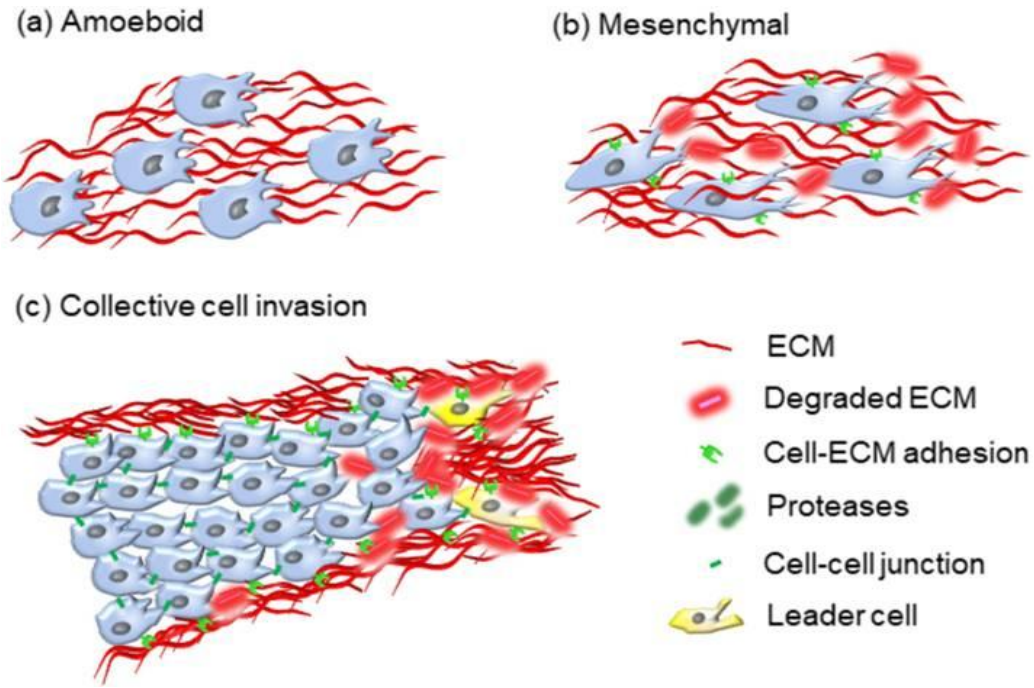

Figure 1.2: Modes of cancer cell migration (Wu *et al*, 2021).

1.2.1.2.1 Single cell invasion

Single cell invasion is the movement of individual cells and can either utilise protease dependent mesenchymal or protease-independent amoeboid cell migration (Melzer *et al*., 2017).

1.2.1.2.1.1 Mesenchymal cell migration

Mesenchymal migration is facilitated by a migration cycle characterised by pseudopod protrusion at the leading edge of the tumour cell, formation of local contacts, focalised proteolysis, actomyosin contraction as the cell interacts with the ECM and lastly, detachment of the trailing edge of the tumour cell as shown in **Figure 1.3** (Melzer *et al*., 2017). It arises in cells undergoing EMT from connective tumour tissue or epithelial cancers (Friedl and Alexander, 2011). Mesenchymal cell migration depends on soluble surface proteases such as cathepsins, serine proteases, and MMPs that remodel and cleave ECM components such as laminin, fibronectin and collagen to allow movement through the extracellular matrix (Friedl and Alexander, 2011; Talkenberger *et al*., 2017). MMPs, particularly MMP-2 and MMP-9, play a key role in tumour cell invasion by degrading collagen type IV at the basolateral site of the epithelium and endothelium (Melzer *et al*., 2017).

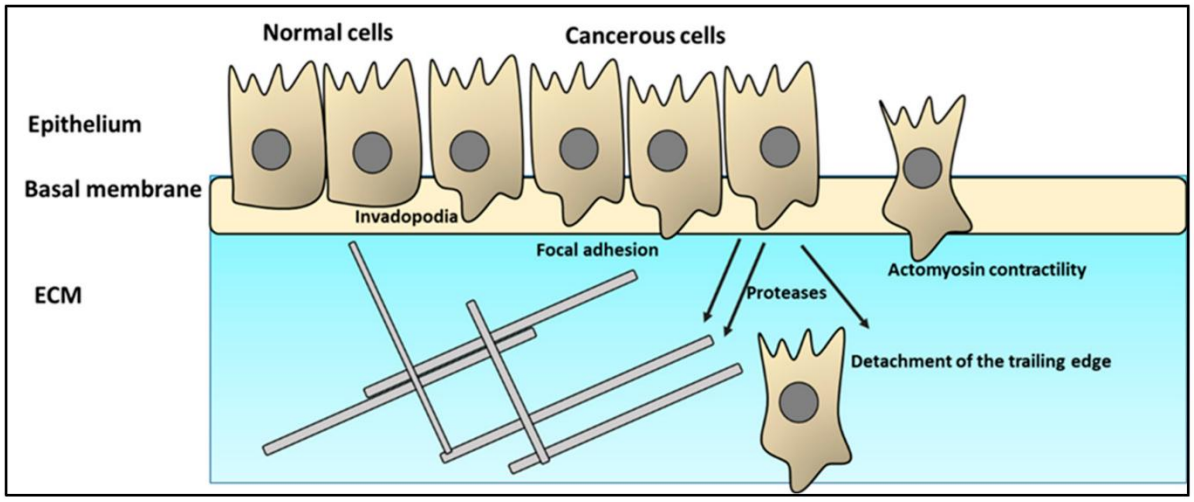

Figure 1.3: Mesenchymal cell migration cycle (Lazarczyk *et al*., 2023).

1.2.1.2.1.2 Amoeboid cell migration

Amoeboid cell migration has a similar functioning and movement to the cellular slime mold *Dictyostelium discoideum* (a cellular slime mold) (Wu *et al*., 2021). Unlike mesenchymal cell migration, this type of movement does not depend on proteases to degrade the ECM (Talkenberger *et al*., 2017) and the migrating cells show reduced capability to remodel the ECM, lower adhesiveness due to missing focal contacts as well as total loss of cell polarity (Melzer *et al*., 2017). Amoeboid cells develop bleb-like protrusions of the cell membrane (**Figure 1.2**), that allow the cells to sense the microenvironment and they also exhibit rapid deformability brought about by reorganization of the cortical actin cytoskeleton allowing them to penetrate the ECM swiftly (Melzer *et al*., 2017; Wu *et al*., 2021).

1.2.1.2.2 Collective cell invasion

Collective tumour cells form sheets, strands, tubes and clusters of tumour cells led by tip or leader cells, as shown in Fig. 1.3 (Lintz *et al*., 2017). The tip or leader cells generally display mesenchymal morphology while the remaining cells retain epithelial cell-to-cell junctions (Lintz *et al*., 2017). The leader cells extend actomyosin-mediated invadopodia that generate integrin-mediated forward traction (Pandya *et al*., 2017). Additionally, these cells release prototypic pericellular proteases towards the tissue structure, which produces remodelled ECM that guides the group (Pandya *et al*., 2017). A key advantage of collective tumour cell invasion not offered to single cells is the protection of the inner cells against immune cells and other defence mechanisms within the tumour microenvironment (Melzer *et al*., 2017).

Tumour cells may utilise amoeboid, mesenchymal and collective migratory modes interchangeably (Taddei *et al*., 2014). By alternating between different migratory modes, the migrating tumour cells can adapt to microenvironment changes and matrix stiffness to resist cancer treatments (Taddei *et al*., 2014). Therefore, the plasticity of cancer invasion modes can be said to be mandatory for tumour cell dissemination and metastasis potential (Taddei *et al*., 2014; Wu *et al*., 2021).

1.2.1.3 Intravasation into the surrounding vasculature system

The endothelial wall or endothelial cells (ECs) normally serve as a barrier to the movement of tumour cells into or out of the bloodstream (Shenoy and Lu, 2016). For tumour cells to cross the endothelial barrier they must enhance the production of new blood vessels from the existing vasculature via tumour angiogenesis to provide oxygen and nutrients as well as to promote dissemination of the migrating tumour cells into the circulatory system (Hapach *et al*., 2019).

1.2.1.4 Tumour angiogenesis

Tumour angiogenesis begins when stroma cells in the tumour microenvironment facilitate the activation of growth factors, inflammatory cytokines, and hypoxic conditions (Ayoub *et al*., 2022). This is followed by the production and release of angiogenic factors in ECs in the wall of an existing small blood vessel (Al-Ostool *et al*., 2021). These factors then activate ECs by binding to their surface receptors leading to proliferation of the ECs, secretion of MMPs that degrade the ECM and directed migration (Al-Ostool *et al*., 2021). Lastly, the new ECs form vesicular tubes and recruit multi-functional cells embedded within the walls of capillaries termed pericytes that aid in the maturation of the new blood vessels (Al-Ostool *et al*., 2021; Hall and Ran, 2010). In contrast to existing blood vessels, the newly formed blood vessels are dynamically unstable and are known for their hyperpermeability, immaturity, poor pericyte coverage and irregularity (Ayoub *et al*., 2022). These features allow tumour cells in the vicinity of the neovasculature to enter the blood vessels under increased pressure of the tumour growth (Guan *et al*., 2020).

1.2.1.5 Survival and transportation through circulation

Following successful entry into the bloodstream through the new blood vessels, circulating tumour cells (CTCs) must survive major hindrances such as frequent collision with blood and immune cells, the mechanical force of blood flow, fluid shear

stress as well as chemotherapy or radiotherapy to reach the secondary site (Melzer *et al*., 2017). Time spent by the CTCs in the circulatory system decreases their proliferative capacity, eventually leading to dormancy if the journey to the new site is prolonged (Guan *et al*., 2020).

There are two classification categories of CTCs, namely, dominant single cells and a minor group of clustered cells (Castrol-Giner and Aceto, 2020). Clustered cells have an adaptive mechanism that enhances survival against challenges encountered in the bloodstream, thus increasing their metastatic potential (Castrol-Giner and Aceto, 2020; Schuster *et al*., 2021). Additionally, CTCs can interact with platelets and acquire a coating shield that prevents their detection by immune cells. This platelet shield around the CTCs further provides the ability to bear the physical stresses of circulation (Fares *et al*., 2020).

1.2.1.6 Extravasation from the vasculature to secondary tissue

Circulating tumour cells that survive circulation will undergo tumour cell extravasation (Riggio *et al*., 2021). The extravasation begins with a decrease in blood flow velocity and a corresponding decrease in CTC circulation in smaller capillaries to facilitate attachment to the endothelium (Melzer *et al*., 2017). Arrested CTCs then modulate the endothelial barrier enabling transendothelial migration (TEM) of the cells to reach the underlying tissues (Strilic and Offermanns, 2017).

1.2.1.7 Metastatic colonisation and mesenchymal-epithelial transition (MET)

The crucial stage in the metastatic cascade involves extravasated tumour cells colonising the secondary site (Hernandez-Caballero, 2013). Steven Paget (1889) proposed the ‘seed and soil’ hypothesis, which suggests that: (1) metastasis can only form in specific organs with biologically compatible microenvironments and (2) metastasis results from the interaction between extravasated tumour cells (the seeds) and the surrounding tissue (the soil). Due to the loss of E-cadherin expression during EMT, CTCs possess limited proliferative capacity and ability to establish cell-cell interactions (Melzer *et al*., 2017). As a result, the majority of extravasated CTCs will die or become dormant, while a small set will fully metastasise (Riggio *et al*, 2021).

Metastatic growth of the tumour cells at the secondary site is facilitated by mesenchymal-to-epithelial transition (MET) - a reverse process of EMT (Jie *et al*., 2017). For extravasated CTCs to initiate MET, activation of epithelial cell-associated

genes and epithelial makers such as E-cadherin must occur (Melzer *et al*., 2017). This re-expression of E-cadherin that was downregulated during EMT favours colonisation of extravasated CTCs at the secondary site and resultant growth of the tumour (Chao *et al*., 2010).

1.2.2 Reactive oxygen species (ROS)

Elevated rates of reactive oxygen species (ROS) have been identified in most cancers, where they have been highlighted to promote many aspects of tumour development and progression (Liou and Storz, 2010; Perillo *et al*., 2020). ROS is a broad term used to define highly reactive oxygen-containing chemical molecules including the superoxide anion, hydrogen peroxide, and hydroxyl radical (Qian *et al*., 2019; Huang *et al*., 2021). They are mostly generated as by-products of the electron transport chain during aerobic metabolism in the mitochondria, peroxisomes and several metabolic enzyme reactions in the cytoplasm (Liang *et al*., 2021). ROS play an important role in the regulation of cellular defense mechanisms that are mandatory for gene expression and G-protein-coupled-receptor activation, which are vital for cell proliferation and survival (Mydin and Okekpa, 2018).

Under normal health circumstances, a balance between reducing and oxidising reactions within cells is ensured via a process called redox homeostasis (Mydin and Okekpa, 2018). This is achieved by equalising the production of these free radicals (oxidants) and the rate of antioxidant consumption by the body cells (Sharifi-Rad *et al*., 2020). Antioxidants are substances that aim to prevent overproduction of free radicals and neutralise or repair the damage they caused (Sharifi-Rad *et al*., 2020).

Oxidative DNA (deoxyribonucleic acid) damage (commonly the formation of hydroxylated DNA bases) has been accepted as one of the driving forces of tumourigenesis by causing genetic mutations and altering gene expression (Klaunig *et al*., 2010; Sharifi-Rad *et al*., 2020). As a consequence, the production of ROS is elevated in tumour cells (Perillo *et al*., 2020). Furthermore, increased levels of ROS can activate NF-κB to promote the expression of Snail. This transcription factor then downregulates E-cadherin and upregulates N-cadherin and vimentin, thus leading to the reduction of cell-to-cell junctions and triggering the EMT process (Huang *et al*., 2021). Increased levels of ROS also favours tumour cell migration via the promotion of cytoskeleton remodeling by modification of proteins and enzymes that regulate

actin dynamics, or by direct oxidisation of the cytoskeleton structural filaments (Aggarwal *et al*., 2019; Huang *et al*., 2021; Valdivia *et al*., 2015). Furthermore, cancer proliferation can initiate ROS-dependent angiogenesis. The elevated metabolic rate of the growing cell mass causes an upsurge in ROS levels leading to oxidative stress in the tumour microenvironment that activates the secretion of angiogenic modulators (Aggarwal *et al*., 2019). As previously stated, antioxidants prevent the accumulation of excess free radicals and repair the damage caused by the radicals (Hassan *et al*., 2017). Natural antioxidants such as flavonoids found in medicinal plants have been shown to be the best exogenous antioxidants (Hassan *et al*., 2017).

1.2.3 Medicinal plants

A medicinal plant refers to any plant that contains substances that can be utilised for therapeutic purposes (Sofowora *et al*., 2013). Medicinal plants have been used for centuries by numerous cultures around the world for their primary healthcare needs (Street and Prinsloo, 2012). Africa has been naturally blessed with extensive biodiversity and approximately 10% of the plant species are used medicinally (Mahomoodally, 2013). This is because Africa is located within the tropical and subtropical climates causing its plants to be subjected to higher radiation and a wide range of pathogenic microbes (Mahomoodally, 2013). Therefore, to survive the harsh environment, these plants accumulate crucial secondary metabolites through evolution (Yang *et al*., 2018). Unlike, primary plant metabolites, secondary plant metabolites are not involved in the growth and development of the plant (Hussein and El-Anssary, 2018). Some classes of compounds such flavonoids, polyphenols, terpenoids and alkaloids have been shown to possess various anticancer activities (Pott *et al*., 2019; Ramakrishna *et al*., 2021). Therefore, due to the severe side effects and ineffectiveness of the current cancer treatment methods (chemotherapy, surgery and radiation therapy), medicinal plants have attracted attention as potential sources for the development of cancer drugs because of their safety, minimal cost and ease of access (Hosseinzadeh *et al*, 2015; Omara *et al*., 2020; Steenkamp and Gouwa, 2006).

1.2.3.1 Dicerocaryum senecioides

Dicerocaryum senecioides is a prostrate perennial plant widely used both as a nutritional source and a traditional herbal remedy in Southern Africa (**Figure 1.5**) (Madiga *et al*, 2009). Numerous studies have shown that *D. senecioides* extracts have antiproliferative, anti-inflammatory and antioxidative activities (Mphahlele, 2008; Madiga *et al*, 2009; Mampuru *et al*, 2012; Rambwawasvika *et al*, 2019). In a study by Mampuru *et al* (2012), antioxidant and free radical scavenging activities of the leaf extracts of *D. senecioides* were evaluated both qualitatively and quantitatively using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) chemical antioxidant assay and the leaf extract exhibited impressive properties of an ideal antioxidant with antiproliferative activity. These properties make *D. senecioides* a potential source for the development of cancer drugs to prevent cancer progression.

Figure 1.5: The *Dicerocaryum senecioides* plant (Rambwawasvika *et al*., 2017)

1.2.3.2 Flaveria trinervia

Flaveria trinervia is a dichotomously branched herb from the Asteraceae family with opposite, oblong, and auricled base leaves (**Figure 1.6**) (Umadevi *et al*, 2006; Rolnik and Olas, 2021). Members of the *Asteraceae* family have a long history in traditional

medicine and have shown hepatoprotective, antioxidative, antimicrobial, and anti-inflammatory activities (Achika *et al*, 2014). An *F. trinervia* leaf methanol extract was proven to contain flavonoids with potent antioxidant properties (Umadevi *et al*, 2006). However, there is a lack of information on the anticancer properties of *F. trinervia* and this study aims to evaluate the possible effects of this plant on metastatic breast cancer.

Figure 1.6: The *Flaveria trinervia* plant (retrieved from powo.science.kew.org)

1.3 Purpose of the study

1.3.1. Aim:

The study aimed to investigate the potential anti-metastatic effects of *Dicerocaryum senecioides* and *Flaveria trinervia* chloroform and methanol extracts on MDA-MB-231 breast cancer cells.

1.3.2. Objectives:

The objectives of the study were to:

i. determine the classes of phytochemicals in the *D. senecioides* and *F. trinervia* chloroform and methanol extracts using standard qualitative chemical tests;

ii. assess the effects of the *D. senecioides* and *F. trinervia* chloroform and methanol extracts on cell viability of MDA-MB-231 breast cancer and HEK-293 human embryonic kidney cells;
iii. evaluate the mode of death of MDA-MB-231 breast cancer cells induced by the *D. senecioides* and *F. trinervia* chloroform and methanol extracts;
iv. assess the effects of the *D. senecioides* and *F. trinervia* chloroform and methanol extracts on morphological changes in MDA-MB-231 cells;
v. analyse the effects of the *D. senecioides* and *F. trinervia* chloroform and methanol extracts on the expression of vimentin;
vi. assess the enzymatic activity of matrix metalloproteinases of MDA-MB-231 breast cancer cells treated with the *D. senecioides* and *F. trinervia* chloroform and methanol extracts;
vii. evaluate the effects of the *D. senecioides* and *F. trinervia* chloroform and methanol extracts on migration of MDA-MB-231 breast cancer cells;
viii. analyse the effects of the *D. senecioides* and *F. trinervia* chloroform and methanol extracts on the attachment of MDA-MB-231 breast cancer cells; and
ix. determine the modification of reactive oxygen species production in MDA-MB-231 breast cancer cells caused by the *D. senecioides* and *F. trinervia* chloroform and methanol extracts.

CHAPTER TWO

2. METHODOLOGY

2.1 Collection and preparation of plant extracts

Dicerocaryum senecioides and *Flaveria trinervia* plants were collected at Mankweng, in the Limpopo province, South Africa. A voucher specimen was deposited at the Larry Leach Herbarium (UNIN) for authentication. The plants were washed and dried at room temperature, ground into fine powder using a Waring commercial blender and exhaustively extracted in either methanol or chloroform (Rochelle Chemicals, RSA) by shaking vigorously overnight at room temperature. The extracts were filtered using Whatman no.1 filter paper (GE Healthcare, UK) and air-dried under a stream of air at room temperature. The dried extracts were redissolved in dimethylsulphoxide for further work.

2.2 Cell culture and maintenance

The MDA-MB-231 human breast cancer cells and HEK-293 human embryonic kidney cells were obtained from Cellonex South Africa (Cellonex, RSA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (HyClone, USA) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, USA). The cells were maintained at 37°C in a humidified incubator (Instrulab cc, Johannesburg, RSA) with 5% carbon dioxide. The cells were sub-cultured at 80-90% confluency after 2-3 days to prevent over confluence.

2.3 Qualitative phytochemical tests

The chloroform and methanol extracts of *D. senecioides* and *F. trinervia* were screened for the presence of phytochemical compounds using various methods, listed in the **Table 2.1** below.

Table 2.1: Tests for the presence of phytochemicals

Phytoconstituent	Test	Observation
Saponins	1 ml of plant extract + 5 ml distilled water	Lather
Terpenoids	5 ml of plant extract + 2 ml chloroform	Reddish-brown colouration
Cardiac glycosides	10% Ferric chloride + 5 ml of plant extract + 1 ml sulphuric acid + 2 ml of glacial acetic acid	Brown ring
Steroids	2 ml acetic acid + 2 ml sulphuric acid + 0.5 ml of plant extract	Purple/violet to blue ring
Alkaloids	1 ml Dragendorff + 1 ml of plant extract	Orange-red precipitation
Phenols	2 ml distilled water + 0.5 ml ferric chloride (10%) + 1 ml of plant extract	Blue or green-black colouration
Tannins	2 ml ferric chloride (10%) + 2 ml of plant extract	Blackish-blue or green-blackish colouration
Flavonoids	3 drops of magnesium shavings + 1 ml ethanol + 1 ml hydrochloric acid + 1 ml of plant extract	Red-pink colouration

2.4 Cell viability assay

The effect of the chloroform or methanol extracts of *D. senecioides* or *F. trinervia* on the viability of MDA-MB-231 and HEK-293 cells was assessed using the Cell Counting Kit 8 (CCK-8) assay. The assay measures cell viability by correlating the production of coloured formazan dye to the number of living cells in the culture. Dehydrogenases from viable cells convert Water-Soluble Tetrazolium 8 (WST-8) into a formazan dye, which produces an easily measurable colour change (Ishiyama *et al*., 1997). The MDA-MB-231 and HEK-293 cells were seeded at 20 x 10^3 cells/well

in 96-well cell culture plates (Nest Biotechnology Co., Ltd, USA) and incubated at 37°C for 24 h. The cells were treated with 50 to 200 µg/ml of *F. trinervia or D. senecioides* methanol and chloroform extracts and incubated for 24 h at 37°C. Eight micrograms per milliliter of tanshinone (Sigma-Aldrich, Shanghai, China) was used as a positive control. After treatment, 10 µl of CCK-8 (Sigma, USA) reagent was added to each well and the plates were incubated at 37°C for 2 h. The absorbance of the coloured formazan dye was measured at 450 nm using a Glomax®-Multi+detection system (Promega Corporation, USA). The percentage viability was calculated using the following formula:

$$Cell\ viability\ (\%) = \frac{The\ absorbance\ of\ treated\ cells}{The\ absorbance\ of\ untreated\ cells} \times 100$$

2.5 Mode of cell death assay

The mode of cell death induced by the chloroform or methanol extracts of *D. senecioides* or *F. trinervia* in MDA-MB-231 cells was assessed using the Muse® Annexin V and Dead Cell assay kit (Thermo Fisher, USA). This assay quantifies four cell populations: live cells, cells undergoing early apoptosis, cells in the late stages of apoptosis, and dead or necrotic cells. The Annexin V assay identifies apoptotic cells by detecting the externalisation of phosphatidylserine (PS) during early apoptosis. Annexin V binds to PS on the outer cell membrane of apoptotic cells but not to living cells, which maintains PS on the inner membrane. When coupled with propidium iodide (PI), the assay distinguishes between living cells, which exclude both Annexin V and PI; early apoptotic cells, which bind Annexin V but not PI; and late apoptotic or necrotic cells, which bind both Annexin V and PI due to membrane damage. This allows for the differentiation of living, early apoptotic, and late apoptotic or necrotic cells based on membrane integrity and PS exposure (Lakshmana and Batra, 2013). Cells were seeded at 1 x 10^5 cells/well in a 24-well cell culture plate and incubated for 24 h at 37°C. The cells were treated with 50 µg/ml (the concentration where 10% of the cells are killed by the extract or IC_{10}) or 200 µg/ml (the concentration where 20% of the cells are killed by the extract or IC_{20}) of the chloroform or methanol extracts of *D. senecioides* and *F. trinervia* or 8 µg/ml of tanshinone as positive control. Following incubation for 24 hours at 37°C, the cells were washed with 500 µl of 1x PBS (phosphate-buffered saline). The cells were then detached by adding 1 ml of 0.25% trypsin-EDTA and incubating the flasks at 37°C for 5 minutes. The contents

of the wells were transferred to 2 ml microcentrifuge tubes. The tubes were centrifuged for 5 min at 200 rpm and the pellets were washed with 300 µl of 1x PBS (Lonza, Basel, Switzerland). Thereafter, 100 µl of Muse® Annexin V and Dead Cell reagent was added to each tube containing 100 µl of cells suspended in PBS then incubated for 20 min at room temperature. The samples were analysed on the Muse® Cell Analyser.

2.6 Morphology transition assay

The epithelial-to-mesenchymal transition (EMT)-related morphological changes of the MDA-MB-231 cells were assessed under a light microscope. The assay is designed to detect EMT by focusing on two key changes: the disruption of cell-cell junctions and the morphological transformation from an epithelial to a fibroblast-like state. These alterations are indicative of the transition from tightly connected epithelial cells to a more loosely associated and elongated fibroblast-like phenotype, making them useful markers for studying EMT. The MDA-MB-231 cells were seeded at 1 x 10^5 cells/well in 24-well cell culture plates using serum-free DMEM and incubated for 24 h at 37°C. The cells were treated with 50 µg/ml or 200 µg/ml of the chloroform or methanol extracts of *D. senecioides* or *F. trinervia*; 8 µg/ml of tanshinone was used as positive control. After treatment, the cells were washed three times with 500 µl of 1x PBS and images of the cells were captured under a 10x objective of an inverted phase-contrast light microscope (Olympus, Japan).

2.7 Protein expression assay

The effect of the chloroform or methanol extracts of *D. senecioides* or *F. trinervia* on vimentin protein expression levels in MDA-MB-231 cells was assessed using the western blot assay. This assay involves separating and identifying proteins through gel electrophoresis, a protein mixture is sorted based on molecular weight. Subsequently, the results are transferred to a Polyvinylidene fluoride (PVDF) membrane, generating a distinct band for each protein (Mahmood and Yang, 2012).

2.7.1 Cell treatment and lysis

MDA-MB-231 cells were cultured in 25 cm^3 culture flask and treated 50 µg/ml or 200 µg/ml of the chloroform or methanol extracts of *D. senecioides* or *F. trinervia*; 8 µg/ml of tanshinone was used as positive control. Following incubation for 24 h at 37°C and the cells washed with 500 µl of 1x PBS, the cells were detached by adding 1 ml

of 0.25% trypsin-EDTA and incubating the flasks at 37°C for 5 min. The detached cells transferred to 2 ml microcentrifuge tubes and centrifuged for 10 min at 1200 rpm. The cell pellets were resuspended in 250 µl RIPA buffer [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 0.1% SDS] and incubated for 30 min on ice. The cell lysates were centrifuged at 12 000 rpm for 20 minutes at 4°C and the supernatant was transferred to clean microcentrifuge tubes. Protein quantification was done using the BCA protein quantification kit (Thermo Scientific, Rockford, USA) proteins were quantified as in Section 2.7.2.

2.7.2 Quantification of Proteins

The procedure for the BCA protein quantification kit involves several steps. Firstly, protein samples and standards were prepared in DMEM. The BCA working reagent was prepared by mixing reagent A (a colourless solution containing BCA and sodium carbonate) and reagent B (a blue-green solution containing copper (II) sulfate) in a 50:1 ratio. This mixture was added to each well of a microplate, followed by the addition of samples, standards, and blanks. The microplate was then incubated at 37°C for 3 h, allowing the colour to develop. During the incubation period, the colour of the solution transitions from green to purple due to the reduction of Cu2+ ions to Cu1+ ions by the proteins present in the samples. This colour change is indicative of the formation of a complex between the proteins and the copper ions in the BCA reagent. The intensity of the purple colour corresponds to the amount of protein present in the samples, with darker shades indicating higher protein concentrations. The calculations for protein concentration were made by first plotting a standard curve with known concentrations of protein standards against their corresponding absorbance values. The absorbance of the samples were then measured using a spectrophotometer at 560 nm, and their protein concentrations were determined by using the standard curve equation.

2.7.3 Western blotting

Samples containing 20 µg of total cellular proteins were boiled in sodium dodecyl sulphate (SDS) sample loading buffer [125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol]. Proteins were then separated on 8 & 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) at 100 Volts (V) for stacking gel and at 150 V when they reached the separating gel for 2 h 30 min. The proteins on the gel were transferred to a PVDF membrane (Thermo Fisher, USA)

using a blotting buffer [20% methanol, 25 mM Tris (pH 8.3), 192 mM glycine] at 200 mA for 1 h 30 min at 4°C. Thereafter, the membrane was blocked with 5% (w/v) non-fat milk in 1x PBST (900 ml dH_2O, 100 ml PBS, and 1 ml Tween-20]) for 1 h at room temperature on a shaker. The membrane was incubated with a 1:500 dilution of GAPDH mouse primary antibody (Thermo Fisher, USA) in 5% non-fat milk at 4°C overnight on a shaker. The membrane was washed three times at 5 min intervals with 1x PBST and thereafter, incubated with of a 1:10 000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Thermo Fisher, USA) in 1x PBST for 1 h at room temperature on a shaker. The membrane was washed again as described previously, then SuperSignal™ West Dura Stable Peroxide Buffer (reagent A) and SuperSignal™ West Dura Enhancer Solution (reagent B) chemiluminescence substrates (Thermo Fisher, USA) were added to the membrane by mixing 1:1 reagent A to reagent B and incubating for 5 min in the dark. After the substrate was added and the membranes were incubated, chemiluminescence occurred due to the reaction between the substrate components and any target molecules on the membrane. This emitted light was then detected and captured using C-DiGit blot scanner. To detect vimentin, western blotting was done as described for GAPDH. The membrane was incubated with a 1:500 dilution of vimentin rabbit primary antibody and 1:10 000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Thermo Fisher, USA) and subsequent procedures conducted as described previously. The intensity of each band relative to control was calculated using ImageJ Version 1.53s software.

2.8 Gelatin-Zymography assay

The gelatin-zymography assay was performed to assess the effect of *D. senecioides* or *F. trinervia* chloroform or methanol extracts on the enzymatic activity of matrix metalloproteinases-2 and -9 in MDA-MB-231 cells. This assay separates proteins based on their molecular weight using SDS-PAGE and quantifies the proteolytic activity of gelatinase A (MMP-2) and gelatinase B (MMP-9) based on their ability to degrade gelatin incorporated into the polyacrylamide gel (Raykin *et al*., 2017). The MDA-MB-231 cells were seeded at 6.5 x 10^5 cells/well in 5 ml of serum-free DMEM in 25 cm^2 cell culture flasks (Nest Biotechnology Co., Ltd, USA) and incubated at 37°C overnight. The cells were treated with 50 µg/ml or 200 µg/ml of the chloroform or methanol extracts of *D. senecioides* and *F. trinervia*; 8 µg/ml of tanshinone was

used as positive control. The conditioned media was collected, and the total protein content therein was quantified using the BCA protein assay kit (ThermoFisher Scientific, USA) as described in **Section 2.7.2**. The protein samples (30µg) were mixed with non-reducing sample buffer [62.5 mM, Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (v/v) SDS, and 0.01% (w/v) bromophenol blue] and electrophoresed using the Novex^{TM} 10% Zymogram Plus (Gelatin) gel (Thermo Fisher Scientific, USA) at 100 V for 120 min at 4°C. Thereafter, the gels were washed with 2.5% (v/v) Triton X-100 for 40 min at room temperature on a rocking shaker to remove the SDS and renature the gelatinases (MMP-2 and -9). The gels were then incubated overnight at 37°C in a developing buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM $CaCl_2$], to allow for the gelatinolytic activity to occur. After incubation, the gels were stained with Coomassie Blue R-250 stain for an hour, then destained for 40 min with destaining solution [50% (v/v) methanol, 10% (v/v) glacial acetic acid] Images of the gels were captured using C-DiGit blot scanner. The densitometric analysis of the bands was done using ImageJ Version 1.53s software.

2.9 Cell migration assay

The trans-well migration assay was performed to evaluate the migrative ability of treated MDA-MB-231 cells *in vitro*. This assay measures the chemotactic capability of cells toward a chemo-attractant (Boyden, 1962). The cells were treated with 50 µg/ml or 200 µg/ml of the chloroform or methanol extracts of *F. trinervia* or *D. senecioides*; 8 µg/ml of tanshinone was used as positive control. MDA-MB-231 cells were seeded at 65 x 10^3 cells/well in 200 µl of serum-free DMEM in the upper chamber of the plate; into the lower chamber, 500 µl DMEM supplemented with 10% FBS was added as a chemoattractant and the plate was incubated at 37°C for 24 h. The cells that migrated to the bottom of the upper chamber were fixed with 4% (w/v) formaldehyde and stained with 0.5% (w/v) crystal violet (Sigma-Aldrich, Shanghai, China). The images of the cells were captured under a 10x objective of an inverted phase-contrast light microscope (Olympus, Japan).

2.10 Cell adhesion assay

The effect of the chloroform or methanol extracts of *D. senecioides* or *F. trinervia* on the adhesion of MDA-MB-231 was assessed using cell adhesion assay. This assay measures cell-binding either to cell monolayers or immobilised ligands under immobile conditions which gives an indication of the ability of the cells to attach to a

secondary site after migrating from the primary organ (Weitz-Schmidt and Chreng, 2012). Briefly, MDA-MB-231 cells were seeded at 75 x 10^3 cells/well in 48-well culture plates and treated as described above for 24 h. After treatment, the cells were detached from the wells by washing with 500 µl of 1x PBS, removing the extract, and adding 80 µl of 0.25% trypsin-EDTA and incubated at 37°C for 5 min. The contents of the wells were transferred to 2 ml microcentrifuge tubes and centrifuged for 10 min at 1200 rpm. The cells were resuspended in DMEM supplemented with 10% FBS and seeded in a 48-well plate for 3 h. Thereafter, the cells were washed with 1x PBS (Lonza, Belgium) and fixed with 4% formaldehyde. The cells were then stained with crystal violet for 3 h and washed 3 times with 1x PBS and the images of the cells were captured under a 10x objective of an inverted phase-contrast light microscope (Olympus, Japan). The absorbance of the crystal violet-stained was measured at 560 nm using a Glomax®-Multi+detection system. The cell adhesion percentage was calculated using the following formula:

$$Cell\ adhesion\ (\%) = \frac{The\ absorbance\ of\ treated\ cells}{The\ absorbance\ of\ untreated\ cells} \times 100$$

2.11 Reactive oxygen species assay

The effect of the chloroform and methanol extracts of *D. senecioides* or *F. trinervia* on reactive oxygen species formation in MDA-MB-231 breast cancer cells was evaluated using the Muse® Oxidative Stress Kit. The assay quantifies cells undergoing oxidative stress based on the intracellular detection of superoxide radicals by the Muse Oxidative Stress Reagent (Lee *et al*, 2021). The cell-permeable reagent, dihydroethidium (DHE), is an important component of the Muse Oxidative Stress Reagent and is thought to undergo oxidation upon reacting with superoxide anions. This reaction produces a DNA-binding fluorophore, possibly ethidium bromide or a structurally similar compound, which then intercalates with DNA, leading to the emission of red fluorescence (Dios *et al*, 2022). The MDA-MB-231 cells were seeded at 1 x 10^6 cells/well in 24-well culture plates and incubated at 37°C for 24 h. The cells were treated with 50 µg/ml and 200 µg/ml of the chloroform and methanol extracts of *D. senecioides* and *F. trinervia*; 8 µg/ml of tanshinone was used as positive control. The cells were harvested by washing with 500 µl of 1x PBS and replacing the extract with 80 µl of 0.25% trypsin-EDTA followed by incubation for 5

min at 37°C. The contents of the wells were transferred to 2 ml microcentrifuge tubes. The tubes were then centrifuged for 5 min at 1200 rpm and the pellets were washed with 300 µl of PBS. The cell pellets were resuspended to 1 x 10^6 cells/well in 1x Assay Buffer. An aliquot of 190 µl of the Muse Oxidative Stress Reagent, diluted with 1x Assay Buffer, was added to 10 µl of cells. The samples were incubated at 37°C for 30 min and analysed on the Muse® Cell analyser.

2.12 Statistical analysis

The data was analysed using GraphPad Prism statistical software (vs 8.4, GraphPad Software Inc., San Diego, USA) and represented as mean ± standard deviation of three independent experiments. Comparisons between two groups were done using the paired student t-test while those among multiple groups were conducted by one-way analysis of variance (ANOVA) with subsequent Tukey-Kramer's multiple comparison test. A value of *p≤0.05, ***p≤0.01, ***p≤0.001, ****p≤0.0001 were considered significant.

CHAPTER THREE

3. RESULTS

3.1 Phytochemical Screening of *D. senecioides* and *F. trinervia* chloroform and methanol extracts showed the presence of secondary metabolites.

Results from qualitative phytochemical tests demonstrated the presence of saponins, steroids, alkaloids, phenols, tannins, and flavonoids in the chloroform extracts of *D. senecioides* and *F. trinervia*, as detailed in **Table 3.1**. Notably, an abundant presence of terpenoids and cardiac glycosides was observed in the chloroform extract of *D. senecioides*, indicating a rich composition of these secondary metabolites. Additionally, the phytochemical screening of the methanol extracts of *D. senecioides* and *F. trinervia* revealed the presence of phenols and an extensive presence of various phytoconstituents, including saponins, terpenoids, cardiac glycosides, steroids, alkaloids, tannins, and flavonoids, as detailed in **Table 3.1**. The marked occurrence of these compounds in the methanol extract suggests a diverse chemical profile within this extract.

Table 3.1: Phytochemical analysis of *D. senecioides* and *F. trinervia* chloroform and methanol extracts.

	D. senecioides Chloroform	*D. senecioides* Methanol	*F. trinevia* Chloroform	*F. trinevia* Methanol
Saponins	+	++	+	++
Terpenoids	++	++	++	++
Cardiac glycosides	++	++	++	++
Steroids	+	++	++	++
Alkaloids	+	++	+	++
Phenol	+	++	+	++
Tannihs	+	++	+	++
Flavonoids	+	++	+	++

+ : presence of phytoconstituents in the extract

++ : higher presence of phytoconstituents in the extract

3.2 The chloroform and methanol extracts of *D. senecioides* and *F. trinervia* exhibits minimal cytotoxicity effects of the MDA-MB-231 breast cancer cells and HEK-293 human embryonic kidney cells.

The effect of the chloroform or methanol extracts of *D. senecioides* and *F. trinervia* on the viability of MDA-MB-231 and HEK-293 cells was assessed using the CCK-8 assay. The MDA-MB-231 cells treated with a range of concentrations of *D. senecioides* extracts exhibited a slight decrease in viability, with non-significant reductions observed for both the chloroform and methanol extracts (Figure 3.2.1A & B). When MDA-MB-231 cells were treated with *F. trinervia* chloroform and methanol extracts, a nonsignificant decrease in cell viability was also noted at all tested concentrations (Figure 3.2.1C & D). Concentrations of 50 and 200 µg/ml were selected as optimal working concentrations for subsequent assays. The IC_{10} and IC_{20} values for all the extracts against the MDA-MB-231 cells were determined to be 50 and 200 µg/ml, respectively. Opting for these sub-lethal concentrations in metastatic studies is to avoid toxicity to cancerous cells, ensuring that any observed effects post-treatment are due to the extracts working rather than cell death. Moreover, a significant increase in viability was observed in HEK-293 cells treated with 50 µg/ml of *D. senecioides* chloroform extract, while the methanol extract at both the concentrations had no significant effect on the cells (Figures 3.2.2A and B). Interestingly, treatment with *F. trinervia* methanol extracts at both 50 and 200 µg/ml resulted in a significant increase in HEK-293 cell viability (Figure 3.2.2D). *F. trinervia* chloroform extract showed no significant effect on HEK-293 cell viability (Figure 3.2.2C). Moreover, treatment with tanshinone (8 µg/ml) showed a nonsignificant effect in inhibiting cell viability.

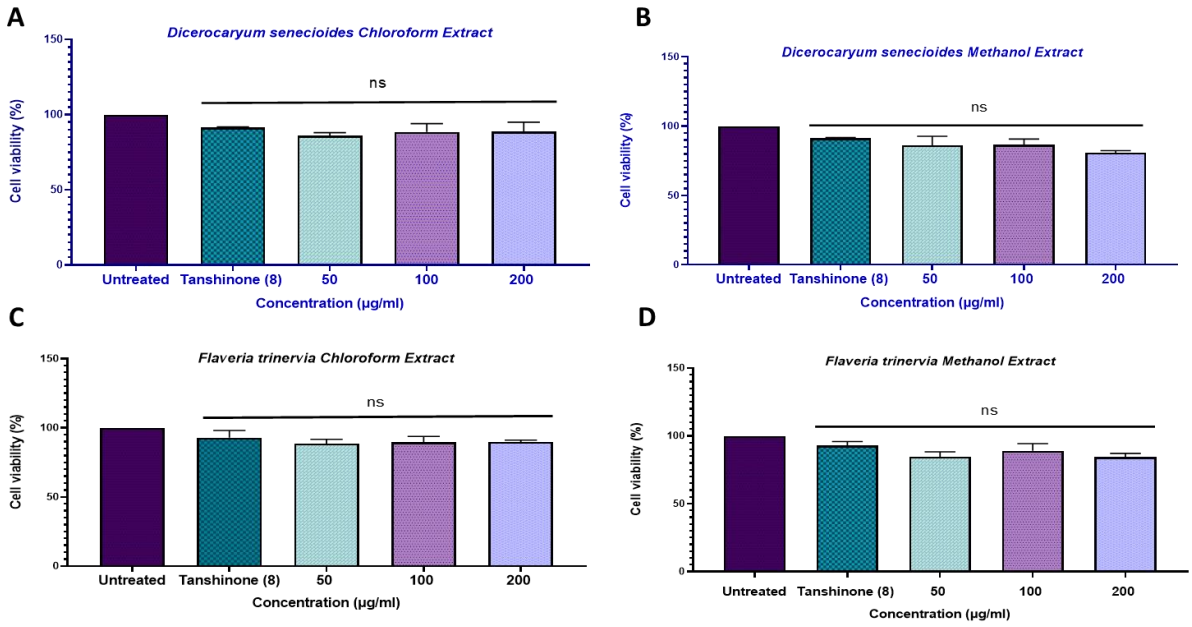

Figure 3.2.1: The effect of *D. senecioides* and *F. trinervia* extracts on the viability of MDA-MB-231 cells. The cells were treated with varying concentrations of methanol or chloroform extracts using CCK-8 kit for 24 h. Tanshinone was used as a positive control. Viability was calculated as percentage of untreated control cells. Data are presented as the mean ± S.D. of three independent experiments, each performed in sextuplicate. The statistical significance of values is indicated by the symbols *p≤0.05, ** p≤0.01, to the untreated control.

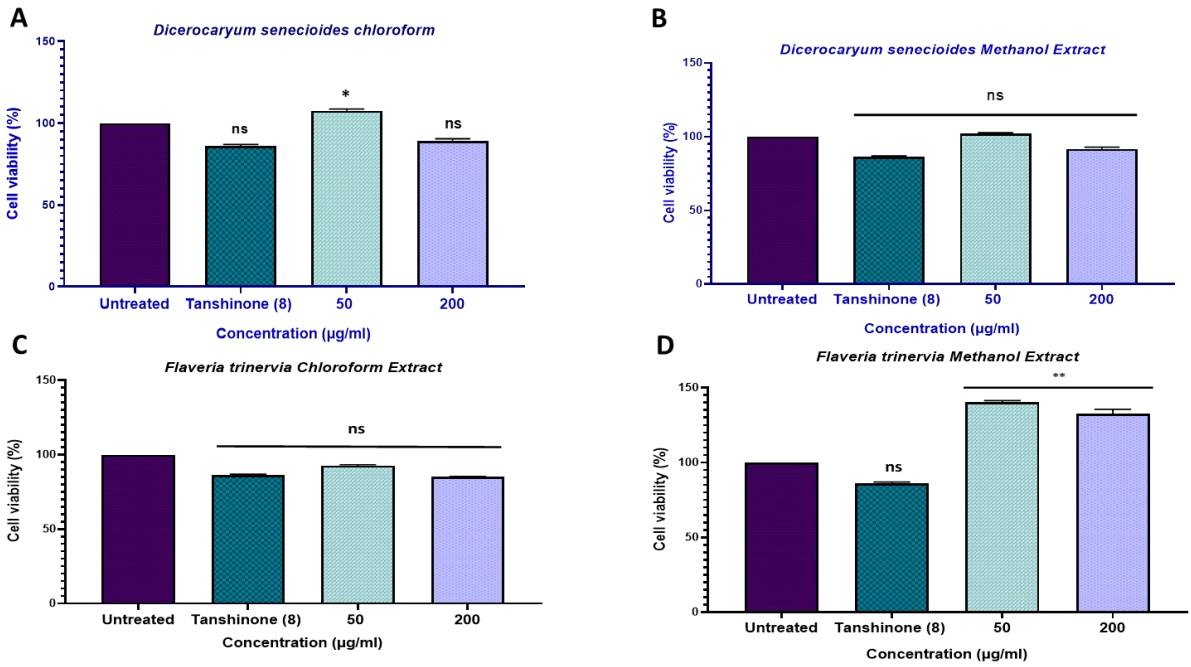

Figure 3.2.2: The effect of *D. senecioides* and *F. trinervia* extracts on the viability of HEK-293 cells. The cells were treated at IC_{10} and IC_{20} values of methanol or chloroform extracts using CCK-8 kit for 24 h. Tanshinone was used as a positive control. Viability was calculated as percentage of untreated control cells. Data are presented as the mean ± S.D. of three independent experiments, each performed in sixtuplicate. The statistical significance of values is indicated by the symbols *p≤0.05, ** p≤0.01, to the untreated control.

3.3 The chloroform and methanol extracts of *D. senecioides* and *F. trinervia* inhibits the phenotypical transition of MDA-MB-231 breast cancer cells.

The epithelial-to-mesenchymal-transition-related morphological changes of the MDA-MB-231 cells were assessed using a light microscope. Typically, MDA-MB-231 cells exhibit a spindle-like shape with numerous protrusions (antennae) that facilitate migration and invasion (Hero *et al*., 2019). However, after treatment with the chloroform or methanol extracts from *D. senecioides* and *F. trinervia,* the cells displayed a rounder morphology with fewer antennae compared to the untreated group, which retained the characteristic spindle-like shape and protrusions. These changes, marked by the red arrows in **Figures 3.3.1** and **3.3.2** suggest that the extracts may inhibit the EMT process in MDA-MB-231 cells.

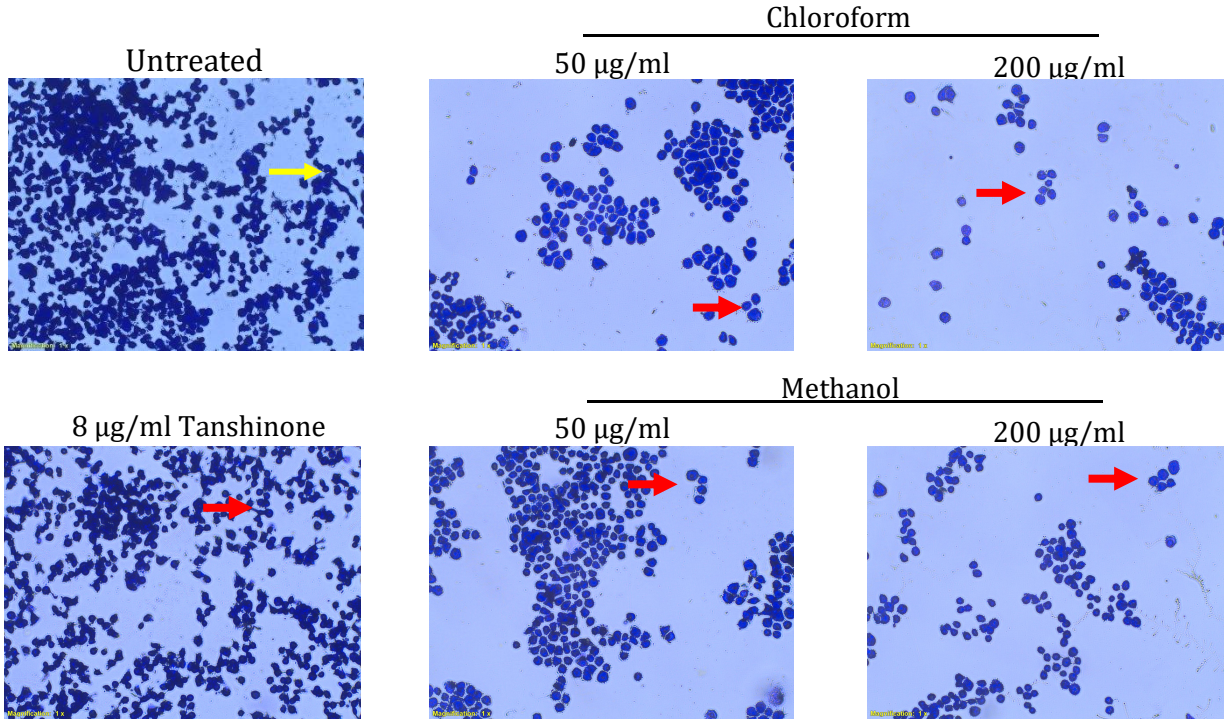

Figure 3.3.1: The effect of *D. senecioides* chloroform and methanol extract on phenotypic transition of MDA-MB-231 cells. Cells were treated with varying concentrations of either extract for 24 h. Tanshinone was used as a positive control. Morphological features associated with EMT changes were assessed and captured under a 10x objective of a light microscope. Photographs represent one of three independent repeats. Yellow arrow– spindle-like shape with antennae cells; Red arrows– round shaped and few antennae cells.

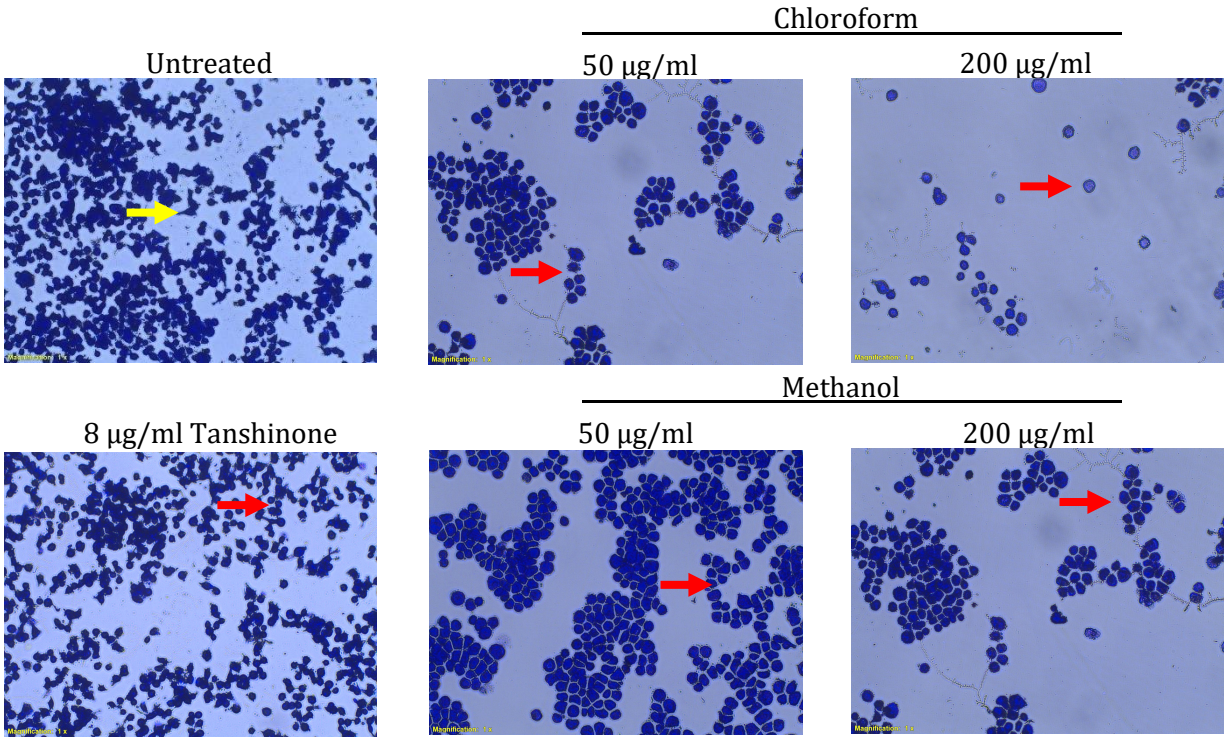

Figure 3.3.2: The effect of *F. trinervia* chloroform and methanol extract on phenotypic transition of MDA-MB-231 cells. Cells were treated with varying concentrations of either extract for 24 h. Tanshinone was used as a positive control. Morphological features associated with EMT changes were assessed and captured under a 10x objective of a light microscope. Photographs represent one of three independent repeats. Yellow arrow– spindle-like shape with antennae cells; Red arrows– round shaped and few antennae cells.

3.4 The effect of the chloroform and methanol extracts of *D. senecioides* and *F. trinervia* downregulate the expression of EMT-related protein vimentin of the MDA-MB-231 breast cancer cells.

The expression of the EMT-related protein vimentin of the MDA-MB-231 cells was assessed using western blot assay. Treatment with *D. senecioides* and *F. trinervia* chloroform or methanol extracts resulted in noticeably reduced vimentin expression in MDA-MB-231, indicated by the fading of the bands corresponding to vimentin protein on the PVDF membrane (**Figure 3.6A**). The intensity of these bands is directly proportional to the level of vimentin expression, thus fainter bands indicate downregulation of the protein expression. Interestingly, in contrast to the other treatments, the western blot analysis revealed that treatment with 50 µg/ml of the *F. trinervia* chloroform extract resulted in upregulation of the expression of vimention when compared to the untreated control, this is shown by **Figure 3.4E**. Additionally, our positive control, tanshinone, exhibited a similar downregulation of vimentin expression.

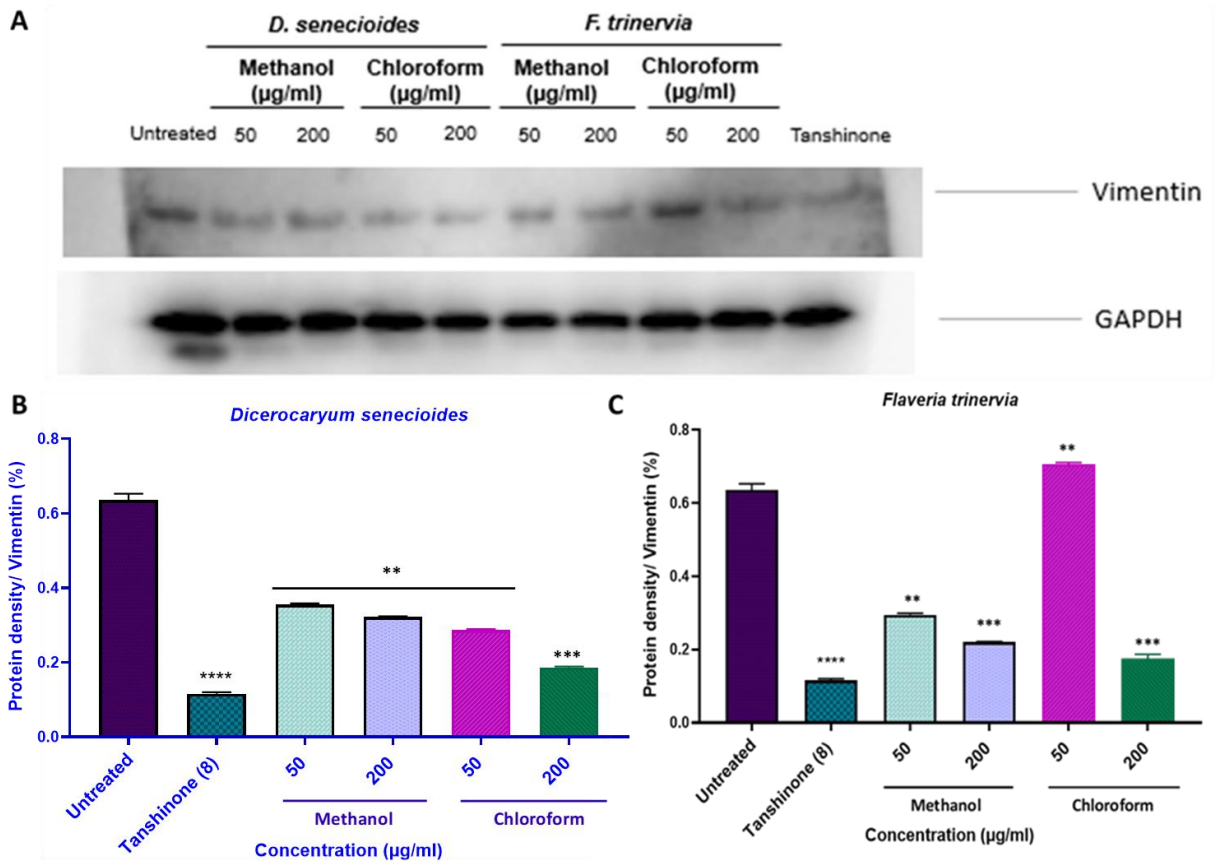

Figure 3.4: The effect of *D. senecioides* and *F. trinervia* chloroform or methanol extracts on the expression of EMT-related protein vimentin of MDA-MB-231. Protein expression level was determined using western blotting, developed and captured using the C-DiGit blot scanner (**A**). The densitometric analysis of the bands was done using ImageJ Version 1.53s software (**B-E**). The statistical significance of values is indicated by the symbols **p≤0.01, *** p≤0.001, to the untreated control.

3.5 The chloroform and methanol extracts of *D. senecioides* and *F. trinervia* extracts induces apoptosis in MDA-MB-231 breast cancer cells.

The Annexin V assay was used to assess the percentage and mode of cell death induced by the chloroform or methanol extracts of *D. senecioides* and *F. trinervia* in the MDA-MB-231 cells. The chloroform or methanol extracts of both *D. senecioides* and *F. trinevia* induced a significant concentration-dependent increase in the percentage of cells in early and late stages of apoptosis (**Figures 3.5.1 & 3.5.2**). A non-significant decrease in the cells undergoing apoptosis was observed in cells treated with 50 µg/ml of the methanol extract of *D. senecioides* (**Figure 3.5.1C**) while a non-significant concentration-dependent increase in total apoptosis was observed in cells treated with both concentrations of methanol extract of *F. trinevia* (**Figure 3.5.2C**).

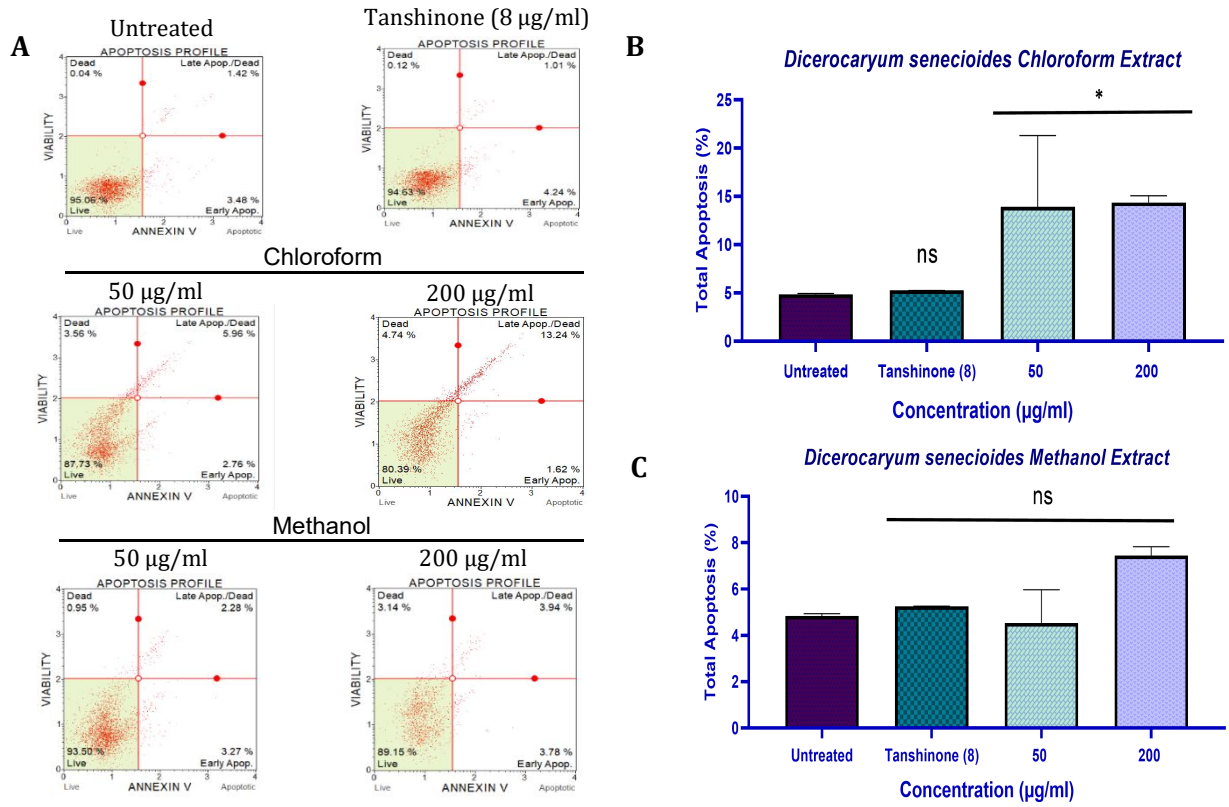

Figure 3.5.1: The effect of *D. senecioides* chloroform and methanol extract on mode of cell death of MDA-MB-231. Cells were treated with varying concentrations of either extract for 24 h. Tanshinone was used as a positive control. The number of cells undergoing cell death was analysed using the Muse analyser (**A**). The percentage of total apoptotic cells was quantified for each sample as the sum of early apoptotic and late apoptotic cells (**B & C**). Data are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. The statistical significance of values is indicated by the symbol ns to the untreated control.

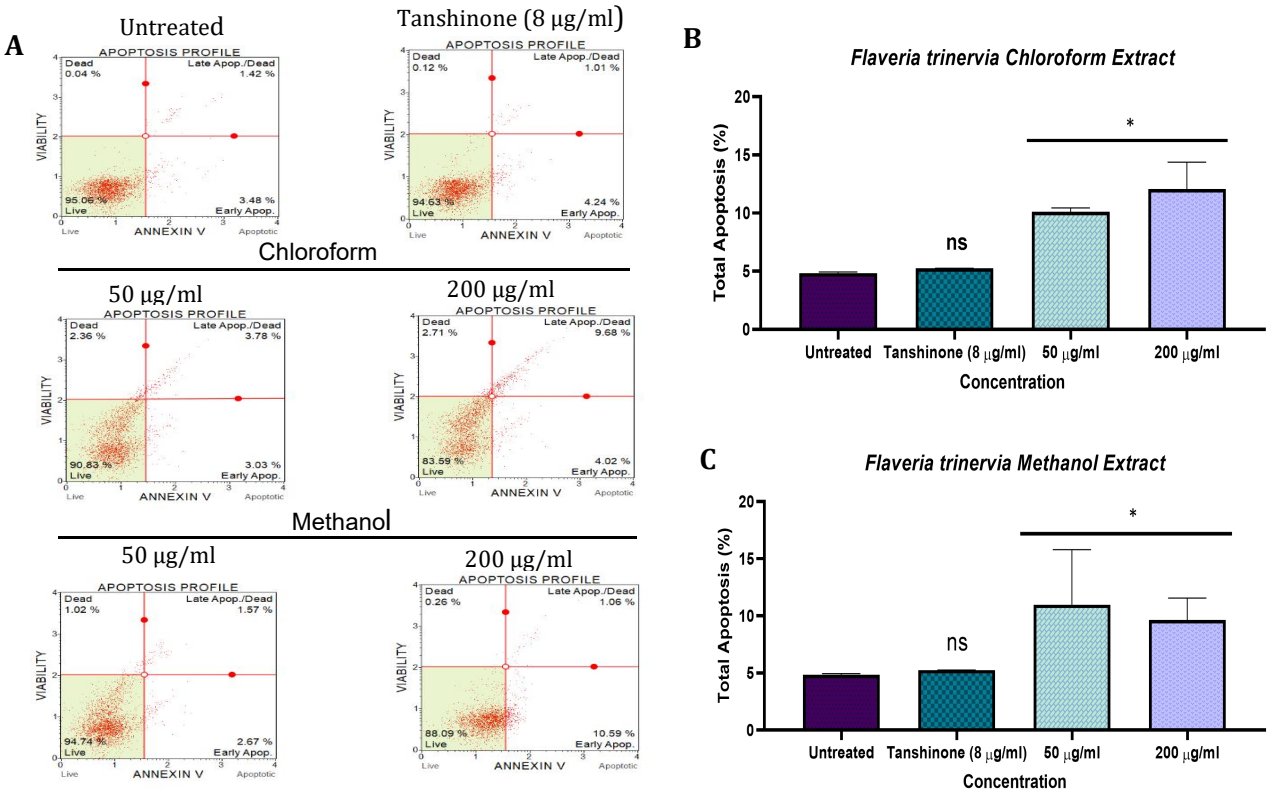

Figure 3.5.2: The effect of *F. trinervia* chloroform and methanol extract on mode of cell death of MDA-MB-231. Cells were treated with varying concentrations of either extract for 24 h. Tanshinone was used as a positive control. The number of cells undergoing cell death was analysed using the Muse analyser (**A**). The percentage of total apoptotic cells was quantified for each sample as the sum of early apoptotic and late apoptotic cells (**B & C**). Data are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. The statistical significance of values is indicated by the symbols *p≤0.05, ** p≤0.01, to the untreated control.

3.6 The chloroform and methanol extracts *D. senecioides* and *F. trinervia* decreases the enzymatic activity of MMP-9 & -2 in the MDA-MB-231 breast cancer cells.

The gelatin-zymography assay was conducted to evaluate the impact of *D. senecioides* and *F. trinervia* chloroform or methanol extracts on the enzymatic activity of MMP-9 and -2. The treatment with both chloroform or methanol extracts of *D. senecioides* and *F. trinervia* resulted in a notable reduction in MMP enzymatic activity, indicated by the fading of the bands corresponding to MMPs on the zymography gel (**Figure 3.6A**). The intensity of these bands is directly proportional to the level of MMP activity, thus fainter bands indicates reduced MMP activity. The significant decrease was also shown by **Figures 3.6B-E**, cells treated with tanshinone (8 µg/ml) exhibited a complete inhibition.

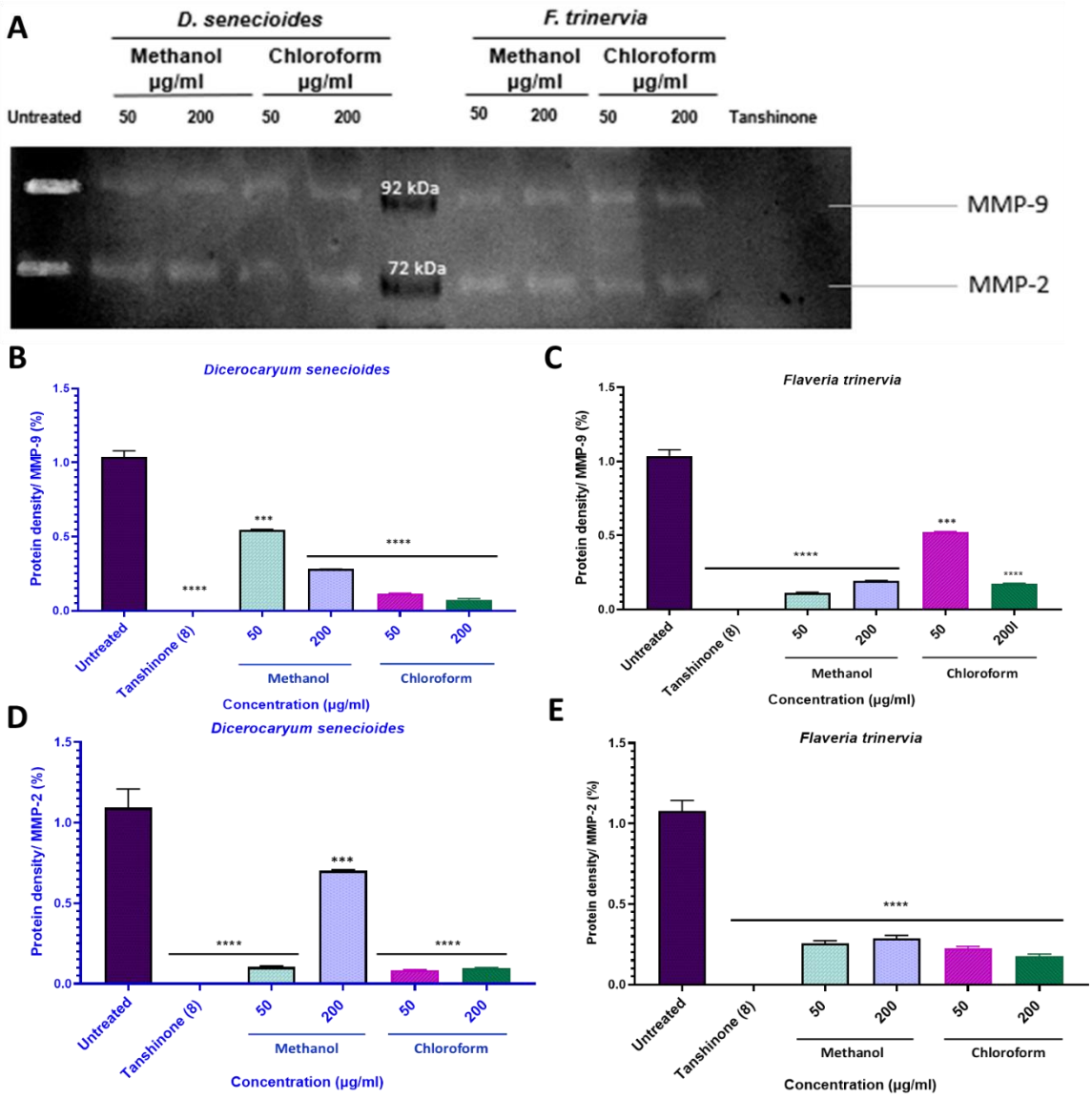

Figure 3.6: The effect of *D. senecioides* and *F. trinervia* chloroform or methanol extract on the enzymatic activity of matrix metalloproteinases. Protein samples (30 µg) were resolved on 8 & 12% SDS-PAGE, electro-blotted onto PVDF, developed and captured using the C-DiGit blot scanner (**A**). The densitometric analysis of MMP-2 and MMP-9 enzyme activity bands was done using ImageJ Version 1.53s software (**B-E**). Data are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. The statistical significance of values is indicated by the symbols ***p≤0.001, **** p≤0.0001, to the untreated control.

3.7 The chloroform and methanol extracts of *D. senecioides* and *F. trinervia* inhibits the migrative ability of MDA-MB-231 breast cancer cells.

The effect of chloroform or methanol extracts of *D. senecioides* and *F. trinervia* on the migrative ability of MDA-MB-231 cells was assessed using the Trans-well migration assay. A decrease in migratory ability was observed in cells subjected to all the treatments, indicated by a lower concentration of crystal violet-stained cells in the treatments compared to the untreated control cells. There was a more marked effect at 200 µg/ml, particularly with the chloroform extract (Figures 3.7.1 & 3.7.2). Treatment with tanshinone also resulted in reduced cell migration, although the effect was not as pronounced as that observed with the 200 µg/ml chloroform extract of *D. senecioides* and *F. trinervia*.

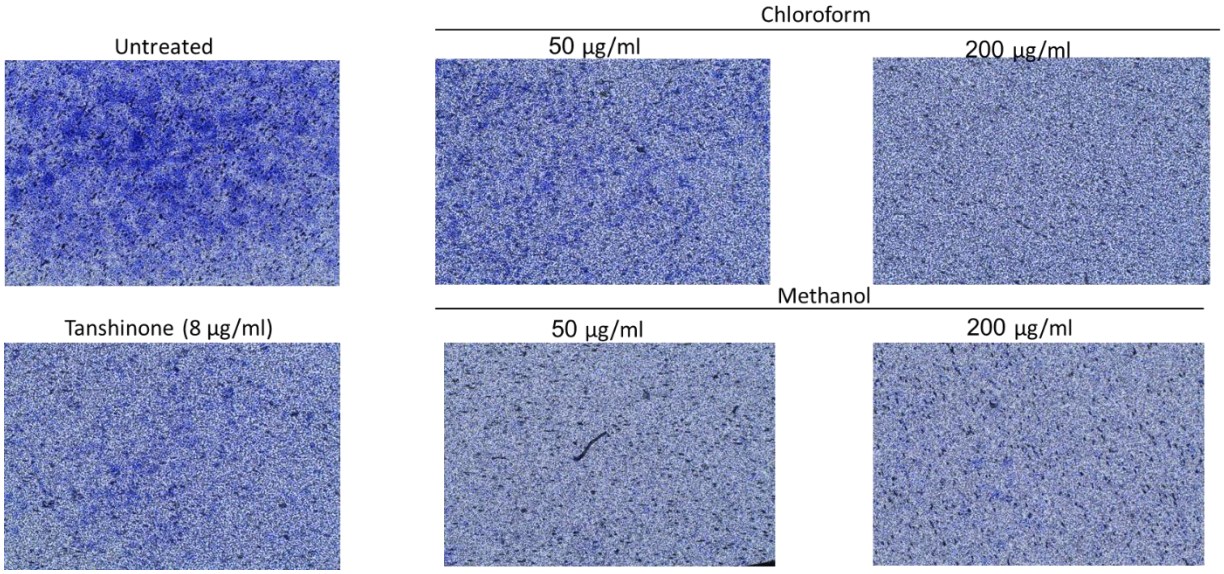

Figure 3.7.1: The effect of *D. senecioides* chloroform or methanol extracts on the migration of MDA-MB-231 cells. Cells were treated with varying concentrations of either extract for 24 h. Tanshinone was used as a positive control. The migrated cells at the lower chamber of the trans-well plate were stained with crystal violet and captured under 10x objective using a light microscope.

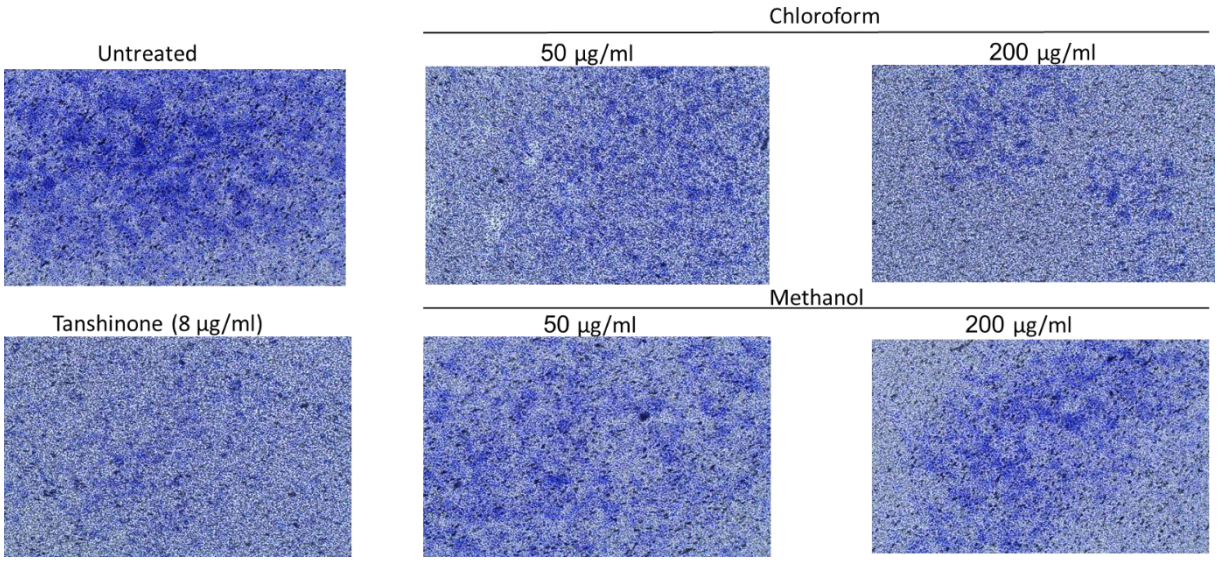

Figure 3.7.2: The effect of *F. trinervia* chloroform and methanol extracts on migration of MDA-MB-231 cells. Cells were treated with varying concentrations of either extract for 24 h. Tanshinone was used as a positive control. The migrated cells from the lower chamber of the trans-well plate were stained with crystal violet and captured under 10x objective using a light microscope.

3.8 The chloroform and methanol extracts of *D. senecioides* and *F. trinervia* inhibits the adhesive ability of MDA-MB-231 breast cancer cells.

The effect of the chloroform or methanol extracts of *D. senecioides* and *F. trinervia* on the adhesion of MDA-MB-231 was assessed using cell adhesion assay. A significant concentration-dependent reduction in the adhesion of MDA-MB-231 cells following treatment with both chloroform and methanol extracts of *D. senecioides* and *F. trinervia* (**Figures 3.8.1 & 3.8.2**). A more prominent decrease in cell adhesion was observed at the concentration of 200 µg/ml for both extracts. The reduction in adhesion was evident from the decrease in the blue density of spindle-shaped cells, which are indicative of migrative and adhesive capabilities in **Figures 3.8.1A & 3.8.2A**. Additionally, a noteworthy decrease in cell adhesion was observed in cells treated with tanshinone (8 µg/ml), indicating its efficacy as a positive control.

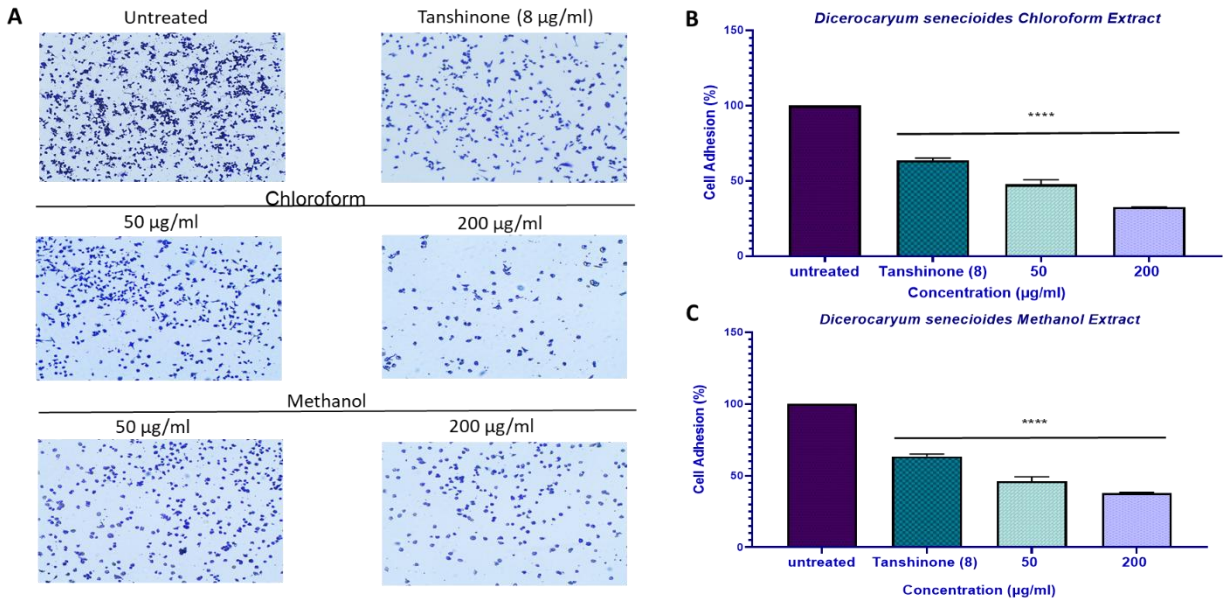

Figure 3.8.1: The effect of *D. senecioides* chloroform and methanol extract on adhesion of MDA-MB-231 cells. Cells were treated with varying concentrations of the extracts for 24 h. Tanshinone was used as a positive control. The attached cells were stained with crystal violet and captured under 10x objective using a light microscope (A). The stained cells were then redissolved in DMSO and quantified at 560 nm using the GloMax®-Multi+ Detection System (B & C). Cell adhesion was calculated as percentage of untreated control cells. Data are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. The statistical significance of values is indicated by the symbols ****p≤0.0001, to the untreated control.

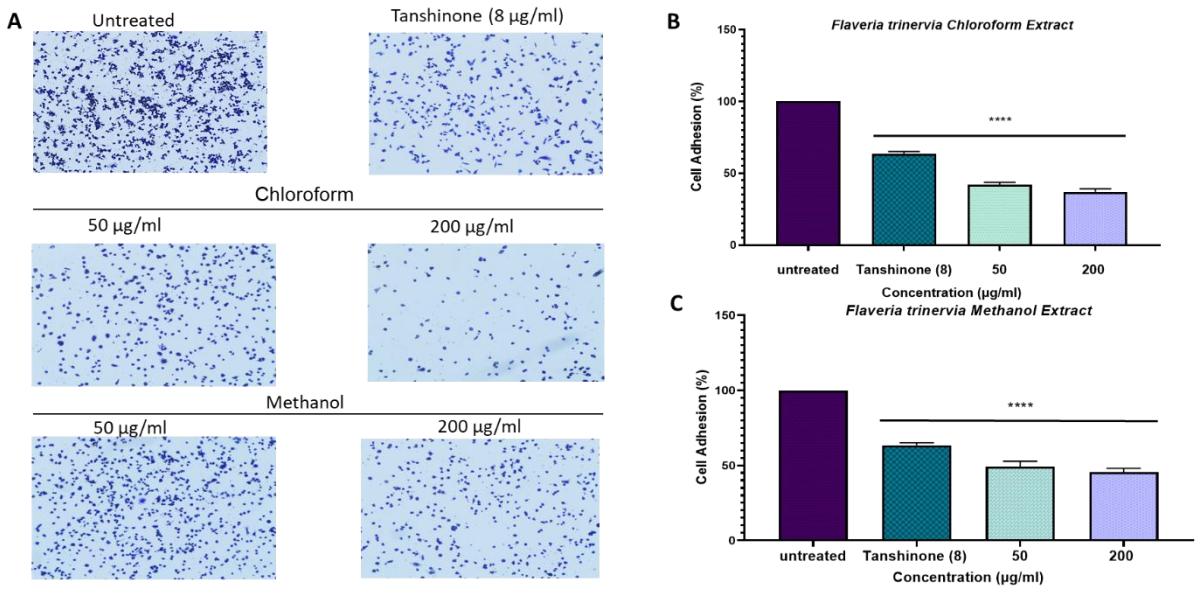

Figure 3.8.2: The effect of *F. trinervia* chloroform and methanol extract on adhesion of MDA-MB-231 cells. Cells were treated with varying concentrations of the extracts for 24 h. Tanshinone was used as a positive control. The attached cells were stained with crystal violet and captured under 10x objective using a light microscope (A). The stained cells were then redissolved in DMSO and quantified at 560 nm using the GloMax®-Multi+ Detection System (B & C). Cell adhesion was calculated as percentage of untreated control cells. Data are presented as the mean ± S.D. of three independent experiments, each performed in triplicate. The statistical significance of values is indicated by the symbols **** $p \leq 0.0001$, to the untreated control.

3.9 The chloroform and methanol extracts of *D. senecioides* and *F. trinervia* downregulate the ROS levels of MDA-MB-231 breast cancer cells.

The effect of the chloroform or methanol extracts of *D. senecioides* and *F. trinervia* on reactive oxygen species formation in MDA-MB-231 breast cancer cells using the Muse® Oxidative Stress Kit. Results obtained revealed a concentration-dependent decrease in ROS levels when cells were exposed to hydrogen peroxide, as depicted in **Figures 3.9.1 & 3.9.2**. The findings indicate that there was a significant decrease in ROS production with an increase in concentration when MDA-MB-231 cells were treated with both chloroform and methanol extracts of *D. senecioides* and *F. trinervia* (**Figures 3.9.1B & C** and **3.9.2B & C**). This shows a concentration-dependent mitigating effect on ROS levels, emphasising their potential role in modulating oxidative stress in breast cancer cells.

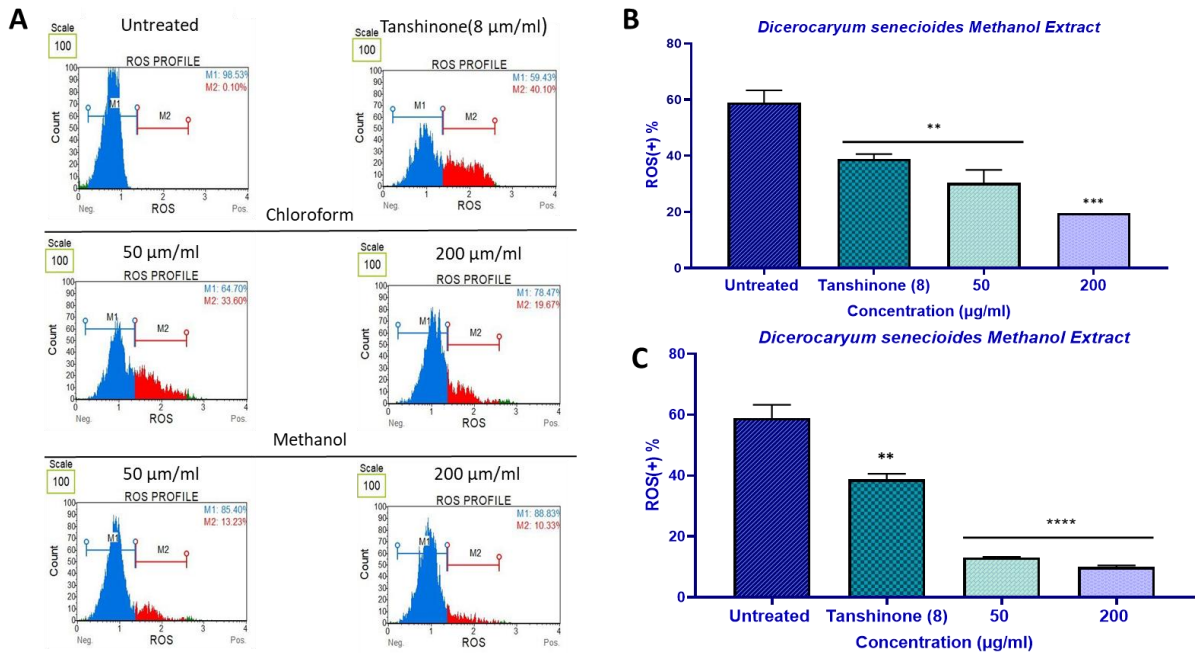

Figure 3.9.2: The effect of *D. senecioides* chloroform and methanol extracts on reactive species of MDA-MB-231 cells. Cells were treated with varying concentrations of the extracts for 22 h, followed by addition of hydrogen peroxide (50 µM) for production of the reactive oxygen species for 2 h. fluorescence probes for ROS generation include DCFH-DA for measuring peroxide radicals or hydrogen peroxide (H_2O_2). The fluorescence intensity was determined using the flow cytometry (A). The percentage of ROS (+) was quantified for each sample using M1 of the chromatograms (B & C). Data are presented as the mean ± S.D. of three independent experiments, each performed in duplicate. The statistical significance of values is indicated by the symbols **p≤0.01, ***p≤0.001, to the untreated control.

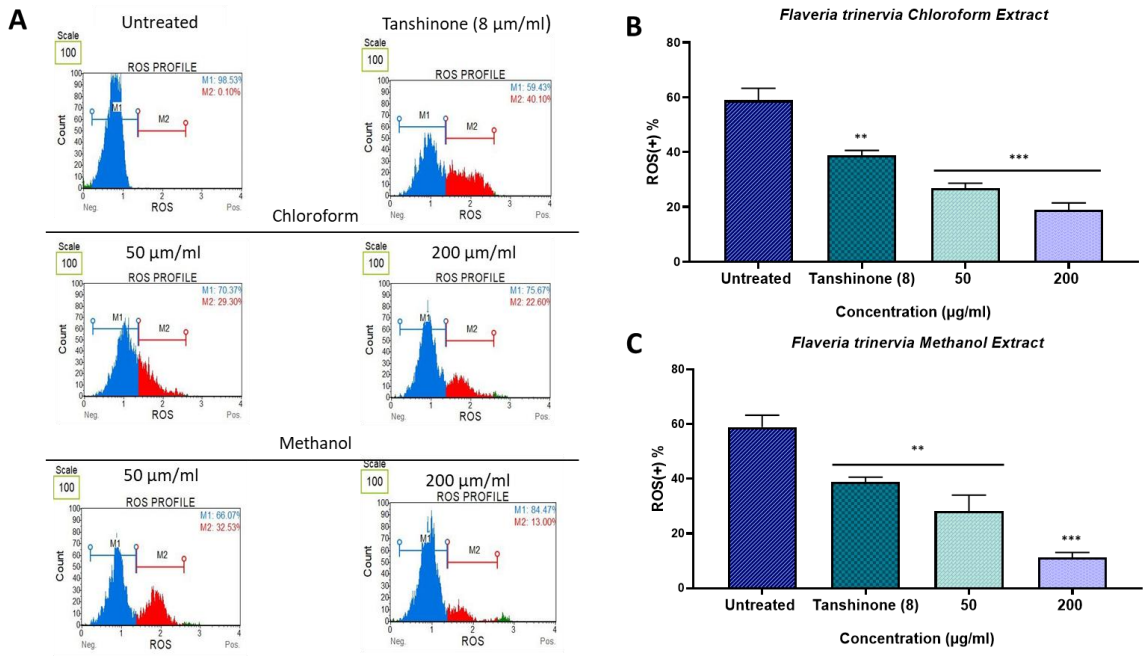

Figure 3.9.2: The effect of *D. senecioides* chloroform or methanol extracts on reactive species of MDA-MB-231 cells. Cells were treated with varying concentrations of the extracts for 22 h, followed by addition of hydrogen peroxide (50 µM) for production of the reactive oxygen species for 2 h. fluorescence probes for ROS generation include DCFH-DA for measuring peroxide radicals or hydrogen peroxide (H_2O_2). The fluorescence intensity was determined using the flow cytometry (A). The percentage of ROS (+) was quantified for each sample using M1 of the chromatograms (B & C). Data are presented as the mean ± S.D. of three independent experiments, each performed in duplicate. The statistical significance of values is indicated by the symbols * *p≤0.01, *** p≤0.001, to the untreated control.

CHAPTER FOUR

Discussion

Triple-negative breast cancer (TNBC) predominantly affects younger women and exhibits more aggressive clinical behaviour compared to other subtypes of breast cancer (Bray *et al*., 2004; Carol *et al*., 2015; Zaidi and Dib, 2019). The aggressive behaviour of TNBC is attributed to the lack molecular targets for endocrine therapy or HER2-targeted therapy, making chemotherapy the primary adjuvant treatment. However, many TNBC tumours exhibit chemotherapy resistance, leading to rapid relapse and metastasis (Agrawal, 2014). As a result, TNBC has a poor prognosis and limited treatment options in terms of efficacy and side effects (Massague and Obenauf, 2016; Madell, 2021).

Plants have been proven to be potential sources of phytochemicals with anti-cancer activity while having minimal impact on the human body (Malathi *et al*., 2012). *Dicerocaryum senecioides* and *Flaveria trinervia* are known for their potential anticancer properties (Maphahlele, 2008; Madiga *et al*., 2009; Mampuru *et al*., 2012; Rambwawasvika *et al*., 2019; Umadevi *et al*., 2006). They contain various bioactive compounds, such as polyphenols, flavonoids, and alkaloids, which have demonstrated anticancer activities (Achika *et al*., 2014; Mampuru *et al*., 2012; Rambwawasvika *et al*., 2017). Furthermore, both plants have been reported to exhibit anti-inflammatory and antioxidative effects (Achika *et al*., 2014; Mampuru *et al.,* 2012), which can contribute to inhibiting metastatic processes such as migration of metastatic cells from their primary site and their subsequent adhesion at a secondary organ. However, it is noteworthy that the exploration of these two plants has not extended to investigating their potential therapeutic effects in breast cancer, a major problem in South African women (Dlamini, Z. *et al*., 2023). Thus, the present study aimed to address part of this knowledge gap by investigating the effects of *D. senecioides* and *F. trinervia* extracts on metastasis in triple-negative breast cancer.

The phytochemical screening of *F. trinervia* and *D. senecioides* chloroform and methanol extracts revealed the presence of diverse bioactive compound classes in both plants (**Table 3.1**). These phytochemicals are known to play crucial roles in various biological processes in plants and are also credited for their potential health benefits (Ashwani *et al*, 2023), making the extracts good candidates for anti-cancer

investigations. Saponins are known for their cytotoxic effects and ability to induce apoptosis in cancer cells (Elekofehinti *et al*, 2021), while terpenoids have anti-inflammatory activity and inhibit of tumour growth through various mechanisms (Thoppil and Bishayee, 2011). Cardiac glycosides are reported to inhibit of cell proliferation, induce apoptosis, and interfere with cellular signalling pathways crucial for cancer progression (Patel, 2016). Steroids, particularly phytosterols, modulate cell cycle progression, induce apoptosis, and inhibit angiogenesis, contributing to their potential as anti-cancer agents (Lossignol, 2016). Many alkaloids interfere with DNA replication, induce apoptosis, and exhibit anti-angiogenic properties, making them relevant in cancer research (Habli *et al*, 2017). Tannins have been studied for their anti-cancer properties, including the ability to induce apoptosis as well as to interfere with angiogenesis and metastasis, which are crucial processes in cancer progression (Youness *et al,* 2021). Flavonoids are well-known for their anti-cancer properties, including antioxidant, anti-inflammatory, and anti-proliferative effects (Abotaleb *et al*, 2019). The diverse phytochemical profile of *D. senecioides* and *F. trinervia* extracts, highlighted in **Table 3.1**, suggests a multifaceted potential for combating metastatic breast cancer.

The methanol and chloroform extracts of *D. senecioides* and *F. trinervia* were shown to induce a slight decrease in the viability of MDA-MB-231 breast cancer cells at low concentrations, both having IC_{10} and IC_{20} values of 50 and 200 µg/ml, respectively. Opting for sub-lethal concentrations is crucial for metastatic studies, as this ensures that observed post-treatment effects are attributed to the extract's efficacy to stop metastatic processes, rather than cell death. An anti-cancer remedy must selectively inhibit cancer cell growth while not being toxic to normal cells (Igbal *et al*, 2017). Concentrations of 50 and 200 µg/ml of the extracts were, therefore, assessed for cytotoxicity in HEK-293 human embryonic kidney cells. The chloroform and methanol extracts of both plants had insignificant toxic effects on the viability of the embryonic kidney cells after 24 h treatment, as shown in **Figure 3.2.2.**

The observed decrease in cell viability necessitated the investigation of the mode in which these cells were dying using the Muse Annexin V and Dead Cell assay. The results obtained revealed an increase in pro-apoptotic MDA-MB-231 cells in a concentration-dependent manner when treated with both *D. senecioides* extracts as well as those of *F. trinervia*. This detection is due to the action of annexin V dye that

selectively binds to phosphatidylserine that has translocated to the outer cell membrane during the process of apoptosis (Kim *et al*., 2020). Despite the higher percentage of cells undergoing apoptosis following treatment with these four extracts, a small population showed necrotic cell death. However, Suzuki *et al*, (2015) stated that when multiple cell death modes occur at the same time, the mechanism involving a higher percentage the cell population prevails. Thus, the findings in **Figures 3.3.1** and **3.3.2** highlight apoptosis as the primary mode of cell death induced by chloroform and methanol extracts of both plant treatments that was associated with the decrease in cell viability (**Figure 3.2.1**) of MDA-MB-231 breast cancer cells.

For the initiation of the metastatic cascade, cancer cells must detach from their primary tumour and invade nearby tissues (Shenoy and Lu, 2016). This involves a morphological shift from the epithelial to the mesenchymal (EMT) phenotype. (Deshmukh *et al*., 2021; Lusby *et al*., 2022). Normally, metastatic MDA-MB-231 cells are spindle-like and have more antennae, characteristics that are beneficial for migration and invasion (Hero *et al*., 2019). However, after treatment with the *D. senecioides* and *F. trinervia* chloroform and methanol extracts, the cells became rounder and the antennae decreased, indicating inhibition of the EMT process (**Figures 3.4.1** and **3.4.2**). Additionally, the expression of vimentin, an important EMT-related protein, was evaluated to further validate the morphology findings. In Figure 3.5, the downregulation of the vimentin protein was evident following treatment of the cells with increasing concentrations of both methanol and chloroform extracts from the plants. This suggests that the cells are unable to transition to their epithelial morphology, a crucial step for invasion and extravasation through blood vessels.

Subsequent to EMT, metastatic cells initiate the synthesis of matrix metalloproteinases that facilitate the degradation of the extracellular matrix (Melzer *et al*., 2017). Consequently, the transitioned cells can efficiently navigate and enter the circulatory system (Melzer *et al*., 2017). This coordinated enzymatic process is crucial for the cellular mechanisms associated with tissue remodelling and migrating within the vasculature (Jiang and Li, 2021). Suppressing the activity of MMP-2 and -9 is essential because these enzymes play a crucial role in degrading the ECM (Jiang and Li, 2021). As depicted in **Figure 3.6**, the untreated MDA-MB-231 breast cancer

cells exhibited a strong invasive ability. In contrast, the cells treated with *D. senecioides* and *F. trinervia* extracts demonstrated a noticeable inhibition of MMP-2 and -9 activity. By inhibiting these MMPs, the tested plant extracts showed potential of preserving the integrity of the ECM, hindering MDA-MB-231 cells from degrading and invading the surrounding tissue, ultimately mitigating the metastatic process.

Once cancerous cells invade the ECM, they embark on a journey through the circulatory system, aiming to adhere to and colonise distant organs (Riggio *et al*., 2021). This migration is a pivotal step in the metastasis of cancerous cells. Notably, a 24 h treatment of the chloroform extract from both plant sources exhibited the most pronounced inhibition of migration compared to the untreated cells and those treated with the methanol extract. This inhibition is visually evident through the decreased intensity of the blue colour, representing cells that successfully migrated through the trans-well plate (**Figures 3.7.1** and **3.7.2**). These findings suggest that the chloroform extracts from *D. senecioides* and *F. trinervia* hold promise in inhibiting the migratory capability of MDA-MB-231 cells, offering potential paths for anti-metastatic interventions in breast cancer. The observed down-regulation of EMT-related vimentin in **Figure 3.5** further supports an inhibition of cell migration resulting in preventing movement of the cells to secondary sites.

Following their migration from the primary site, metastatic cells complete the metastatic cascade by attaching to the cells at a secondary organ (Riggio *et al*., 2021). This was best explained by Steven Paget (1889) as a "seed and soil" hypothesis, suggesting that specific tumour cells (the seed) thrive well in particular microenvironments of chosen organs (the soil). According to this concept, metastasis only occurs if the seed is planted in suitable soil (Melzer *et al*., 2017). The findings of the study revealed that the extracts effectively inhibited the attachment of cells to the cell culture plates after a 24 h treatment period (**Figures 3.8.1 and 3.8.2**). The plant extracts created an unfavourable microenvironment for the seeded cancerous cells, resulting in inhibition of their attachment and growth. Such inhibition at this stage in the metastatic process is promising, suggesting that these extracts may interfere with the vital step of cell adhesion during metastasis. Consequently, if the cancerous cells fail to attach to an organ, they will be directed towards apoptosis (Murthy *et al*., 2021).

The growth and spread of tumour cells are often a consequence of the overproduction of reactive oxygen species. However, ROS can play a dual role in regulating cell growth and apoptosis. While moderate levels of ROS are involved in normal cellular signalling and can contribute to the initiation of apoptosis (Manish *et al*., 2021; Kumari *et al*., 2018) excessively high ROS can lead to cell damage and apoptosis resistance. To assess if the apoptotic effects observed with the Annexin V and Dead Cell assay could be attributed to modulation of ROS production, the effects of both plant extracts on ROS production were evaluated. The observed decrease in ROS levels in **Figures 3.9.1 & 3.9.2**, coupled to the apoptosis, migration, and invasion findings, suggests an interesting interplay between oxidative stress and cellular behaviour. Oxidative stress (often associated with elevated ROS) has been linked to processes such as apoptosis, cell migration and invasion by influencing pathways that involve nitrogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K/AKT) (Chen *et al*., 2015; Aggarwal *et al*., 2019). Therefore, a reduction in ROS levels could potentially influence these cellular activities and contribute to the regulation of signalling pathways involved in apoptosis, migration and invasion (Aggarwal *et al*., 2019). By mitigating oxidative stress, the *D. seneciodes* and *F. trinervia* plant extracts could modulate these pathways, potentially affecting the cell death, migratory and invasive properties of the cells. Moreover, the decrease in reactive oxygen species (ROS) levels with an increase in concentration after exposing cells to hydrogen peroxide suggests a potential antioxidant effect. This could indicate that the extracts from *D. senecioides* and *F. trinervia* possess the ability to reduce the oxidative stress induced by hydrogen peroxide. Antioxidants are known for their capacity to neutralise ROS and protect cells from oxidative damage. Therefore, it is important to note that a concentration-dependent response, where higher concentrations lead to a greater decrease in ROS, may indicate a dose-dependent antioxidant effect. This observation aligns with the concept that higher concentrations of antioxidants may provide more protection against oxidative stress. However, the relationship between ROS, cell death, migration, and invasion is complex and context dependent. However, while excessive ROS can promote migration, invasion and resistance to apoptosis allowing cancer cells to evade cell death, a delicate balance is necessary for proper cellular function (Mydin and Okekpa, 2018). Therefore, the observed decrease in ROS levels indicated in

Figures 3.9.1 and **3.9.2** reflect a regulatory mechanism that impacts apoptosis, migration and invasion.

Conclusion

This study on the effects of *Dicerocaryum senecioides* and *Flaveria trinervia* chloroform and methanol extracts on MDA-MB-231 triple-negative breast cancer cells revealed significant findings with potential implications for breast cancer treatment. The extracts were shown to suppress cell invasion by inhibiting the activity of MMP-9 & -2, also inhibiting migration and adhesion of the cells. The findings collectively suggest that *D. senecioides* and *F. trinervia* extracts, particularly those obtained with chloroform, may hold promise as anti-metastatic agents in TNBC. The observation between the chloroform and methanol extracts raises intriguing possibilities for the diverse nature of compounds involved. With methanol's ability to extract a wide range of compounds with varying polarities, extracts prepared with the solvent were expected to show higher mitigation of metastasis. However, the observed effectiveness of chloroform in the inhibition of migration, MMP-9 & -2 enzyme activity and adhesion of the MDA-MB-231 cells suggests a complex interplay of compounds of intermediate polarity. The observed differences might be attributed to potential antagonistic interactions among compounds within the methanol extract, impacting their collective inhibitory effect. Future studies should focus on identifying the specific compounds responsible for these inhibitory effects to provide a clearer understanding of their individual and interactive roles. A detailed analysis of the phytochemical composition of both extracts, could shed light on the specific compounds contributing to the observed inhibitions. This would not only enhance our understanding of the plant extracts' mechanisms but also guide future studies in optimising and refining these extracts for potential therapeutic applications.

CHAPTER FIVE

References

Abotaleb, M., Liskova, A., Kubatka, P., and Busselberg, D. (2020). Therapeutic potential of plant phenolic acids in the treatment of cancer. *Biomolecules*, 10(2): 221.

Abotaleb, M., Samuel, S., Varghese, E., Varghese, S., Kubatka, P., Liskova, A., and Busselberg, D. (2019). Flavonoids in cancer and apoptosis. *Cancer (basel)*, 11(1): 28.

Achika, J.I., Arthur, D.E., Gerald, I., and Adedayo, A. (2014). A review on the phytoconstituents and related medicinal properties of plants in the Asteraceae family. *IOSR Journal of Applied Chemistry*, 7(8), pg. 1–8.

Aggarwal, V., Tuli, H. S., Varol, A., Thakral, F., Yerer, M. B., Sak, K., Varol, M., Jain, A., Khan, M. A., & Sethi, G. (2019). Role of Reactive Oxygen Species in Cancer Progression: Molecular Mechanisms and Recent Advancements. *Biomolecules*, *9*(11), 735.

Al-Ostool, F.H., Salah, S., Khamees, H.A., and Khanum, S.A. (2021). Tumour angiogenesis: current challenges and therapeutic opportunities. *Cancer Treatment and Research Communications,* 28:100422.

Ashwani, K., Nirmal, P., Mukul, K., Anina, J., Vidisha, T., Emel, O., Charalampos, p., Maomao, Z., Tahra, E., Sneha, K., and Fatih, O. (2023). Major phytochemicals: recent advances in health benefits and extraction method. *Molecules*, 28(2): 887.

Ayoub, N.M., Jaradat, S.K., Al-Shami, K.M., and Alkhalifa, A.E. (2022). Targeting Angiogenesis in Breast Cancer: Current Evidence and Future Perspectives of Novel Anti-Angiogenic Approaches. *Frontier in Pharmacology,* 13:838133.

Azevedo, A.S., Follian, G., Patthabhiraman, S., Harlepp, S., and Goetz, J.G. (2015). Metastasis of circulating tumour cells: favorable sol or suitable biomechanics, or both?. *Cell Adhesion & Migration*, 9(5).

Babaei, G., Aziz, S.G.G., Jaghi, N.Z.Z. (2021). EMT, cancer stem cells and autophagy; The three main axes of metastasis. *Biomedicine & Pharmacotherapy*, 133:110909.

Begum, H., Murugesan, P., and Tangutur, A.D. (2022). Western blotting: a poweful stable in scientific and biomedical research. *Biotechniques,* 73(1), pg. 59–69.

Boyden S. (1962): The chemotactic effect of mixture of antibody and antigen on polymorphonuclear leukocytes. *J Exp Med*; 115:453-466.

Castro-Giner, F., and Aceto, N. (2020). Tracking cancer progression: from circulating tumour cells to metastasis. *Genome Medicine*, 12:31.

Chao, Y.L., Shepard, C.R., and Wells, A. (2010). Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Molecular Cancer*, 9(179).

Chen, W-T., Ebelt, N.D., Stracker, T.H., Xhemalca, B., Van den Berg, C.L., and Miller, K.M. (2015). ATM regulation of IL-8 links oxidative stress to cancer cell migration and invasion. *elife* 4:e07270.

Das K., and Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science*, 2:53.

Debnath, P., Huirem, R.S., Dutta, P., Palchaudhuri, S. (2022). Epithelial-mesenchymal transition and its transcription factors. *Bioscience Reports,* 42 (1).

Deng, Z., Wang, H., Liu, J., and Deng, Y. (2021). Comprehensive understanding of anchorage-independent survival and its implication in cancer metastasis. *Cell Death & Disease,* 12:629.

Deshmukh, A.P., Vasaikar, S.V., Tomczak, K., Tripathi, S., den Hollander, P., Arslan, E., Chakraborty, P., Soundararajan, R., Jolly, M.K., Rai, K., Levine, H., and Mani, S.A. (2021). Identification of EMT signaling cross-talk and gene regulatory networks by single-cell RNA sequencing. *PNAS*, 118(19).

Dlamini, Z., Molefe, T., Khanyile, R., Mkhabele, M., Damane, B., Kokoua, A., Bida, M., Saini, K.S., Chauke-Malinga, N., Luvhengo, T.E., and Hull, R. (2023). From incidence to intervention: A comprehensive look at breast cancer in South Africa. Oncology and Therapy, 12(1), 1-11.

Dios, L., Collazo, C., and Inostroza-Nieves, Y. (2022). Renin-angiotensin-system increases phosphorylated tau and reactive oxygen species in human cortical neuron cell line. *Biochemistry Biophysics Reports*, 101355.

Elekofehinti, O., Iwaloye, O., Olawale, F., and Ariyo, E. (2021). Saponins in cancer treatment: current progress and future prospects. *Pathophysiology*, 28(2), pg. 250–272.

Foroni, C., Broggini, M., Generali, D., and Damia, G. (2012). Epithelial-mesenchymal transition and breast cancer: Role, molecular mechanisms and clinical impact. *Cancer Treatment Reviews*, 38, pg. 689–697.

Friedl, P., and Alexander, S. (2011). Cancer invasion and microenvironment: plasticity and reciprocity. *Cell,* 147(5), pg. 992–1009.

Guan, Y., Xu, F., Tian, J., Chen, H., Yang, C., Huang, S., Gao, K., Wan, Z., Li, M., He, M., and Chong, T. (2020). Pathology of circulating tumour cells and the available capture tools (review). *Oncology Reports*, 43(5), pg. 1355–1364.

Gout, S., Tremblay, P.L., and Hout, J. (2007). Selectins and selectin lingands in extravasation of cancer cells and organ selectivity of metastasis. *Clinical & Experimental Metastasis*, 25(4), pg. 335–44.

Habli, Z., Toumieh, G., Fatfat, M., Rahal, O., and Gali-Muhtasib, H. (2017). Emerging cytotoxic alkaloids in the battle against cancer: overview of molecular mechanisms. *Molecules*, 22(2):250.

Hall, K.L., and Ran, S. (2010). Regulation of tumour angiogenesis by the local environment. *Frontiers in Bioscience*, 15(1), pg. 195–212.

Hapach, L.A., Mosier, J.A., Wang, W., and Reinhart-King, C.A. (2019). Engineered models to parse apart the metastatic cascade. *npj Precision Oncology.* 3, Article no. 20.

Hassan, W., Noreen, H., Rehman, S., Gul, S., Kamal, M. A., Kamdem, J. P., Zaman, B., and da Rocha, J. B. T. (2017). Oxidative Stress and Antioxidant Potential of One Hundred Medicinal Plants. *Current topics in medicinal chemistry*, *17*(12), pg. 1336–1370.

Hejmadi, M. (2013). Introduction to cancer biology, 2nd Ed. Denmark; Bookboon 2009.

Hero, T., Buhler, H., Kouam, P.N., Priesch-Grzeszowiak, B., Lateit, T., and Adamietz, I.A. (2019). The triple-negative breast cancer cell line MDA-MB 231 is specifically inhibited by the ionophore salinomycin. *Anticancer Research*, 39(6), pg. 2821–2827.

Hernandez-Caballero, M. (2013). Molecular Mechanisms of Metastasis: Epithelial-Mesenchymal Transition, Anoikis and Loss of Adhesion. in K. Tonissen (ed.), Carcinogenesis, IntechOpen, London.

Hosseinzadeh, S., Jafarikukhdan, A., Hosseini, A., and Armand, R. (2015). The Application of Medicinal Plants in Traditional and Modern Medicine: A Review of *Thymus vulgaris*. *International Journal of Clinical Medicine*, 6(9), pg. 635–642.

Huang, R., Chen, H., Liang, J., Li, Y., Yang, J., Luo, C., Tang, Y., Ding, Y., Liu, X., Yuan, Q., Yu, H., Ye, Y., Xu, W., and Xie, X. (2021). Dual Role of Reactive Oxygen Species and their Application in Cancer Therapy. *Journal of Cancer*, 12(18), pg. 5543–5561.

Hussein, R. A., and El-Anssary, A. A. (2018). Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants. in P. F. Builders (ed.), Herbal Medicine, IntechOpen, London.

Igbal, J., Abbasi, B.A., Mahmood, T., Kanwal, S., Ali, B., Shah, S.A., and Khalil, A.T. (2017). Plant-derived anticancer agents: a green anticancer approach. *ScienceDirect*, 7(12), pg. 1129–1150.

Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y., and Ueno, K. (1997). A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator of NADH as well as cell viability. *Talanta*, 44(7), 1299-1305.

Janiszewska, M., Primi, M. C., and Izard, T. (2020). Cell adhesion in cancer: Beyond the migration of single cells. *The Journal of biological chemistry*, *295*(8), pg. 2495–2505.

Jiang, H., and Li H. (2021). Prognostic values of tumoral MMP2 and MMP9 overexpression in breast cancer: a systematic review and meta-analysis. *BMC Cancer*, 21, article no. 149.

Jie, X. X., Zhang, X. Y., and Xu, C. J. (2017). Epithelial-to-mesenchymal transition, circulating tumour cells and cancer metastasis: Mechanisms and clinical applications. *Oncotarget*, *8*(46), pg. 81558–81571.

Kim, H., Kim, H.Y., Lee, E.Y., Choi, B.K., Jang, H., and Choi, Y. (2020). A quenched annexin V-fluorophore for the real-time fluorescence imaging of apoptotic processes in vitro and in vivo. *Advanced Science*, 7(24).

Klaunig, J.E., Kamenduls, L.M., and Hocevar, B.A. (2010). Oxidative stress and oxidative damage in carcinogenesis. *Toxicologic Pathology*, 38, pg. 96–109.

Kleiner, D.E., and Stetlerstevenson, W.G. (1994). Quantitative zymography: detection of picogram quantities of gelatinases. *Analytical Biochemistry*, 218(2), pg. 325–329.

Kumar, S., Kapoor, A., Desai, S., Inamdar, M.M., and Sen, S. (2016). Proteolytic and non-proteolytic regulation of collective cell invasion: tuning by ECM density and organization. *Scientific Reports*, 6:19905.

Lai, X., Li, Q., Wu, F., Lin, J., Chen, J., Zheng, H., and Guo, L. (2020). Epithelial-mesenchymal transition and metabolic switching in cancer: lessons from somatic cell reprogramming. *Frontiers in Cell and Development Biology,* 8:760.

Lazarczyk, M., Mickael, M. E., Skiba, D., Kurzejamska, E., Lawinski, M., Horbanczuk, J. O., Radziszewski, J., Fraczek, K., Wolinska, R., Paszkiewicz, J., Religa, P., and Sacharczuk, M. (2023). The Journey of Cancer Cells to the Brain: Challenges and Opportunities. *International journal of molecular sciences*, *24*(4), 3854.

Lakshmanan, I., and Batra, S.K. (2013). Apoptosis assay by flow cytometry using annexin V staining method. Bio-Protocol, 3(6).

Lee, S-O., Joo, S., Kwak, A-W., Lee, M-H., Seo, J-H., Cho, S-S., Yoon, G., Chae, J., and Shim, J-H. (2021). Podophyllotoxin induces ROS-mediate apoptosis and cell

cycle arrest in human colourectal cancer cells via p38 MAPK signaling. *Biomolecules & Therapeutics (Seoul),* 29(6), pg. 658–666.

Leonard, A.K., Loughran, E.A., Klymenko, Y., Liu, Y., Kim, O., Asem, M., McAbee, K., Ravosa, M.J., and Stack, M.S. (2018). Chapter 4 – methods for the visualization and analysis of extracellular matrix protein structure and degradation. *Methods in Cell Biology*, 143, pg. 79–95.

Liang., W., He, X., Bi, J., Hu, T., and Sun, Y. (2021). Role of reactive oxygen species in tumours based on the 'seed and soil' theory: A complex interaction (Review). *Oncology Reports*, 46(3).

Liou, G. Y., and Storz, P. (2010). Reactive oxygen species in cancer. *Free radical research*, *44*(5), pg. 479–496.

Lintz, M., Munoz, A., and Reinhart-King, C.A. (2017). The mechanics of single cell and collective migration of tumour cells. *Journal of Biomechemical Engineering*, 139(2).

Logue, S.E., Elgendy, M., and Martin, S.J. (2009). Expression, purification and use of recombinant annexin V for the detection of apoptotic cells. *Nuture Protocols*, 4, pg. 1383–1395.

Lossignol, D. (2016). A little help from steroids in oncology. *Journal of Translational internal medicine*, 4(1), pg. 52–54.

Lugano, R., Ramachandran, M., and Dimberg, A. (2020). Tumour angiogenesis: causes, consequences, challenges and opportunities. *Cellular and Molecular Life Sciences*, 77(9), pg. 1745–1770.

Lusby, R., Dunne, P., and Tiwari, V.K. (2022). Tumour invasion and dissemination. *Biochemical Society Transactions*, 50(3), pg. 1245–1257.

Madiga, M.C., Cockeran, R., Mokgotho, M.P., Anderson, R. and Mampuru, L.J. (2009). Dichloromethane extract of *Dicerocaryum senecioides* leaves exhibits remarkable anti-inflammatory activity in human T-lymphocytes. *Natural Product Research*, 23(11), pg. 998–1006.

Mahmood, T., and Yang, P-C. (2012). Western blot: technique, theory, and trouble shooting. *North American Journal of Medicine Science*, 4(9), 429–434.

Mahomoodally, M.F. (2013). Traditional medicine in Africa: An Appraisal of ten potent African medicinal plants. *Evidence-Based Complementary and Alternative Medicine*, 2013:617459.

Malathi, M., Sundarshana, M.S.S., and Niranjan, M.H. (2012). Antioxidant activity of *flaveria trinervia* (sprengel) C. Mohr. *International Journal of Medicinal Plants Research,* 6(42), pg. 5519–5521.

Mampuru, L.J., Chokoe, P.K., Madiga, M.C., Theron, A., Anderson, R. and Mokgotho, M.P. (2012). Antioxidant and anti-proliferative capacity of a dichloromethane extract of *Dicercaryum senecioides* leaves. in V. Rao (ed.), Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health, IntechOpen, London.

Martin, T.A., Ye, L., Sanders, A.J., Lane, J., and Jiang, W.G. (2013). Cancer invasion and metastasis: molecular and cellular perspective. in: madame curie bioscience database [internet]. Austin (TX): Landes Bioscience; 2000-2013.

Masi, I., Caprara, V., Bagnato, A., and Rosano, L. (2020). Tumour cellular and microenvironment clues controlling invadopodia formation. *Frontiers of Cell and Development Biology*, 8:584181.

Melzer, C., Von der ohe, J., and Hass, R. (2017). Breast carcinoma: from initial tumour cell detachment to settlement at secondary sites. *BioMed Research International*, Vol. 2017.

Micalizzi, D.S., Farabaugh, S.M. and Ford, H.L. (2010). Epithelial-Mesenchymal Transition in Cancer: Parallels Between Normal Development and Tumour Progression. *Journal of Mammary Gland Biology and Neoplasia,* 15, pg. 117–134

Moussa, Z., Judeh, Z.M., Ahmed, S.A. (2019). Nonezymatic exogenous and endogenous antioxidants. In K. Das et al. (eds.), Free Radicals Medicine and Biology, IntechOpen, London.

Mydin, R. B. S., and Okekpa, S. I. (2018). Reactive Oxygen Species, Cellular Redox Homeostasis and Cancer. in F. Lasakosvitsch, S. D. A. Garnes (eds.), Homeostasis - An Integrated Vision, IntechOpen, London.

Na, T.-Y., Schecterson, L., Mendonsa, A.M., and Gumbiner, B.M. (2020). The functional activity of E-cadherin controls tumour cell metastasis at multiple steps. *PNAS,* 117(11), pg. 5931–5937.

Omara, T., Kiprop, A.K., Ramkat, R.C., Cherutoi, J., Kagoya, S., Nyangena, M., Tebo, T.A., Nteziyaremye, P., Karanja, L.N., Jepchirchir, A., Maiyo, A., Kiptui, B.J., Mbabazi, I., Nakiguli, C.K., Nakabuye, B.V., Koske, M.C. (2020). Medicinal Plants Used in Traditional Management of Cancer in Uganda: A Review of Ethnobotanical Surveys, Phytochemistry, and Anticancer Studies. *Evidence-Based Complementary and Alternative Medicine*, vol. 2020, Article ID 3529081.

Paget, S., 1889. The distribution of secondary growths in cancer of the breast. *The Lancet*, 133(3421), pp.571-573.

Pandya, P., Orgaz, J.L., and Sanz-Moreno, V. (2017). Modes of invasion during tumour dissemination. *Molecular Oncology,* 11(1), pg. 5–27.

Patel, S. (2016). Plant-derived cardiac glycosides: role in heart ailments and cancer management. *Biomedicine & Pharmacotherapy*, 84, pg. 1036–1041.

Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G., and Migliaccio, A. (2020). ROS in cancer therapy: the bright side of the moon. *Experimental & Molecular Medicine,* 52, pg.192–203.

Poltavets, V., Kochetkova, M., Pitson, S.M., and Samuel, M.S. (2018). The Role of the Extracellular Matrix and Its Molecular and Cellular Regulators in Cancer Cell Plasticity. *Frontiers in Oncol*ogy 8:431.

Pott, D.M., Osorio, S., and Vallarino, J.G. (2019). From Central to Specialized Metabolism: An Overview of Some Secondary Compounds Derived From the Primary Metabolism for Their Role in Conferring Nutritional and Organoleptic Characteristics to Fruit. *Frontiers of Plant Science*, vol. 10.

Qian, Q., Chen, W., Cao, Y., Cao, Q., Cui, Y., Li, Y., and Wu, J. (2019). Targeting Reactive Oxygen Species in Cancer via Chinese Herbal Medicine. *Oxidative Medicine and Cellular Longevity*, 2019:924026.

Querzoli, P., Coradini, D., Pedriali, M., Boracchi, P., Ambrogi, F., Raimondi, E., Sorda, R.L., Lattanzio, R., Rinaldi, R., Lunardi, M., Frasson., Modesti, F., Ferretti, S., Piantelli, M., Lacobelli, S., Biganzoli, E., Nenci, I., and Alberti, S. (2010). An immunohistochemically positive E-cadherin status is not always predictive for a good prognosis in human breast cancer. *British Journal of Cancer,* 103, pg. 1835–1839.

Ramakrishna, W., Kumari, A., Rahman, N., and Mandave, P. (2021). Anticancer Activities of Plant Secondary Metabolites: Rice Callus Suspension Culture as a New Paradigm. *Rice Science*, 28(1), pg. 13–30.

Rambwawasvika, H., Dzomba, P., and Gwatidzo, L. (2019). Hair growth promoting effect of *Dicerocaryum senecioides* phytochemicals. *International Journal of Medicinal Chemistry,* Vol. 2019.

Rambwawasvika, H., Parekh, C.T., Naidoo, B., and Chiririwa, H. (2017). Extraction and characterisation of mucilage from the herb *Dicerocaryum senecioides* and its use a potential hair permanent. *International Journal of Applied Chemistry*, 13(3), pg. 691–705.

Ribatti, D., Tamma, R., and Annese, T. (2020). Epithelial-Mesenchymal Transition in Cancer: A Historical Overview. *Translational Oncology*, Vol. 13.

Riggio, A.I., Varley, K.E., and Welm, A.L (2021). The lingering mysteries of metastatic recurrence in breast cancer. *British Journal of Cancer*, 124, pg.13–26.

Rolnik, A., and Olas, B. (2021). The plants of the Asteraceae family as agents in the protection of human health. *International Journal of Molecular Sciences*, 22(6).

Santiago-Medina, M., palmer, T., and Yang, J. (2016). Cancer cell invasion through tissue barriers. *Encyclopedia of Cell Biology*, 3, pg. 223–229.

Schuster, E., Taffat, R., Reduzzi, C., Albert, M.K., Romero-Calvo, I., and Liu, H. (2021). Better together: circulating tumour cell clustering metastatic cancer. *Trends in Cancer*, 7(11), pg. 1020–1032.

Seyfried, T. N., and Huysentruyt, L. C. (2013). On the origin of cancer metastasis. *Critical reviews in oncogenesis*, 18(1–2), pg. 43–73.

Sharifi-Rad, M., Kumar, N.V., Zucca, P., Varoni, E.M., Dini, L., Panzarini, E., Rajkovic ,J., Fokou, P.V., Azzini, E., Peluso, I., Mishra, A., Nigam, M., El Rayess, Y., Beyrouthy, M.E., Polito, L., Iriti, M., Martins, N., Martorell, M., Docea, A.O., Setzer, W.N., Calina ,D., Cho, W.C., and Sharifi-Rad, J. (2020). Lifestyle, Oxidative Stress, and Antioxidants: Back and Forth in the Pathophysiology of Chronic Diseases. *Frontiers in Physiology.* 11:694

Shenoy, A.K., and Lu, J. (2016). Cancer cells remodel themselves and vasculature to overcome the endothelial barrier. *Cancer Lett*, 380(2), pg. 534–544.

Simu, S., Marcovici, I., Dobrescu, A., Malita, D., Dehelean, C.A., Coricovac, D., Olaru, F., Draghici, G.A. and Navolan, Dan. (2021). Insights into the behavior of Triple-Negative MDA-MB-231 breast carcinoma cells following the treatment with 17β-ethinylestradiol and levonorgestel. *Molecules,* 26(9).

Sofowora, A., Ongubodede, E., and Onayade, A. (2013). The role and place of medicinal plants in the strategies for disease prevention. *African Journal of Traditional, Complementary and Alternative Medicines,* 10(5), pg. 210–229.

Song, W., Mazzieri, R., Yang, T., and Gobe, G.C. (2017). Translational Significance for Tumour Metastasis of Tumour-Associated Macrophages and Epithelial-Mesenchymal Transition. *Frontiers in Immunology.* 8:1106.

Steenkamp, V., and Gouws, M.C. (2009). Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer. South African Journal of Botany, 72(4), pg. 630-633.

Street, R.A., and Prinsloo, G. (2012). Commercially important medicinal plants of South Africa: a review. *Journal of Chemistry*, 2013:205048.

Szczurek, E., Krüger, T., Klink, B., and Beerenwinkel, N. (2020). A mathematical model of the metastatic bottleneck predicts patient outcome and response to cancer treatment. *PLoS Computational Biology*, 16(10).

Taddei, M.L., Giannoni, E., Morandi, A., Ippolito, L., Ramazzotti, M., Callari, M., Gandellini, P., and Chiarugi, P. (2014). Mesenchymal to amoeboid transition is

associated with stem-like features of melanoma cells. *Cell Communication and Signaling*, 12:24.

Talkenberger, K., Cavalcanti-Adam, E. A., Voss-Böhme, A., and Deutsch, A. (2017). Amoeboid-mesenchymal migration plasticity promotes invasion only in complex heterogeneous microenvironments. *Scientific reports*, *7*(1), 9237.

Thhoppil, R., and Bishayee, A. (2011). Terpenoids as potential chemoprentive and therapeutic agents in liver cancer. *World Journal of Hepatology*, 3(9), 228–249.

Umadevi, S., Mohanta, G.P., Kalaichelvan, V.K., and Manavalan, R. (2006). Studies on wound healing effect of *Flaveria trinervia* leaf in mice*. Indian Journal of Pharmaceutical Sciences*, 68(1), pg. 106–108

Weitz-Schmidt, G., and Chreng, S. (2012). Cell adhesion assays. Methods Molecular Biology, 757(15–30).

Valastyan, S., and Weinberg, R. A. (2011). Tumour metastasis: molecular insights and evolving paradigms. *Cell*, *147*(2), pg. 275–292.

Valdivia, A., Duran, C., and San Martin, A. (2015). The Role of Nox-Mediated Oxidation in the Regulation of Cytoskeletal Dynamics. *Current Pharmaceutical Design*, 21(999).

Van Ziji, F., Krupitza, G., and Mikulits, W. (2011). Initial steps of metastasis: Cell invasion and endothelial transmigration. *Mutation Research*, 728(1–2), pg. 23–34.

Vella, V., De Francesco, E.M., Lappano, R., Muoio, M.G., Manzella, L., Maggiolini, M., and Belfiore, A. (2020). Microenvironmental Determinants of Breast Cancer Metastasis: Focus on the Crucial Interplay Between Estrogen and Insulin/Insulin-Like Growth Factor Signaling. *Frontiers in Cell Developmental Biology.* 8:608412.

Weiss, L., and Ward, P. M. (1983). Cell detachment and metastasis. *Cancer metastasis reviews*, *2*(2), pg.111–127.

Wendt, M. K., Taylor, M. A., Schiemann, B. J., and Schiemann, W. P. (2011). Down-regulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer. *Molecular biology of the cell*, *22*(14), pg. 2423–2435.

Yang, L., Wen, K,-S., Ruan, X., Zhao, Y,-X., Wei, F., and Wang, Q. (2018). Response of Plant Secondary Metabolites to Environmental Factors. *Molecules,* 23(4), 762.

Youness, R., Kamel, R., Elkasabgy, N., Shao, P., and Farag, M. (2021). Recent advancesin tannic acid (gallotannini) anticancer activities and drug delivery systems for efficacy improvement; a comprehensive review. *Molecules,* 26(5): 1486.

Yu, H., Shen, Y., Hong, J., Xia, Q., Zhou, F., and Liu, X. (2015). he contribution of TGF-β in Epithelial–Mesenchymal Transition (EMT): Down-regulation of E-cadherin via snail. *Neoplasma*, 62(1).

Zavyalova, M.V., Denisov, E.V., Tashireva, L.A., Savelieva, O.E., Kaigorodova, E.V. Krakhmal, N.V., and Perelmuter, V.M. (2019). Intravasation as a key step in cancer metastasis. *Biochemistry (Moscow*), 84, pg. 762–772.