Phytochemical analysis of *Momordica cardiospermoides* crude acetone and methanol leaf extracts and their effects on MDA-MB-231 cell migration and invasiveness

by

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DECLARATION

I declare that the study entitled **Phytochemical analysis of *Momordica cardiospermoides* crude acetone and methanol leaf extracts and their effects on MDA-MB-231 cell migration and invasiveness** is my work. This report is being submitted for the degree of Master of Science in Biochemistry at the University of Limpopo. This report has not been submitted to any other University and I further declare that all sources quoted are indicated and acknowledged using a comprehensive list of references.

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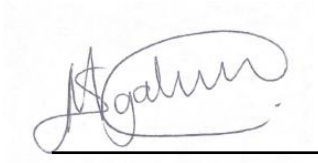
Surname and Initials

20 October 2021

Date



Signature

DEDICATION

I would like to dedicate this work to **myself**. Completing this has been the most taxing thing I had to do in my entire academic life. I have learned a lot about myself and have grown immensely through this process. It’s been a long fight and I just had to have a warrior’s spirit.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CEF	Chloroform: ethyl acetate: formic acid
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
EMW	Ethyl acetate: methanol: water
FBS	Foetal bovine serum
MMPs	Matrix metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
TIMPs	Tissue inhibitor of metalloproteinases
TLC	Thin layer chromatography
TSP	Thrombospondin
uPA	Urokinase-type plasminogen activator
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

ABSTRACT

Drug discovery from medicinal plants continues to play an important role in the development of anticancer agents, this is because medicinal plants are reservoirs of bioactive compounds that exert a plethora of pharmacological effects on human beings. This study aimed to analyse the phytochemical constituents of the *Momordica cardiospermoides* crude acetone and methanol leaf extracts as well as investigate their potential anti-metastatic effects on the MDA-MB-231 breast cancer cell line. *Momordica cardiospermoides* leaves were extracted with absolute methanol or acetone to produce crude methanol and acetone extracts, respectively. The extracts were then screened and analysed for phytochemicals using thin layer chromatography, qualitative and quantitative phytochemical tests, and their antioxidant activity was determined using the quantitative 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. The fingerprint profiles of the *M. cardiospermoides* leaf extracts revealed that compounds of the acetone extracts were optimally separated in the nonpolar mobile phase (TAE), whereas those of the methanol extract separated best in the polar mobile phase (EMW), thereby suggesting that the crude acetone and methanol extracts had more non-polar and polar compounds present, respectively. Furthermore, the qualitative phytochemical analysis indicated the presence of various phytochemicals such as flavonoids, steroids, coumarins, and tannins in both plant extracts, however, saponins were found present in the methanol extract and not in the acetone extract. Moreover, quantification of major phytochemicals revealed that the acetone extract had the highest total phenolic content (23.0683 mg GAE/g), total tannin content (22.0442 mg GAE/g) and total flavonoid (32.6933 mg QE/g) content as compared to the methanol extract (14.2349 mg GAE/g, 11.3164 mg GAE/g and 7.692 mg QE/g respectively). The DPPH free radical scavenging activity assay revealed that the extracts exhibited an increase in percentage inhibition/ DPPH scavenging effect, with an increase in extract concentration. The results also revealed that the acetone extract possessed a higher radical scavenging activity as compared to the methanol extract. These results are in correlation with the quantitative analysis of the extracts, as all the major phytochemicals found in higher amounts in the acetone extract have antioxidant properties. The extracts were then assessed *in vitro* for their cytotoxic effects on MDA-

MB-231 breast cancer cells and HEK 293 cells using the cell count and viability assay and the results obtained revealed a concentration-dependent decrease in the viability of MDA-MB-231 cells at 24 hours of treatment with either the acetone or methanol extract. Comparatively, treatment of HEK 293 cells with the acetone extract resulted in a significant decrease in the percentage of viable cells, whereas treatment with the methanol extract had no significant effect on the viability of HEK 293 cells, as the percentage of viable cells was maintained at 85–98% at 24 hours of treatment. These results also revealed that the methanol extract is more selective to cancer cells in comparison to the acetone extract, suggesting that the methanol extract is a better antineoplastic candidate. The mode of cell death induced by the methanol or acetone extracts was assessed using the acridine orange and ethidium bromide dual staining assay and the annexin V and dead cell kit. The results from the acridine orange/ethidium bromide dual staining assay showed that both extracts induced nuclei and cellular morphological changes in a concentration-depended manner, at 24 hours of treatment. Moreover, the annexin V and dead assay kit results revealed that the acetone extract induced necrotic cell death, while the methanol extract induced apoptotic cell death. Since the acetone extract was shown to be non-selective towards normal cells and induced necrotic cell death, it was discontinued for further assays. The effect of the methanol extract on MDA-MB-231 cell migration and attachment was determined using the wound healing assay and the adhesion assay. The results revealed that treatment with 150 or 300 µg/ml significantly suppressed MDA-MB-231 cell migration, associated with serpin E1 downregulation and TIMP-1 upregulation, at 24 hours of treatment. Moreover, treatment with the methanol extract also significant inhibited MDA-MB-231 cell adhesion in a concentration-dependent manner, as evident by the decrease in the number of crystal violet stained cells. The effect of the methanol extract on the expression of matrix metalloproteinase-2 and -9 was assessed using western blotting, and the results revealed that the extract significantly downregulated the expression of both MMP-2 and -9, suggesting that the methanol extract has inhibitory effects on MDA-MB-231 cell invasion. The human angiogenesis antibody array kit was then used to determine the effect of the extract on the expression of angiogenesis-related proteins. Treatment with 150 or 300 µg/ml of the extract significantly upregulated the expression levels of tissue inhibitor of metalloproteinases (TIMP) -1 and thrombospondin-1 in a concentration-dependent manner. The results also revealed a significant downregulation in the expression of serpin E1, in a

concentration-dependent manner, in comparison to the untreated control. However, the expression of uPA, VEGF, and IGFBP-1, 2 and -3 was upregulated following treatment with 150 and 300 µg/ml of the extract. In conclusion, the current study demonstrated the potential of *M. cardiospermoides* crude methanol extract as an effective anti-metastatic agent or a source of compounds with anti-metastatic properties.

CHAPTER 1

INTRODUCTION

According to the International Agency for Research on Cancer (IARC) report of 2018, there has been an estimated 18.1 million new cancer cases and 9.6 million deaths in 2018 (Ferlay *et al.,* 2019). This means that cancer continues to be a burden to communities around the globe. Breast cancer is the second commonly diagnosed cancer type worldwide, following lung cancer. In 2018 alone, there was a reported 2,088,849 (11.6%) new breast cancer cases and 626,679 (6.6%) cancer deaths (Bray *et al.,* 2018). In 2014, the Cancer Association of South Africa reported that breast cancer accounted for 22.28% of all cancer cases (DeSantis *et al.,* 2015). Although these statistics have not been updated, the incidence of breast cancer in the year 2020 is estimated to be much higher.

Breast cancer often exhibits inclined metastasis towards distant sites including lung, liver, bone, and brain, which may result in varied responses to treatment and patient prognosis (Singletary *et al.,* 2003). In solid tumour cancer, drug resistance is a common cause of treatment failure that results due to mutations that may occur in the target. Moreover, solid tumours are often accompanied by local invasion and migration. Candidate drugs for solid tumours are predominantly evaluated based on their ability to induce tumour shrinkage (Gandalovia *et al.,* 2017), which is rarely sustained and is not predictive of an anti-metastatic effect. Treatment strategies for metastatic breast cancer take into consideration its specific type, the parts of the body to which it has spread as well as patient preferences (daCosta *et al.,* 2014). Widely used chemotherapeutic drugs, although very effective, are not devoid of their intrinsic problems. For instance, cyclophosphamide, a chemo drug used to treat many malignant conditions has been reported to have bladder toxicity in the form of haemorrhagic cystitis, immunosuppression and at higher doses cardiotoxicity (Srivastava *et al.,* 2019; Ogino and Tadi, 2020). For that reason, treatments of solid tumours need to be complemented with drugs that not only have lessened side effects but those that can inhibit the cancer cell's ability to invade through the extracellular matrix and establish secondary tumours elsewhere. These drugs are termed migrastatics (Gandalovia *et al.,* 2017). This is because the ability to invade and metastasise is a hallmark for cancer that distinguishes malignant from benign tumours.

Therefore, deepened research on the molecular heterogeneity of metastatic breast cancer, which is a cause of a significant number of therapeutic failures, will grant the ability to explore more effective metastasis-targeting agents. Despite the development of anticancer drugs and/or treatment strategies, such as oestrogen antagonists (Anthony 2006), radiation or chemotherapy, that shrink or attenuate solid tumour growth, no treatment that permanently eradicates metastasis exists at present (Meirson *et al.,* 2018). The ability of these treatments to reduce tumour size is indicative of their ability to only target proliferating cells. This approach does not prevent the initial invasive process and dissemination of tumour cells from the primary tumour (Crane *et al.,* 2002). There is a need for the development of anti-metastatic agents or strategies that will interfere with one or more of the processes, such as cell migration and invasiveness, involved in the spread of cancer cells.

Drug discovery from medicinal plants continues to play an important role in the development of anticancer agents, as several agents derived from plant secondary metabolites have been applied towards combating cancer (Balunas and Kinghorn, 2005). In essence, out of all available anti-cancer drugs between 1940 and 2002, 40% are natural products or derivatives thereof (Newman *et al.,* 2003). This is because plants are reservoirs of bioactive compounds such as tannins, alkaloids, flavonoids, and phenolic compounds (Jasuja *et al.,* 2012; Koul and Walia, 2009). Most of these plant-based compounds have shown to possess therapeutic effects such as anticancer (pro-apoptosis, anti-carcinogenic, anti-mutagenic, anti-angiogenesis), anti-inflammatory and anti-bacterial (Silva *et al.,* 2006; Rafieian-Kopaei, 2012).

Momordica cardiospermoides, commonly known as ntwe (Setswana) or inshubaba (siSwati) is a plant species found abundantly in eSwatini and the Limpopo and Mpumalanga provinces of South Africa (Banana and Mozambique*,* 2010). Although this plant is traditionally used to treat a variety of diseases including diabetes (Behera *et al.,* 2011), there is no reported scientific data on the plant's phytochemical composition, antioxidant activity and anticancer activity. Therefore, the current study aims to analyse the phytochemicals present in the acetone and methanol extracts of *Momordica cardiospermoides* leaves, as well as investigate its potential anticancer effect on MDA-MB-231 breast cancer cells.

CHAPTER 2
LITERATURE REVIEW

2.2. Metastasis

Metastasis is a complex, multistep and multifunctional cascade through which cancer cells spread from primary sites to distant secondary anatomical sites (Valastyan *et al.,* 2011) (**Figure 2.1)**. Initiation of the metastatic cascade relies on tumour cells undergoing an epithelial-mesenchymal transition (EMT). Through transitioning to a mesenchymal phenotype, tumour cells acquire the ability to detach from adjoining tumour cells and expand migratory and invasive capacities (Ribatti *et al.,* 2020). This multistep process also encompasses transendothelial migration of tumour cells into vessels known as intravasation, survival in the circulatory system by acquiring platelet shielding and immunosuppression by negating the activity of suppressor immune cells, such as natural killer (NK) cells and extravasation (exit from the circulatory system) (Majidpoor and Mortezaee*,* 2021). Once tumour cells exit the circulatory system, a reverse transition from mesenchymal-epithelial phenotype (MET) occurs. Subsequently, tumour cells proliferate in the competent organs leading to colonisation. The cells grow and survive at the secondary site by stimulating the formation of new blood vessels (angiogenesis) for nutrients and oxygen supply, as well as for waste excretion (Bersini *et al.,* 2014; Rankin *et al.,* 2016).

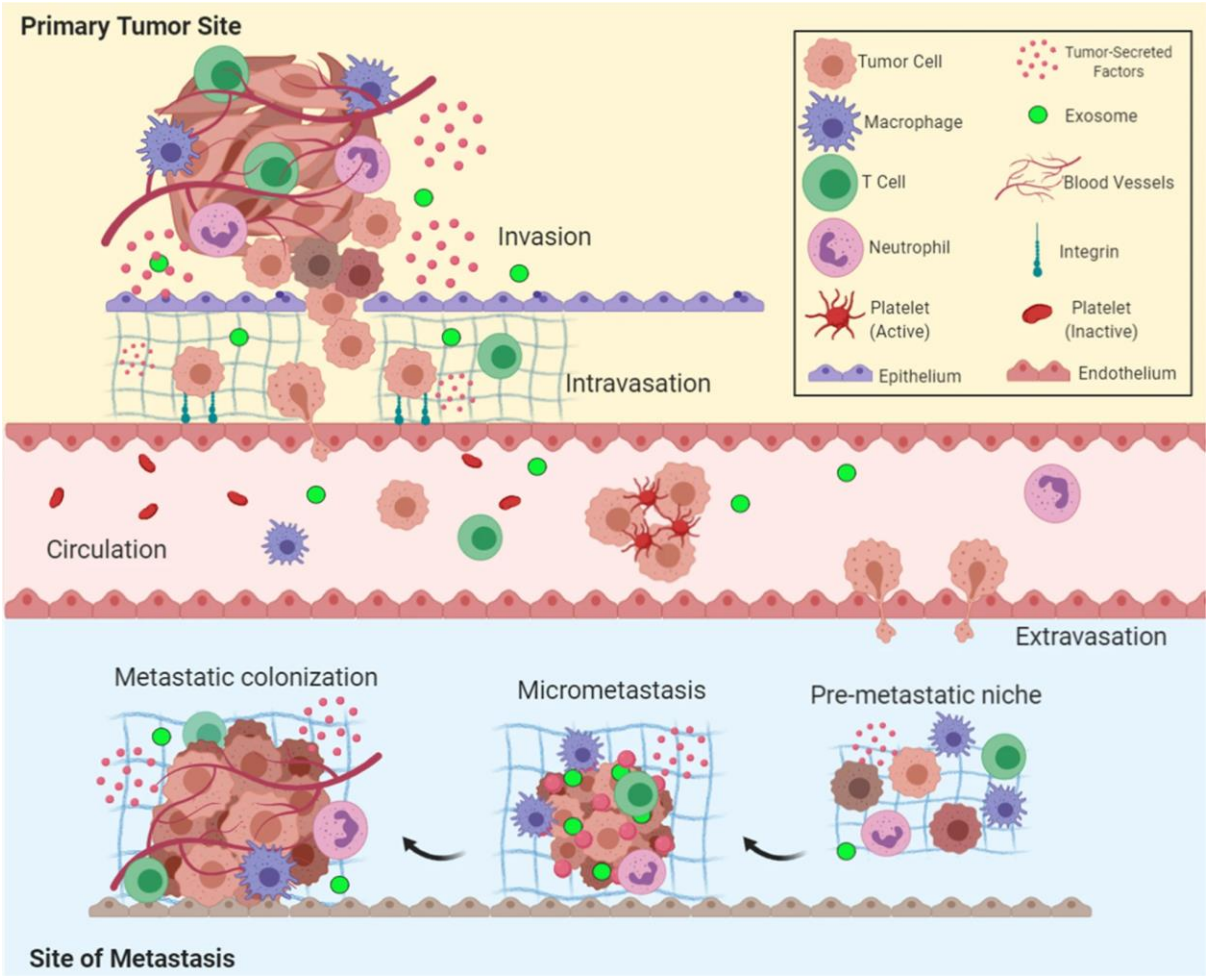


Figure 2.1: Schematic representation of the metastatic cascade. Tumour cells detach from the primary site and invade the surrounding tissues. They then infiltrate the blood or lymphatic system (intravasation). In the vasculature, tumour cells survive by association with immune cells (lymphocytes and platelets) and attachment to the endothelial lining. The tumour cells then exit the capillary beds into the parenchyma of the secondary site/organ (extravasation), they induce the formation of new blood vessels for nutrient supply and proliferation and colonisation (Lahdenranta 2004).

2.1.1. Epithelial to mesenchymal transition

Progression towards malignancy in many carcinomas is accompanied by the loss of epithelial differentiation and a shift towards a mesenchymal phenotype. This morphological transition known as epithelial-mesenchymal transition (EMT) is a reversible process that is considered a pre-requisite to cancer metastasis (Gloushankova *et al.,* 2018) and describes a transition in which epithelial cells differentiate into a highly mobile and invasive mesenchymal phenotype (Voulgari and

Pintzas, 2009). Epithelial to mesenchymal transition is observed during normal embryonic development (Eastham *et al.,* 2007; Marcucci *et al.,* 2016), wound healing and tissue inflammation (Heinrich *et al.,* 2012) in response to stimuli including cytokines, hypoxia and growth factors that are secreted to the microenvironment (Gonzalez and Medici, 2016). However, tumour cells that undergo EMT become resistant to chemotherapy and acquire the ability to suppress an immune response. Physiologically, epithelial cells display apical-basal polarity and are held together laterally by adherens and tight junctions, the former of which are formed on the surface of epithelial cadherin (E-cadherin) molecules (Dongre and Weinberg, 2019). Consequent to EMT activation, the expression of E-cadherin molecules is repressed resulting in the loss of the typical morphology of epithelial cells. The cells acquire a mesenchymal morphology and express markers such as neural cadherin (N-cadherin), vimentin and fibronectin, associated with the mesenchymal cell state (Mrozik *et al.,* 2018). Furthermore, EMT activation also results in the loss of cell polarity, disruption of cell-cell and cell-ECM adhesion, basement membrane degradation and the remodelling of the extracellular matrix by matrix metalloproteinases (Polyak and Weinberg, 2009; Iwatsuki *et al*., 2010).

2.1.2. Extracellular matrix remodelling and matrix metalloproteinases

The extracellular matrix is a structural support network made up of diverse proteins that play a vital role in tissue architecture and homeostasis (Sonbol 2018). Numerous proteinaceous components contribute to the specialized structure of the ECM, such as collagens that are produced and secreted by various stromal cells, fibroblasts, and the basement membrane, including laminin, entactin, and various growth factors and proteases (Popharitov *et al.,* 2015; Meyer 2019). These components help in maintaining tissue integrity, regulate cell migration and invasion, and provide a reservoir of growth factors and cytokines. Remodelling of the ECM requires the synthesis and deposition of ECM components and their proteolytic breakdown. Several proteases have been implicated in ECM degradation, most prominent among which are members of the matrix metalloproteinase (MMP) family (Winer *et al.,* 2018).

Matrix metalloproteinases are a group of zinc-dependent endopeptidases that are involved in the disruption of the extracellular matrix (Xue and Jackson, 2015). In cancer, MMPs support several tumourigenic processes including cell proliferation,

tumour cell invasiveness and angiogenesis (Conlon *et al.,* 2019). They also partake in regulating non-matrix targets such as cytokines, chemokines, cell-cell adhesion molecules, cell surface receptors etc. The structure of MMPs consists of three domains viz. pro-peptide, catalytic and hemopexin-like domains. The pro-peptide domain contains cysteine-switch motifs that are responsible for keeping the MMPs in inactive forms (Visse and Nagase*,* 2003). Therefore, a catalytic cleavage is necessary to activate the enzymes through disruptions of the bonds between cysteine in the pro-peptide domain and zinc ion in the catalytic domain. The catalytic domain has a zinc-binding site and methionine residue which chemically supports zinc-binding. Lastly, the C-terminal hemopexin-like domain is linked by a flexible proline-rich hinge region to the catalytic domain (Visse and Nagase 2003; Nagase *et al.,* 2006). The structure of the hemopexin-like domain supports several activities including substrate specificity and activation, inhibition and dimerisation (Morgunova *et al.,* 2002).

Matrix metalloproteinases are classified into seven groups based on substrate organisation or domain organisation: collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and -26), membrane-type MMPs (MMP-14, -15, -16, -17 and -24), metalloelastase (MMP-12) and other non-classified MMPs (Yadav *et al.,* 2014; Cui *et al.,* 2017). Matrix metalloproteinases -2 and -9 are the two most extensively studied MMPs and are primarily involved in the remodelling of the extracellular matrix and degradation of basement membrane components for successful tumour cell invasion (Stamenkovic 2003; Folgueras *et al.* 2004; Martins *et al.,* 2020). Matrix metalloproteinase-2 (gelatinase A) is a 72 kDa type IV collagenase that is strongly expressed in tumour and stromal cells. It is synthesised as a 631 amino acid pro-enzyme and its activation involves the removal of the first 80 amino acids (Henriet and Emonard*,* 2019). On the other hand, matrix metalloproteinase-9 (gelatinase B) is an 84 kDa gelatinase that is expressed in some normal cells as well as in tumour cells (Ugarte-Berzal *et al.,* 2019). These two gelatinases are distinguished from other MMPs by a fibronectin-type II domain within the catalytic domain, the presence of which influences the size and shape of the active site and, therefore, substrate specificity. The fibronectin domains render MMP-2 and MMP-9 the ability to bind to denatured collagen type I (gelatin), thereby, facilitating its cleavage (Johansson *et al.,* 2000; Hannocks *et al.,* 2019). Matrix metalloproteinase-2 and -9 influence collagen remodelling by inducing the degradation

of extracellular matrix components such as collagen (I, IV, V, Vll, X, XI), elastin, tenascin and fibronectin as well as collagen (I, III, IV, V), vitronectin, entactin, pro-TNF-α and elastin, respectively (Pardo and Selman*,* 2006; Wang *and Khalili,* 2018). The ability of these enzymes to remodel the ECM enables cancer cells to migrate through and invade neighbouring structures, and enter into the circulatory system into the bloodstream (intravasation) or move out of a blood vessel into the tissue to form a secondary tumour (extravasation) (Rua and McGavern*,* 2015; Ireland 2018).

Matrix metalloproteinase expression is regulated by naturally occurring inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). To date, four TIMPs have been characterised in humans and designated as TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (Kathamuthu *et al.,* 2020). These are 21-23 kDa proteins possessing 12 conserved cysteine residues. Although they all inhibit MMPs proteolytic activity, they differ in several aspects including interaction with proenzymes (pro-MMPs), solubility, and regulation of expression (Brew *et al.,* 2000; Nagase, *et al.,* 2006; Vandenbroucke and Libert*,* 2014). Tissue inhibitor of metalloproteinase-3 is tightly bound to the matrix while TIMP-1, TIMP-2, TIMP-4 are present in their soluble forms. Tissue inhibitor of metalloproteinase-1, which has shown a cytokine-like effect, forms a preferential complex with pro-MMP-9 (Clarke *et al.,* 2015), whereas TIMP-2 associates with MMP-2 at the cell surface to regulate its activity (Cheng *et al.,* 2017). On the other hand, TIMP-3 and TIMP-4 can bind and inhibit the activity of either pro-MMP-2 or pro-MMP-9 (Verstappen and Von den Hoff*,* 2006; Jackson *et al.,* 2017). The MMPs inhibitory activity of TIMPs has been evidenced in tumour progression *in vitro* as well as *in vivo*. Downregulation of both TIMP-1 and TIMP-2 expression is associated with increased invasiveness (i.e. higher levels of MMPs). However, increased TIMPs expression may denote a stromal response to tumour invasion and could reflect host response, leading to the control of MMPs activities and maintenance of the ECM integrity. Tissue inhibitors of matrix metalloproteinases have also shown involvement in angiogenesis (Bajbouj *et al.,* 2021). This was first demonstrated in chick embryo yolk-sac membranes where polyamines-induced angiogenesis was inhibited by TIMP-1 and TIMP-2 (Honkavuori-Toivola 2014). Furthermore, TIMP-1 overexpression HepG2 cells were reported to exhibit increased migration by chemotaxis, which can be reduced by MMP-2 and -9 inhibition (Ordonez *et al.,* 2014).

2.1.3. Tumour cell migration

Cell migration is defined as the movement of individual cells, cell sheets, and clusters from one location to another, which is fundamental to establishing and maintaining the proper organisation of multicellular organisms (Van Helvert *et al.,* 2018). The cell migration process is central to a variety of physiological processes that are essential for proper immune response, wound repair, and tissue homeostasis, while aberrant cell migration is found in various pathologies including metastatic cancer. The movement of cells from a primary tumour to the secondary site occurs by a simple form of locomotion known as chemotaxis, which transpires in response to chemoattractant gradients (Roussos *et al.,* 2011). During chemotaxis, cells enter a motility cycle where they polymerise new actin-rich filaments and extend protrusions which direct their movement towards a chemoattract (Bailly *et al.,* 1998; Weijer *et al.,* 2020). The process is primarily regulated by the formation of; F-actin stress fibres (that provide a force for movement), filopodia and lamellipodia (dictate directional movement) (Parker *et al.,* 2002; Innocenti 2018) and focal adhesions (attachment points required for movement) (Xia *et al.,* 2008). Many cell types migrate as individual cells (making use of amoeboid or mesenchymal-type movement) or as cell sheets, clusters and strands using collective migration (Friedl and Wolf*,* 2003). Collective cell migration is the synchronised movement of multiple cells that retain cell-cell adhesion while protruding along or within tissues (Mayor and Etienne-Manneville *,* 2016). Coherent groups of cells are held together by cell-cell adhesion molecules including cadherins and integrins. This type of cell migration can be seen in the migration of endothelial cells and epithelial cells, for example, in wound healing and angiogenesis (Van Helvert *et al*., 2018). On the other hand, amoeboid migration is a mode of rapid motility driven by actin-rich pseudopods and highly contractile uropods (Lammermann and Sixt*,* 2009). It mimics features of the single-cell behaviour of the amoeba *Dictyostelium discoideum*, an ellipsoid cell with fast deformability that translocates through rapidly alternating cycles of morphological expansion and contraction (Friedl *et al.,* 2001). This type of locomotion can be seen in leukocytes as well as in cancers arising from connective tissue cells, thus leading to their higher rate of metastasis (Mierke 2013). In contrast to cells that use amoeboid migration, mesenchymal migration is characterised by cell polarisation to form a leading-edge protrusion such as lamellipodia, which leads to adhesive interactions with the substratum (i.e. a surface to which a cell attaches) and proteolytic extracellular matrix remodelling,

followed by retraction of the contractile cell rear to achieve cellular movement (Talkenberger *et al.,* 2017).

2.2.4. Tumour cell adhesion

Metastatic tumour cells need to interact with the extracellular matrix at the new anatomical site to form a secondary tumour, this process is known as cell adhesion (Koller and Papoutsakis*,* 2018). During this process, tumour cells revert to the epithelial phenotype (mesenchymal-to-epithelial transition or MET) to regain epithelial cell-to-cell junctions, proliferate and become a clinically relevant and detectable tumour mass. These interactions are facilitated by cell adhesion molecules (CAM) composed of intracellular, trans-membrane and extracellular domains. Cell adhesion molecules are categorised as either calcium-independent (e.g. lymphocyte homing receptor) or most prominently, calcium-dependent (e.g. selectins and integrins) (Bian 2013). Selectins are vascular adhesion molecules that mediate physiological responses such as immunity, inflammation, wound healing and homeostasis (Borsig 2018). During cancer progression, selectins promote various steps enabling the interactions between tumour cells and blood constituents such as platelets, endothelial cells and leukocytes (Noh *et al.,* 2019), following intravasation. Numerous studies have shown that increased selectin ligand expression on tumour cells correlates with enhanced metastasis and poor prognosis for cancer patients (Borsig 2018). Integrins are a large family of homologous cell surface receptors that integrins adhere to extracellular matrix glycoproteins such as laminins and collagens in basement membranes or connective tissue components like fibronectin to promote cell-substratum adhesion, whereas others recognise integral membrane proteins of immunoglobin family on other cells and mediate cell-cell adhesion (Rizwan *et al.,* 2019). Integrin-mediated adhesion is evident in physiological processes such as embryonic development and maintenance of tissue organisation (Klose and Artis*,* 2016), wound healing (Bielefeld *et al.,* 2013) and inflammation (Sorokin 2010). Integrins are also involved in tumour cell penetration of adjacent extracellular matrices and blood vessels, the interaction of tumour cells with platelets and leukocytes in the circulation, as well as arrest and extravasation in the target organ (Cooper *et al.,* 2019).

2.2.5. Angiogenesis

For successful metastasis to occur, extravasated tumour cells need to acquire the ability to survive in the foreign microenvironment that they encounter in the parenchyma of distant tissues (Lambert *et al.,* 2017). Microenvironment differences such as types of stromal cells, available growth factors and cytokines and the microarchitecture of the tissue itself affect the ability of the cells to adapt to the new site (Peinado *et al.,* 2011). Therefore, tumour cells often establish a 'pre-metastatic niche' until the microenvironment becomes hospitable for tumour cells to colonize (Liu, 2016). Following successful seeding in the pre-metastatic niche, tumour cells interact with their environment to promote their growth. These interactions are necessary to evade immune destruction, activate growth signalling, and gain access to nutrients that support their proliferation (Doglioni *et al.,* 2019).

Angiogenesis is a process by which tumour-associated neo-vessels sprout from existing blood vessels (Olsen *et al.,* 2017). It is broadly involved in several physiological events such as wound healing, reproductive cycle, and skeletal morphogenesis, however, it also occurs in several pathological conditions including chronic inflammation and cancer (Wyss *et al.,* 2015a). The formation of new blood vessels is crucial to support growing tumours with oxygen and nutrients and to allow for recirculation of immune surveillance cells (Wyss *et al.,* 2015b). Angiogenesis eventuates by sprouting, migration and proliferation of endothelial cells from pre-existing blood vessels. Numerous crucial pro-angiogenic factors have been identified such as vascular epithelial growth factor (VEGF), urokinase-type plasminogen activator (uPA) and serpin E1 (Zheng *et al.,* 2013; Su *et al.,* 2016; McHale *et al.,* 2019). Inhibitors of angiogenesis (anti-angiogenic factors) can either be direct or indirect (Gacche and Meshram, 2014). Direct angiogenesis inhibitors, such as endostatin and angiostatin, act directly on the endothelial cell by blocking the motility and proliferation of the cell regardless of the stimulator. Contrary, indirect angiogenesis inhibitors, including antibodies to growth factors like anti-VEGF, block growth factors or pathways of cells other than epithelial cells (Bielenberg and Zetter, 2015). The switch to the angiogenic phenotype in specific physiological and pathological conditions is determined by the balance between pro- and anti-angiogenic factors. The angiogenic switch is a hallmark of malignant tumour progression (Baeriswyl and Christofori, 2009; Kazerounian and Lawler, 2018).

2.4. Reactive oxidative species and Cancer

Reactive oxygen species (ROS) are small chemically reactive species containing oxygen (Mates 2020). These species are present in the environment and some are produced by metabolic activity within cells triggered by endogenous sources such as cytokines and growth-factor receptors (Sies and Jones, 2020). Maintaining homeostasis between oxidants and antioxidants is crucial to sustaining optimal physiological conditions. When an equilibrium lacks between the concentration of ROS and the activation of antioxidant mechanisms, a pathological condition known as oxidative stress occurs (Khansari *et al.,* 2009). At moderate concentrations, free radicals regulate cell-signalling cascades. However, at high concentrations, they are reported to induce DNA damage (Davalli *et al.,* 2018), lipid peroxidation (Yadav and Ramana, 2013), and protein modification resulting in the disruption of signal transduction (Brown *et al.,* 2015), which may lead to cancer (Rani *et al.,* 2016). Depending on the quantity of the disturbance, normal cells can either restore equilibrium or activate pathways of cell death such as apoptosis. On the other hand, cancer cells sustain a proliferative and aggressive phenotype by exploiting this phenomenon. This is because the overproduction of ROS or its reduced disposal influences hallmarks of cancer including invasion and angiogenesis (Greenhough *et al.,* 2009; Pavlova and Thompson, 2016). Antioxidants, which can be obtained from food or medicinal plants containing phytochemicals such as phenolic compounds are essential in the prevention of oxidative stress (Zhang *et al.,* 2015). These compounds may protect cellular systems from oxidative damage, which may in turn lower the risk of chronic diseases such as cancer (Bhattacharya 2015).

2.5. Medicinal plants and their role in cancer treatment

Many chronic diseases, including cancer, have been shown to develop as a result of the production and accumulation of free radicals or reactive oxygen species (Rafieian-Kopaei *et al.,* 2013). Medicinal plants can, therefore, be used in the treatment or prevention of these diseases mainly because they are a source of natural antioxidants, are readily available, and are inexpensive (Xu *et al.,* 2017; Akbar *et al.,* 2018). The medicinal effect of plants is attributed to their metabolites, especially secondary metabolites. Secondary metabolites are synthesized from primary metabolites (sugars, lipids, proteins) through processes such as methylation, glycosylation, and

hydroxylation, and may be activated when plants are under environmental stress due to the limitation of nutrients, etc. (Kasote *et al.,* 2015).

In humans, plant secondary metabolites have been shown to protect against the build-up of free radicals, to have anti-aging, anti-inflammatory, and cholesterol-lowering properties as well as fight against infections caused by fungi, bacteria, and some viruses (Mamabolo 2018). The therapeutic relevance of secondary metabolites is attributed to their structural variations and chemical components. Among the great structural diversity of phytochemicals, phenolic compounds have attracted considerable attention for their wide variety of bioactivities. Phenolics are compounds possessing one or more hydroxyl groups with more than 8000 structural variants. In plants, phenolic compounds act as a defence mechanism against pathogens, parasites and predators, as well as contribute to the colour of plants (Dai and Mumper*,* 2010). In humans, phenolic compounds possess complementary and overlapping mechanisms of action including antioxidant activity, regulation of gene expression of proto-oncogenes and tumour suppressor genes (such as p53), modulation of carcinogen metabolism, induction of apoptosis through cell-cycle arrest, inhibition of signal transduction pathways including nuclear factor-kappa-B and activator protein 1; suppression of angiogenesis and many more (Chen and Kong*,* 2005; de Kok *et al.,* 2008; Huang *et al.,* 2009; Khan *et al.,* 2020). Phenolics are generally categorised as phenolic acids, tannins, flavonoids, coumarins and others, based on the number of phenolic rings and structural elements that link these rings (Cai *et al.,* 2004).

Phenolic acids are the largest group of secondary metabolites produced by plants. They are classified based on parameters such as hydroxyl groups, aromatic rings and substitutes present in the carbon skeleton, and most phenolic compounds possess one or more hydroxyl groups attached to an aromatic hydrocarbon chain (Vermerris and Nicholson *et al.* 2007; Shahidi and Ambigaipalan*,* 2015). Predominant phenolic acids include hydroxycinnamic acids (e.g. ferullic acid and caffeic acid) and hydroxybenzoic acids (e.g. vanillic acid and gallic acid) (Lee *et al.* 2013). This group of secondary metabolites has been reported to have health-promoting properties to humans such as antioxidants, anti-inflammatory, anti-hypertensive, antimicrobial and anti-cancer (Alhakmani *et al.,* 2013). Major types of phenolics include phenols, coumarins, tannins, flavonoids (Luna-Guevara *et al.,* 2018).

Flavonoids are naturally occurring glycosides that are water-soluble and possess more than one hydroxyl group substituted into the aromatic ring (Kumar and Pandey, 2013). The main subclasses of flavonoids are flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins (Hui *et al.,* 2013). Quercetin, a flavonol, is one of the major dietary flavonoids found in a broad range of fruits, vegetables, and beverages. Evidence has shown that long term intake of herbs or food containing flavonoids has positive effects on incidences of type II diabetics (Bi *et al.,* 2017) and cardiovascular diseases (Zarfeshany *et al.,* 2014) as they possess properties such as antioxidant activity, anti-inflammation and anti-microbial activity (Griffiths *et al.,* 2016). Furthermore, compelling data from epidemiological investigations, laboratory studies and human clinical trials indicate that flavonoids have important effects on cancer chemoprevention and chemotherapy (Chahar *et al.,* 2011). Numerous mechanisms of action have been identified and include anti-proliferation, carcinogen inactivation, cell cycle arrest, induction of apoptosis and differentiation, angiogenesis inhibition and multidrug resistance reversal (Tosetti *et al.,* 2002; G Mercader *et al.,* 2012; Pratheeshkumar *et al.,* 2012).

Tannins are also water-soluble polyphenolic compounds and are capable of forming complexes with nucleic acids, minerals, and polysaccharides. They are located in the bark, root, stem and outer layers of the plant tissue (Ashok and Upadhyaya, 2012). Tannins are divided into two main groups viz. hydrolysable (casuarictin) and condensed tannins (proanthocyanidins) (Ekambaram *et al.,* 2016). Many plants used in traditional Chinese medicine for the treatment of cancer have been reported to contain high tannin constituents. Furthermore, tannins are the most abundant and multipotent molecules exhibiting antioxidant, proapoptotic and anti-angiogenic activity, and are therefore effective in the treatment of degenerative diseases including initiation and progression of tumours (Rekha *et al.,* 2015). Tannin-rich medicinal plants are considered toxic to fungi, yeast and bacteria and have therefore been traditionally used as healing agents in illnesses such as diarrhoea and leucorrhoea (Doughari 2012).

Drug discovery from medicinal plants continues to play a vital role in the development of anticancer agents, as several agents derived from plant secondary metabolites

have been applied towards combating cancer (Khan *et al.,* 2019). Moreover, novel natural products provide opportunities for innovation in drug discovery. A considerable number of anti-tumour agents currently used are of natural origin. For instance, over half of internationally approved anti-cancer prescription drugs between 1940 and 2006 were natural products or their derivatives (Behzad *et al.,* 2014; Tewari *et al.,* 2019), this is because plant-derived therapeutic drugs are highly effective, possess low toxicity, and have a minor environmental impact.

2.5.2. *Momordica cardiospermoides*

The genus *Momordica* comprises 60 recorded species, 47 of which are found in Africa (Schaefer and Renner, 2010). Species belonging to this genus have been shown to have therapeutic properties. *Momordica charantia* has been found to have some therapeutic activities and has been used in traditional medicine since ancient times (Grover and Yadav, 2004). It has been used and recommended for centuries in many parts of the world to treat diabetes and other diseases, including cancer (Raman and Lau, 1996; Real 2011). Another related plant species that is under extensive research in our laboratory is *Momordica balsamina* and findings suggests anti-metastatic properties against HT-29 colon cancer cells (Serala *et al*, 2021).

Momordica cardiospermoides (formerly known as *Momordica clematidea* or Otto Wilhelm Sonder) is a plant species found abundantly in eSwatini and around the Limpopo and Mpumalanga provinces of South Africa (Dlamini and Geldenhuys *et al.,* 2011b). This bitter gourd is a herbaceous climber that possesses a perennial tuberous rootstock and several thickened, spindle-shaped roots covered with a yellow bark (Welman 2005). The leaves of this plant species are up to 120 mm long, hairless, short-stalked, and are a dark green colour above and paler below (**Figure 2.3**). Both male and female flowers (on the same plant) are all stalked and solitary (Behera *et al.,* 2011).

This plant species was listed as a medicinal plant in a publication on the Swaziland Flora by Dlamini (1981). *Momordica cardiospermoides* was also found to be one of the top ten predominant indigenous species that are most preferred and frequently harvested for both food and medicinal purposes in villages such as Mlumati, Emoti and Madvuma in Swaziland (Dlamini *et al.,* 2011a). The leaves of *M. cardiospermoides*

are regarded as edible and are cooked like spinach. The fresh or dried roots of this plant are also cooked and drunk for boils. Although traditionally used to treat a variety of diseases including diabetes (Behera, John *et al.,* 2011), there is no reported scientific data on the plant's phytochemical composition, antioxidant activity and anti-cancer/anti-metastasis activity. Hence this study aims to investigate the potential of this plant as a source of anti-metastasis compounds.

Figure 2.2: *Momordica cardiospermoides* plant.

2.5. Purpose of the study

2.5.1. Aim

To investigate the anti-metastatic potential of *Momordica cardiospermoides* crude acetone and methanol leaf extracts on MDA-MB-231 breast cancer cells.

2.5.2. Objectives

The objectives of this study were to:

I. determine the fingerprint profiles of crude acetone and methanol extracts of *M. cardiospermoides* using thin-layer chromatography.

II. determine the phytochemicals present in crude acetone and methanol extracts of *M. cardiospermoides* leaves using different tests for phytochemicals.

III. evaluate the cytotoxic effects of crude acetone and methanol extracts of *M. cardiospermoides* on MDA-MB-231 breast cancer cells and Hek 239 kidney cells using Muse™ count & viability assay kit assay.

IV. determine the mode of cell death induced in MDA-MB-231 cells by crude acetone and methanol extracts of *M. cardiospermoides* using the acridine orange and ethidium bromide dual staining assay.

V. determine the percentage of MDA-MB-231 cells undergoing apoptosis following treatment with crude acetone and methanol extracts of *M. cardiospermoides* using Muse™ annexin-V & dead cell kit.

VI. determine the effect of crude methanol extract of *M. cardiospermoides* on the migration of MDA-MB-231 breast cancer cells using the wound healing assay.

VII. determine the effect of crude methanol extract of *M. cardiospermoides* on MDA-MB-231 breast cancer cell adhesion using the cell adhesion assay.

VIII. determine the effect of crude methanol extract of *M. cardiospermoides* on expression of MMP-2 and MMP-9.

IX. determine the effect of crude methanol extract of *M. cardiospermoides* on expression of angiogenesis regulatory proteins using the Proteome Profiler™ human angiogenesis antibody array kit

CHAPTER 3

METHODS AND MATERIALS

3.1. Materials and Equipment

- Waring Commercial Blender (Model 32BL79, Dynamics Corporation, New Hartford, Connecticut, USA)
- Microtiter-Plate Multimode Detector (Model 550 and Model DTX 800, Bio-Rad Laboratories, California, USA)
- CO_2 incubator (NAPCO model, Instrulab cc, Johannesburg, RSA)
- New Brunswick G-25 Shaker Incubator (New Brunswick Scientific, New Jersey, USA) Centrifuge (Model GS-15R, Beckman Coulter, Germany)
- Inverted light and fluorescence microscope (Nikon, Japan)
- Methanol and Acetone (Rochelle Chemicals, SA)
- Dimethyl sulfoxide (DMSO) (Darmstadt, Germany)
- MDA-MB-231 (ATCC® HTB-26™) (ATCC, Rockville, USA)
- HEK 293 (ATCC® CRL-1573)
- Dulbecco's Modified Eagle Medium (DMEM) and foetal bovine serum (FBS) (HyClone, Cramlington, UK)
- Phosphate buffered saline (PBS) and 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA) (Lonza, Basel, Switzerland)

3.2. Plant collection and extraction

Momordica cardiospermoides plant leaves were collected from Annadale, Polokwane (GPS coordinates S 23°52'23'' E 29°27'9''), Limpopo province of South Africa. The leaves were air-dried at room temperature and ground to powder using a commercial blender. The plant powder (20 g) was then mixed with 200 ml of absolute methanol or acetone, shaken overnight, and filtered using a Whatman No.2 filter paper. The filtered extracts were then air-dried under an industrial fan to obtain the most stable, non-volatile compounds. The dried extracts were dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 100 mg/ml and stored at –20 °C until use.

3.3. Phytochemical screening and analysis

3.3.1 Thin-layer chromatography

Thin-layer chromatography (TLC) was used to determine the fingerprint profile of the *M. cardiospermoides* crude extracts. Thin-layer chromatography is based on the relative affinity of compounds towards the stationary and mobile phases. Compounds with a higher affinity for the stationary phase travel slowly compared to those of a lower affinity (Valle Jr *et al.,* 2015). Dried extracts were reconstituted in acetone or methanol to 10 mg/ml and 10 µl of each extract was spotted on aluminium-baked TLC plates. The TLC plates were allowed to dry and then developed in three solvent systems of varying polarity, namely, ethyl acetate/methanol/water [EMW] (10:1.3:1) (polar/neutral); chloroform/ethyl acetate/formic acid [CEF] (10:8:2) (intermediate polarity/acidic); toluene/ethyl acetate/ammonium (18:2:0.2) (non-polar/basic). To detect the presence of non-fluorescing phytochemicals, vanillin-sulphuric acid reagent [0.1 g vanillin (Sigma®): 28 ml methanol: 1 ml concentrated sulphuric acid] or p-anisaldehyde-sulphuric acid [0.5 ml p-anisaldehyde: 15 ml glacial acetic acid: 85 ml methanol: 5 ml sulphuric acid] were sprayed on the TLC plates. The plates were subsequently heated at 110°C for 10 minutes for optimal colour development and photographed.

3.3.2. Qualitative phytochemical tests using standard chemical tests

The methanol and acetone extracts were screened for phytochemicals using various methods described by Yadav *et al*. (2014) as depicted in the table below.

Table 1: Tests for the presence of phytochemicals.

Phytoconstituent	**Test**	**Observation**
Tannins (Braymers' Test)	2 ml extract + 2 ml H_2O + 2-3 drops of $FeCl_3$ (5%)	Green precipitate
Flavonoids	1 ml extract + 1 ml $Pb(Ac)_4$ (10%)	Yellow colouration
Saponins	5 ml of extract + olive oil (few drops)	Emulsion forms
Phlabatannins (precipitate test)	2 ml extract + 2 ml HCl (1%) + heat	Red precipitate
Coumarins	2 ml extract + 3 ml NaOH (10%)	Yellow colouration
Anthocyanins	2 ml extract + 2 ml HCl (2N) + NH_3	Pinkish red to bluish violet Colouration

3.3.3. Quantitative phytochemical test

3.3.3.1 Determination of total phenolic content

Determination of the total phenolic content (TPC) was done using the Folin-Ciocalteau method as described by Singleton *et al.* (1999) with minor modifications. The assay is based on the reduction of the Folin-Ciocalteu reagent in the presence of phenolics resulting in the production of molybdenum–tungsten blue (Swain and Hillis 1959). A volume of 66.6µL of 10 mg/ml of either the acetone or methanol extract was added into a test tube. This was followed by the addition of 250 µL of 10% (v/v) Folin-Ciocalteau phenol reagent. The mixture was shaken and kept in the dark for 5 minutes. A volume of 250 µL of 7% (w/v) sodium carbonate (Na_2CO_3) solution was then added to the mixture and incubated for a further 90 minutes at room temperature. A set of standard solutions (ranging from 0 – 0.5 mg/ml) of gallic acid in methanol were

prepared in the same manner. Absorbance was measured against the reagent blank at 750 nm using the GloMax®-Multi+ Detection System (Promega, Madison, USA). Total phenolic content was calculated using the formula below and expressed as mg of GAE/g of extract.

$$TPC = C \times \frac{V}{M}$$

Where ´C´ is the concentration of gallic acid mg/ml, ´V´ is the volume of plant extract in ml and ´M´ is the weight of the extract in grams.

3.3.3.2. Determination of total flavonoid content

Determination of the total flavonoid content (TFC) was done using the aluminium chloride colourimetric assay as described by Gorinstein *et al.* (2007) with minor modifications. In this assay, aluminium chloride forms acid-stable complexes with the ortho-dihydroxyl groups in A- or B-rings of flavonoids. Briefly, 50µL of 10 mg/ml of either the acetone or methanol extract was added into a test tube. This was followed by the addition of 1.4 ml of distilled water, 50 µL of 1 M potassium acetate and 50µL of 10% (w/v) aluminium chloride into the mixture which was then incubated at room temperature for 30 minutes. A set of reference standard solutions (ranging from 0 – 0.5 mg/ml) of quercetin dissolved in methanol were prepared in the same manner. The absorbance of the test and standard solutions was measured against the reagent blank at 420 nm using the GloMax®-Multi+ Detection System (Promega, Madison, USA). Total flavonoid content was calculated using the formula mentioned in **section 3.3.3.1.** and expressed as mg of QE/g of extract.

3.3.3.3. Determination of total tannin content

Determination of the total tannin content (TTC) was done using the Folin-Ciocalteau reagent method as described by Gorinstein *et al.* (2007) with minor modifications. The assay is based on the reduction of the Folin–Ciocalteu reagent in the presence of tannins resulting in the production of molybdenum–tungsten blue (Swain and Hillis 1959). A volume of 20 µL of 10 mg/ml of either the acetone or the methanol extract was added to a test tube containing 1.5 ml of distilled water. To this mixture, 0.1 ml of 10% Folin-Ciocalteau phenol reagent and 1 ml of 35% sodium carbonate (Na_2CO_3) solution were added. This was made up to a final volume of 2 ml with distilled water. The mixture was shaken and kept at room temperature for 30 minutes. A set of

standard solutions (ranging from 0 – 0.5 mg/ml) of tannic acid dissolved in acetone were prepared in the same manner. Absorbance for test samples and standard solutions was measured against the blank at 725 nm using a GloMax®-Multi+Detection System (Promega, Madison, USA). Total tannin content was calculated using the formula mentioned in **section 3.3.3.1.** and expressed as mg of TAE/g of extract.

3.3.3.4. DPPH free radical scavenging activity

Analysis of the free radical scavenging activity of the methanol or acetone extract was done using the DPPH free radical scavenging assay as described by Brand-Williams *et al*. (1995) with some modifications. This assay makes use of the organic chemical compound 2, 2-diphenyl-1-picryhydrazyl, which is a purple coloured stable free radical that provides an easy and rapid way to evaluate antioxidant activity. When an odd electron becomes paired in the presence of a free radical scavenger, the DPPH solution is decolourised as the colour change from deep violet to light yellow (Madhu 2013). The degree of reduction in absorbance measured is indicative of the radical scavenging (antioxidant) power of extract. Sample stock solutions (1 mg/ml) of the acetone or methanol extract were diluted to concentrations of 0, 20, 40, 60, 80 and 100 µg/ml, in 96-well plates. In each well, 100 µL of 200 µM DPPH dissolved in methanol was added. L-ascorbic acid (1 mg/ml) was prepared in the same manner. Methanol was used as blank while the DPPH solution was used as a standard control. The plates were shaken and incubated in the dark for 30 minutes. The absorbance reading was measured at 515 nm using the GloMax®-Multi+ Detection System (Promega, Madison, USA). The inhibitory effect of the extracts was calculated according to the following formula.

$$\%\, Inhibition = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample) \times 100}{Aborbance\ of\ control}$$

3.4. Cell culture, maintenance, and treatment

Breast MDA-MB-231 cancer cells and kidney HEK 293 cells were purchased from the American Type Culture Collection (ATCC, USA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and maintained in a tissue culture incubator at 37°C in humidified air containing 5% CO_2. The cells were sub-cultured every 48 hours and used for seeding for experiments when at 70–80% confluency. For the treatment of cells, the stock solution of the acetone extract or methanol extract dissolved in DMSO was diluted to various concentrations using DMEM. Curcumin at 25 µM was used as a positive control.

3.5. Cell viability assay

The Muse count and viability assay kit (Merck, Germany) was used to determine the effect of the *M. cardiospermoides* crude acetone or methanol extracts on the viability of MDA-MB-231 cells and HEK 293 cells according to the manufacturers' protocol. The muse count and viability reagent differentially stains and non-viable cells based on their permeability to two DNA binding dyes present in the reagent (Nynca *et al.* 2016). Briefly, MDA-MB-231 cells and HEK 293 cells were seeded in 96-well plates at a density of 5×10^3/well and allowed to attach overnight in a tissue culture incubator. The attached cells were then treated with 0 to 300 µg/ml of the acetone or methanol extract or 25 µM of curcumin (positive control). Following 24 hours of treatment, the cells were collected and centrifuged at 6000 rpm for 10 minutes in micro-centrifuge cells. Pelleted cells were then resuspended in 25 µL of 1x PBS and mixed with 225µL of the muse count and viability reagent, incubated at room temperature for 5 minutes and analysed using the Muse® Cell Analyser (Merck, Germany). Data were expressed as graphs showing the percentage of viable cells.

3.6. Analysis of pro-apoptotic effects

3.6.1. Acridine orange/ethidium bromide dual staining

Changes in cellular and nuclear morphology associated with apoptosis in MDA-MB-231 cells treated with *M. cardiospermoides* crude methanol or acetone extracts were assessed using the acridine orange/ethidium bromide dual staining assay as described by Baskic *et al.* (2006) with slight modifications. Acridine orange (AO) is a membrane-permeable dye that stains the nucleus and live cells green. Ethidium

bromide which serves as a counter-stain is a membrane-impermeable dye that fluoresces red after staining the nuclei of cells that have lost their membrane integrity (Lakshmi *et al.,* 2008). Briefly, MDA-MB-231 cells were seeded in a 24-well plate at a density of 3×10^4 /well and allowed to attach overnight. The cells were then treated as described in **section 3.5.** for 6 or 24 hours. Following treatment, the cells were centrifuged for 10 minutes at 5000 rpm. The culture medium was discarded, and the cells were washed with 500 µL of 1x PBS. A mixture of acridine orange (10 µg/ml) and ethidium bromide (10 µg/ml) solutions at a ratio of 1:1 was added into each well and incubated for 10 minutes in the dark, at room temperature. Subsequently, the cells were washed twice with 1x PBS, to remove excess dye. Changes in cell and nuclear morphology were observed and captured using an inverted fluorescence microscope (Nikon, Japan).

3.6.2. Annexin V and Dead cell assay

To quantify the percentage of cells undergoing extract-induced cell death, the Annexin V and Dead cell assay kit was used following the manufacturer's protocol (Merck, Germany). This kit utilises annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine to detect phosphatidylserine on the external membrane of apoptotic cells, as well as 7-AAD, a dead cell marker that serves as an indicator of cell membrane structural integrity. MDA-MB-231 cells were seeded in 48-well plates at a density of 3×10^5 /well and allowed to attach overnight. The cells were then treated as described in **section 3.5**. for 6 or 24 hours. Following treatment, detached cells were collected and attached cells were harvested using 1x trypsin-EDTA. The cells were then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the pellet was re-suspended in 1 ml of DMEM supplemented with 1% FBS. About 50 µL of the cell suspension was added to 50µL of the Muse Annexin V & Dead cell reagent in microcentrifuge tubes, incubated at room temperature for 20 minutes and analysed using the Muse® Cell Analyser (Merck, Germany). Data were expressed as graphs showing early and late apoptotic rates.

3.7. Analysis of anti-metastatic effects

3.7.1. Scratch wound healing assay

The effect of the *M. cardiospermoides* crude methanol extract on the migratory abilities of MDA-MB-231 cells was assessed as described by Liang *et al.* (2007) with slight modifications. This is a standard *in vitro* technique used for probing two-dimensional cell migration and evaluating wound closure (Ashby *et al.,* 2012). MDA-MB-231 cells were seeded in 6-well plates and allowed to attach and form monolayers overnight. The cell monolayers were scratched with a 200 µL sterile pipette tip to create a wound and then washed three times with 1x PBS to remove suspended cells and debris. The wounded cell monolayers were exposed to 150 µg/ml or 300 µg/ml of the methanol extract in serum-free media, or 25 µM of curcumin. To monitor wound closure, cell monolayers were viewed and captured under an inverted light microscope (Nikon, Japan) using 4x magnification at 0, 6 and 24 hours of incubation. The wound sizes were measured using the LC-micro software and data expressed as percentage wound closure using the formula below:

$$Wound\ closure\ (\%) = \frac{Wound\ Size\ (T0) - Wound\ Size\ (Tn)}{Wound\ Size\ (Tn)} \times 100$$

3.7.2. Cell attachment assay

The effect of the *M. cardiospermoides* crude methanol extract on MDA-MB-231 cell attachment was assessed using the cell attachment assay as described by Humphries (2009) with minor modifications. This assay measures the ability of cells to attach to pre-coated culture plates. Briefly, MDA-MB-231 cells (3×10^6 /well) were seeded in 6-well plates, incubated overnight and treated with 150 or 300 µg/ml of *M. cardiospermoides* crude methanol extract or 25 µM curcumin in serum-free media for 6 or 24 hours. Cells were harvested with 1x trypsin-EDTA and centrifuged for 5 minutes at 3000 rpm. The cells were then seeded in 6-well plates for 2 hours and washed with 300 µL of 1x PBS. The attached cells were stained with 0.1% (w/v) crystal violet, viewed and captured under a 10x objective of a phase-contrast inverted light microscope. Following the capturing, 100 µL of DMSO was added to each well and absorbance was measured at 565 nm using a GloMax®-Multi+ Detection System

(Promega, Madison, USA). Data were expressed as percentage cell adhesion using the following formula:

$$Cell\ adhesion\ (\%) = \frac{Absorbance\ of\ treated}{Absorbance\ of\ untreated\ cell} \times 100$$

3.8. Determination of protein expression

3.8.1. Western blotting

The effect of the *M. cardiospermoides* methanol extract on the expression of MMP-2 and MMP-9 was assessed using western blotting as described by (Iqbal *et al.,* 2016) with minor modifications. This is a sensitive and rapid assay that separates proteins on a polyacrylamide gel according to their molecular weight. The separated proteins are then electroblotted onto a nitrocellulose membrane and detected using specific primary antibodies and secondary enzyme labelled antibody and substrate. MDA-MB-231 cells were treated with 150 or 300 µg/ml of the methanol extract or with 25 µM of curcumin for 24 hours. Following treatment, the cells were washed twice with 1x ice-cold PBS before being harvested with 1x trypsin-EDTA and transferred into a microcentrifuge tube. The cells were then centrifuged at 1500 rpm for 10 minutes, 250 µL of chilled RIPA lysis buffer [50 nM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM EDTA, 0.1% SDS] added to the pelleted cells and incubated for 30 minutes on ice. The cell lysates were centrifuged at 14000 rpm for 10 minutes and the supernatant was transferred to new tubes. The proteins were then quantified using the BCA protein quantification kit (Thermo Scientific, Rockford, USA), according to the manufacturer's protocol. Aliquots containing total cellular proteins (30 µg) were boiled in 2x 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) 2- mercaptoethanol] before being resolved on 8–12% sodium dodecyl sulphate-polyacrylamide gels . Proteins on the gels were then electro-blotted using a trans-blotter (Bio-rad, USA) onto PVDF membranes (Thermo Fisher, USA) using a blotting buffer (20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3) at 200 mA for 30 minutes at room temperature. Following electro-blotting, the membranes were blocked with 0.05% TBS-Tween (25 mM Tris, 125 mM NaCl, pH 7.4) containing 5% (w/v) fat-free milk for 1 hour at room temperature. After blocking, the membranes were washed three times for 10 minutes each with 0.05% TBS-Tween and then incubated with specific primary antibodies (Novus Biologicals, USA): mouse

anti-β-actin (1:1000), mouse anti-MMP-2 (1:500), and mouse anti-MMP-9 (1:500) overnight at 4°C on a rocking platform. The membranes were washed as described above and incubated with goat anti-mouse IgG HRP-conjugated secondary antibody for 2 hours at room temperature, on a rocking platform. The membranes were washed again as described above and 1 ml of chemiluminescent reagent mix was pipetted onto each membrane and incubated for 5 minutes. The membranes were then developed and captured using the C-DiGit blot scanner. Densitometric analysis of the bands was done using the Image Studio Digits Version 5.2 software to determine the relative change in protein expression between samples and represented as percentage pixel density.

3.8.2. Proteome Profiler™ human angiogenesis antibody array kit

The effect of the *M. cardiospermoides* methanol extract on the expression of angiogenesis-related proteins was analysed using the Proteome Profiler™ human angiogenesis antibody array kit (R&D Systems, USA) following the manufacturer's instructions. The assay uses nitrocellulose membranes each spotted with different primary antibodies specific to proteins involved in angiogenesis and metastasis. MDA-MB-231 cells were treated with 150 or 300 µg/ml of the methanol extract or 25 µM of curcumin for 24 hours. The cells were then washed twice with 1x PBS, centrifuged and then the cell pellet was lysed using the RIPA buffer [50 nM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM EDTA, 0.1 % SDS] for 30 minutes on ice. The cell lysates were centrifuged at 14000 rpm for 10 minutes and the supernatant was transferred to new tubes. The proteins were then quantified using the BCA protein quantification kit, according to the manufacturer’s protocol (Thermo Scientific, Rockford, USA). Membranes were blocked using 2 ml of array buffer 7 for an hour on a rocking platform. While blocking, protein samples (300 µg) were mixed with array buffer 4, array buffer 5 and 15µL of reconstituted detection antibody cocktail and incubated for an hour at room temperature. Following the blocking of membranes, the array buffer 7 was aspirated, replaced with the protein/antibody mixture, and incubated overnight at 4°C on a rocking platform. After incubation, the membranes were washed 3 times with 1x wash buffer for 10 minutes and streptavidin-HRP was added onto each membrane and incubated for an hour at room temperature. The membranes were washed again as described above and 1 ml of chemiluminescent reagent mix was pipetted onto each membrane and incubated for 5 minutes. The membranes were then developed and

captured using the C-DiGit blot scanner. Densitometric analysis of the bands was done using the Image Studio Digits Version 5.2 software to determine the relative change in protein expression between samples and represented as percentage pixel density.

3.7. Statistical analysis

Statistical differences between treatments and the controls were calculated using the GraphPad Instat 3 software by one-way ANOVA followed by Dunnett's comparison tests. The data were expressed as mean ± standard deviation (SD) of three independent experiments done in duplicate and the $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.1$ were considered significant.

CHAPTER 4

RESULTS

4.1. Phytochemical screening and analysis

4.1.1. Fingerprint profile of *Momordica cardiospermoides* crude acetone and methanol extracts

Plant crude extracts contain numerous phytochemical constituents that may vary in polarities; therefore, thin-layer chromatography was performed to separate the phytochemicals present in *M. cardiospermoides* crude acetone and methanol extracts. **Figure 4.1** below shows that the mobile phase TAE (nonpolar) was able to better separate the compounds present in the acetone extract (Ace), suggesting that the compounds in the acetone extract were mostly non-polar. Compounds present in the methanol extract (MeOH) were better separated in the polar mobile phase (EMW), suggesting that the compounds present in this extract were mostly polar.

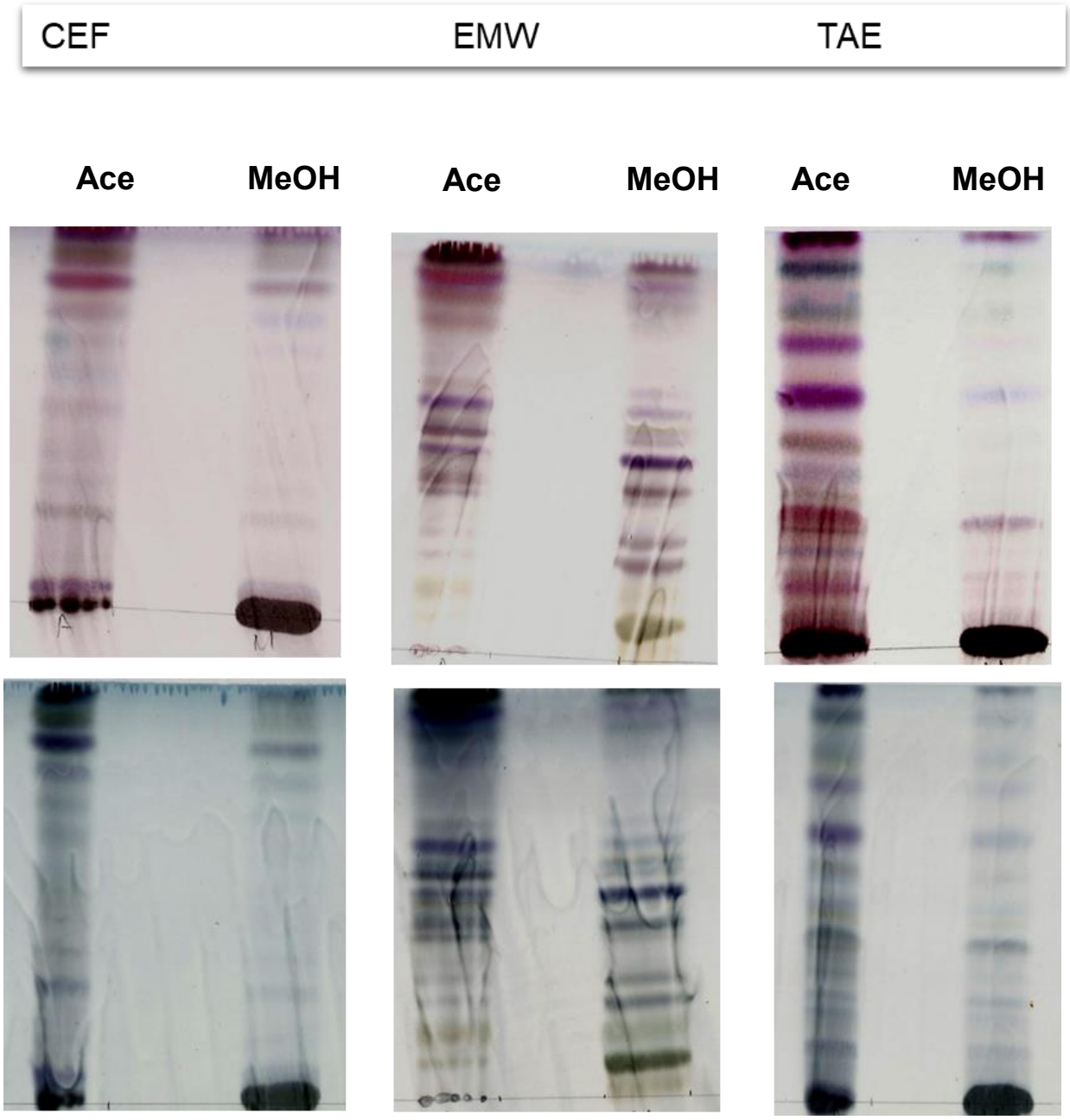


Figure 4.1: Chromatograms of *M. cardiospermoides* crude acetone and methanol extracts. Thin-layer chromatography plates were developed in solvents of different polarities viz. chloroform/ethyl acetate/formic acid (CEF), ethyl acetate/methanol/water (EMW) and toluene/acetone/ethyl acetate (TAE). The plates were then sprayed with either p-Anisaldehyde spray (top) or general vanillin and sulphuric acid spray (bottom) and heated at 110°C. Ace: acetone extract; MeOH: methanol extract.

4.1.2. Phytochemical Screening of *Momordica cardiospermoides* methanol and acetone extracts showed the presence of secondary metabolites.

Preliminary phytochemical analysis of crude extracts is important as it aids in identifying chemical compounds with pharmacological activity. Therefore, qualitative phytochemical screening was done to determine the presence of tannins, flavonoids, terpenoids, saponins, phlabatannins and coumarins in *M. cardiospermoides* acetone and methanol extracts. The results obtained revealed the presence of tannins, flavonoids, and coumarins and the absence of phlabatannins and terpenoids, in both extracts. Saponins were found present only in the methanol extract (**Table 4.1**).

Table 4.1: Qualitative phytochemical screening of *M. cardiospermoides* crude acetone and methanol extracts.

Phytochemicals	Occurrence	
	Methanol extract	Acetone extract
Tannins	+	+
Flavonoids	+	+
Terpenoids	-	-
Saponins	+	-
Phlabatannins	-	-
Coumarins	+	+

Key: + present/ - absent

4.1.3. Quantification of major phytochemicals

Quantification of phytochemicals is useful for ascribing an extent of activity to specific phytochemicals. Therefore, major phytochemicals (flavonoids, tannins, and phenolics) found present in the extracts were quantified as mentioned in **sections 3.3.3.1. to 3.3.3.3.** The results obtained (**figure 4.2A, B and C**) revealed that the acetone extract had higher total phenolic (23.0683 mgGAE/g), total flavonoid (32.6933 mgQE/g) and total tannin (22.0442 mgTAE/g) contents as compared to the methanol extract total phenolic (14.2349 mgGAE/g), total flavonoid (7.692 mgQE/g) and total tannin (11.3164 mgTAE/g) contents.

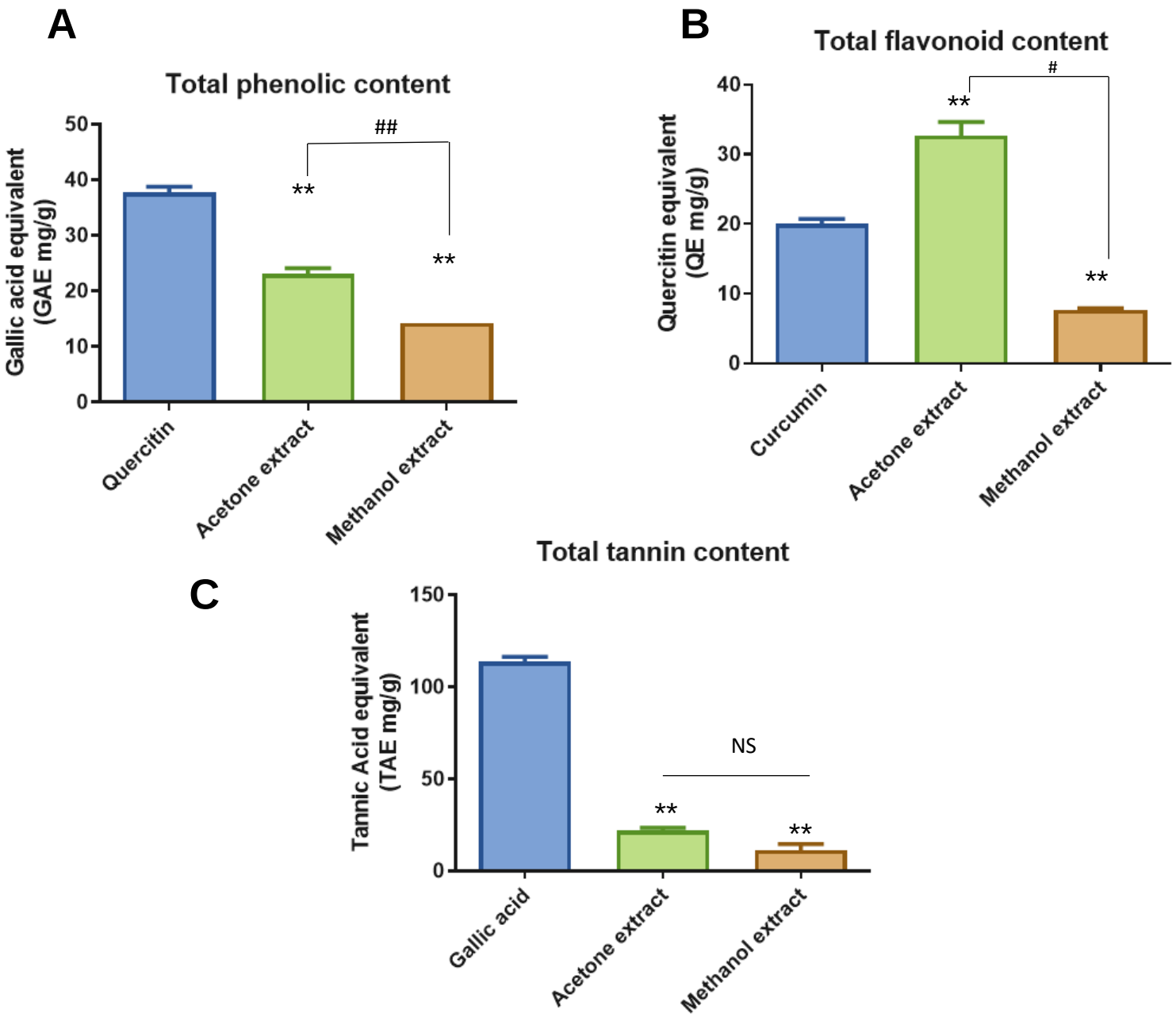


Figure 4.2: Quantitative analysis of phenolic, flavonoid, and tannin contents of *M. cardiospermoides* acetone and methanol extracts. The total phenolic **(A)**, flavonoid **(B),** and tannin **(C)** content were assessed using the assay Folin-Ciocalteau and aluminium assays. Absorbance readings were obtained at 750 nm, 420 nm, and 725 nm, respectively, using the GloMax®-Multi+ Detection System. Data are expressed as the mean ± S.D three independent experiments, performed in sextuplicates, with ** $p \leq 0.001$ indicating a significant difference to the respective control (quercetin, curcumin, or gallic acid), and # $p \leq 0.05$ and ## $p \leq 0.01$ indicate a significant difference between the extracts

4.1.4. DPPH free radical scavenging activity of *Momordica cardiospermoides* acetone and methanol leaf extract.

Phytochemicals such as phenolic acids, flavonoids and tannins are considered antioxidant compounds which scavenge free radicals in living organisms. To this point, quantitative antioxidant activities of *M. cardiospermoides* crude acetone and methanol extracts were assessed by the DPPH free radical scavenging activity assay using L-ascorbic acid as a reference standard. The zero inhibition was considered for the solution which contained only DPPH without any plant extract. **Figure 4.5** below shows that the acetone extract had a greater % inhibition activity comparative to the methanol extract.

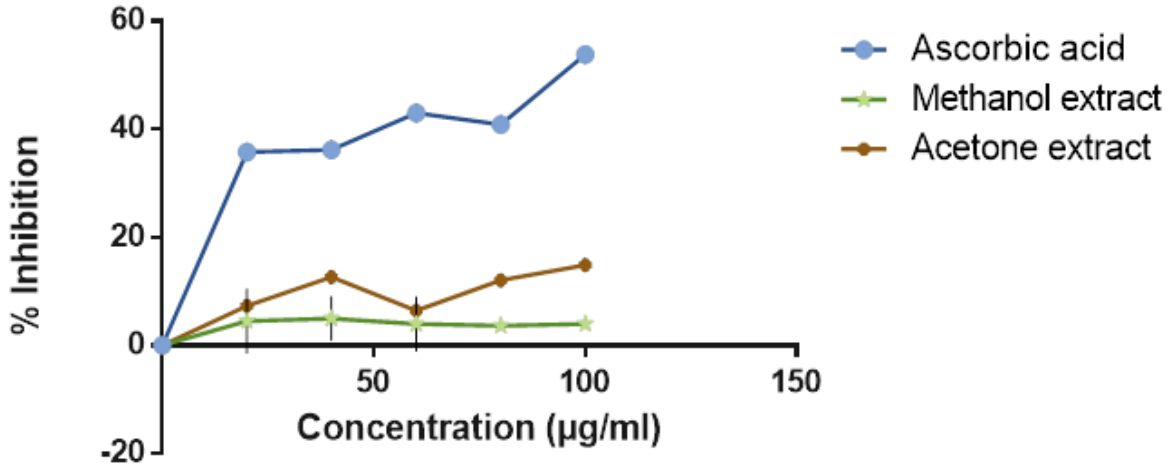


Figure 4.3: Quantitation of the antioxidant activity of *M. cardiospermoides* acetone and methanol extracts. Free radicle scavenging activity was assessed using 2,2-diphenyl-1-picryhydrazyl (DPPH) assay, with ascorbic acid used as the positive control. Absorbance readings were obtained at 515 nm using the GloMax®-Multi+ Detection System spectrophotometer and the percentage inhibition was calculated. Data are expressed as mean ± SD of three independent experiments performed in sextuplicate.

4.2. Cytotoxic effects of *Momordica cardiospermoides* acetone and methanol leaf extracts on MDA-MB-231 breast cancer and HEK 293 kidney cell viability.

An important characteristic of an ideal anticancer agent is its ability to reduce the viability of cancer cells without affecting the viability of normal cells in the body. Therefore, the effect of *M. cardiospermoides* acetone or methanol extracts on the viability of MDA-MB-231 breast cancer and HEK 293 kidney cells was determined using the Muse count and Viability kit. **Figure 4.4. A** show that treatment with 100 to 300 µg/ml of the acetone extract for 24 hours decreased the percentage viability of MDA-MB-231 cells. A significant ($p \leq 0.001$) decrease in the percentage viability of MDA-MB-231 cells was observed in cells treated with concentrations between 200 µg/ml and 300 µg/ml of the extract with the percentage of viable cells ranging between 51–53%. Furthermore, a significant ($p \leq 0.01$) difference in MDA-MB-231 cell viability was observed among the three treatments. On the other hand, treatment of MDA-MB-231 cells with the methanol extract exhibited a significant ($p \leq 0.001$) concentration-dependent decrease in viability, with the percentage of viable cells ranging between 66–76%.. Moreover, a significant difference in viability was observed among the three treatments as well. Treatment with 25 µM of curcumin resulted in a non-significant decrease of MDA-MB-231 cell viability.

Figure 4.4. B shows the effect of the extracts on HEK 293 cell viability and as observed, the acetone extract significantly ($p \leq 0.001$) decreased the percentage of viable cells at 100, 200, and 300 µg/ml. The percentage of viable cells following treatment with the acetone extract ranged between 27–30%. Comparatively, the methanol extract did not have a significant negative effect on the viability of HEK 293 cells, as the percentage of viable cells was maintained at 85–98% at 24 hours of treatment. On the other hand, treatment with 25 µM of curcumin resulted in a significant decrease in HEK 293 cell viability when compared to the untreated control.

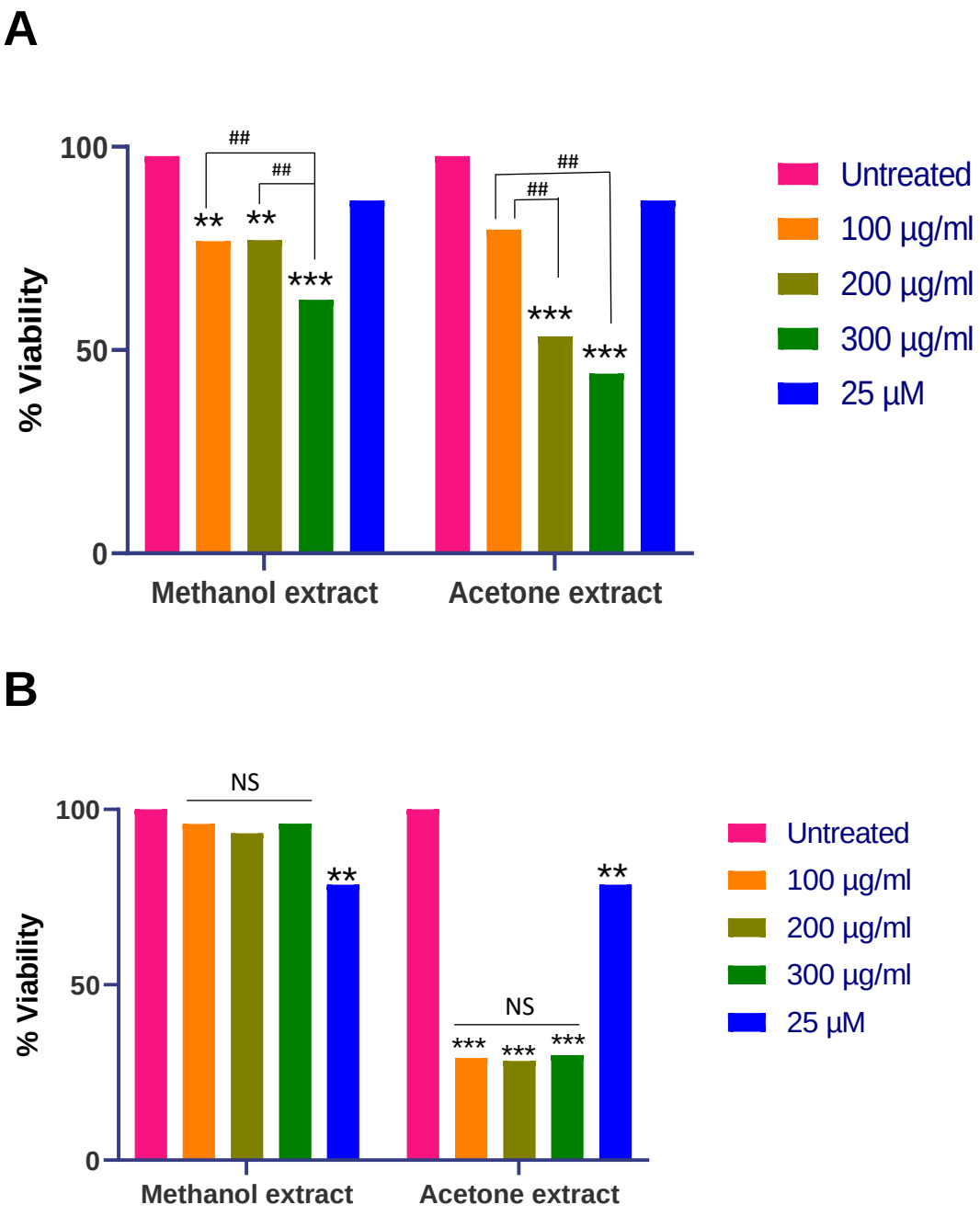


Figure 4.4: Effect of *M. cardiospermoides* methanol or acetone extract on MDA-MB-231 (A) and HEK 293 (B) cell viability. The cells were treated with varying concentrations of either the methanol or acetone leaf extract or 25 µM of curcumin (positive control) for 24 hours. The percentage of viable cells was assessed using the muse count and viability assay kit. Data are expressed as the mean± S.D. of three independent experiments, performed in octuplicates, with p≤ 0.05 and *** p ≤ 0.001 indicating a significant difference to the untreated control and ## p ≤ 0.01 indicates a significant difference between treatments.

4.3. Effect of *Momordica cardiospermoides* methanol and acetone leaf extracts on cellular and nuclei morphology of MDA-MB-231 cells.

Dying cells often exhibit nuclei and cell morphological changes that may be used to characterise the mode of cell death. The acridine orange and ethidium bromide dual staining assay was used to determine the effect of the extracts on the MDA-MB-231 cell and nuclei morphology. **Figure 4.5** shows that both extracts induced nuclei and cell morphological changes in a concentration-depended manner at 24 hours of treatment. The observed morphological changes included chromatin condensation (early apoptosis) and loss of membrane function (late apoptosis), which are indicated by cells fluorescing a bright green colour and a faint orange or red colour, respectively. Treatment with 25 µM of curcumin for 24 hours also meagerly induced chromatin condensation and loss of membrane function in MDA-MB-231 cells.

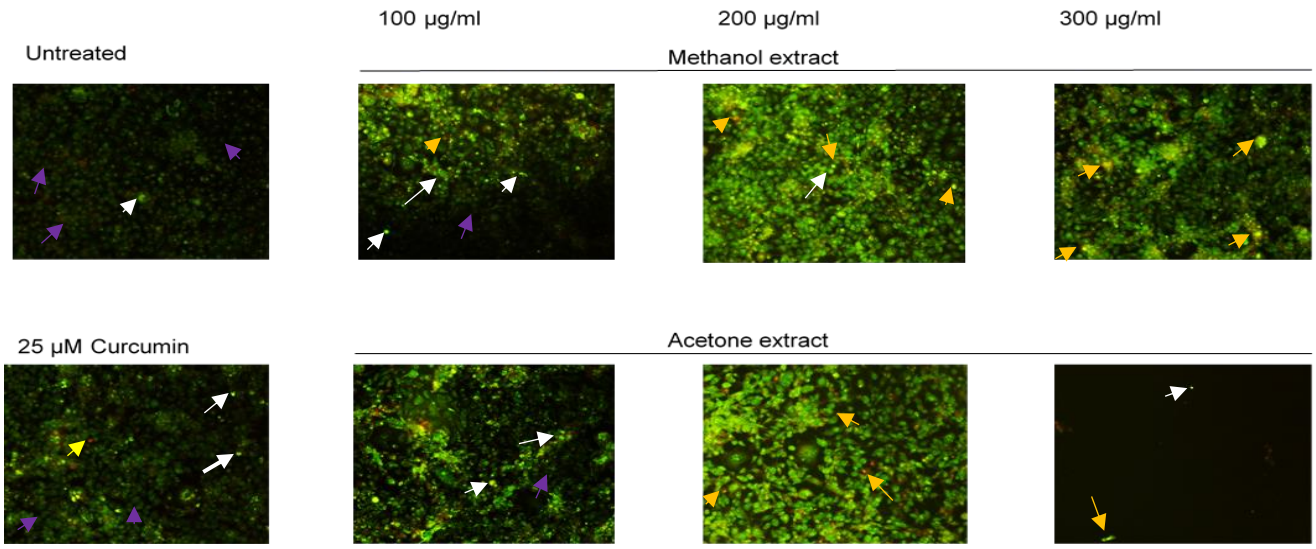


Figure 4.5: Effect of the *M. cardiospermoides* methanol or acetone extracts on cell and nuclei morphology of MDA-MB-231 cells. Cells were treated with varying concentrations of either extract or 25 µM of curcumin for 24 hours. Cell and nuclei morphological changes were assessed using acridine orange/ ethidium bromide dual stains and captured under a 10x objective of a fluorescent microscope. Photographs represent one of three independent repeats. Purple arrows–viable cells; White arrows –early apoptotic; Orange – late apoptotic cells.

4.4. Effect of *Momordica cardiospermoides* on extract-induced apoptosis in MDA-MB-231 breast cancer cells.

The translocation of the phosphatidylserine (PS) from the inner surface to the outer surface of the cell membrane is a key indicator of early apoptosis. Therefore, the percentage of cells undergoing extract-induced cell death was quantified. As shown in **figure 4.6 A,** treatment for 6 and 24 hours with the acetone extract revealed a significant ($p \leq 0.01$) concentration- and time-dependent increase in the percentage of PI-positive cells (dead cell quadrant), when compared to the untreated controls. Moreover, a non-significant increase in the percentage of MDA-MB-231 cells undergoing apoptosis after treatment with the acetone extract for 6 or 24 hours was observed, as compared to the untreated control (0 µg/ml).

On the other hand, 6 and 24 hours of treatment with the methanol extract (**figure 4.6 B**) revealed an increase in the percentage of annexin V-FITC stained cells (early apoptosis quadrant), compared to the untreated controls. Furthermore, a concentration- and time-dependent increase in total apoptotic cells was observed. Moreover, a significant ($p \leq 0.5$) increase in MDA-MB-231 total apoptotic cells was observed following 24 hours of treatment with 300 µg/ml of the methanol extract. Treatment of MDA-MB-231 cells with 25 µM of curcumin for 6 and 24 hours revealed a time-dependent increase in total apoptotic cells.

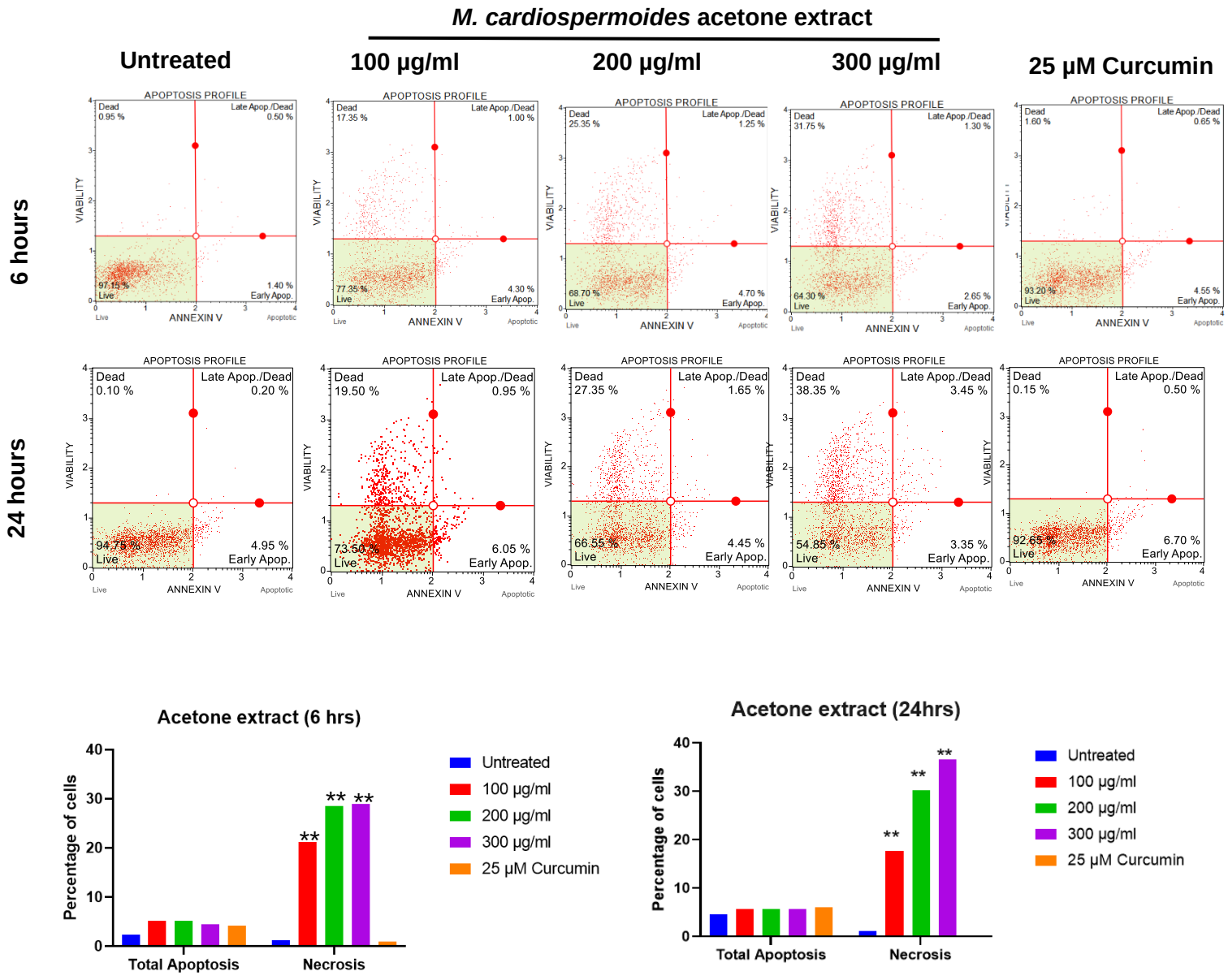


Figure 4.6A: Quantitation of cell death by the *M. cardiospermoides* acetone extract in MDA-MB-231 cells. Cells were treated with varying concentrations of either extract or with 25 µM of curcumin (positive control), for 6 or 24 hours. The number of cells undergoing cell death was quantified using the Annexin V and Dead cell assay kits and analysed using the Muse analyser. Data are expressed as mean ± SD of three independent experiments performed in duplicates, with ** $p \leq 0.01$ indicating a significant difference to the untreated control.

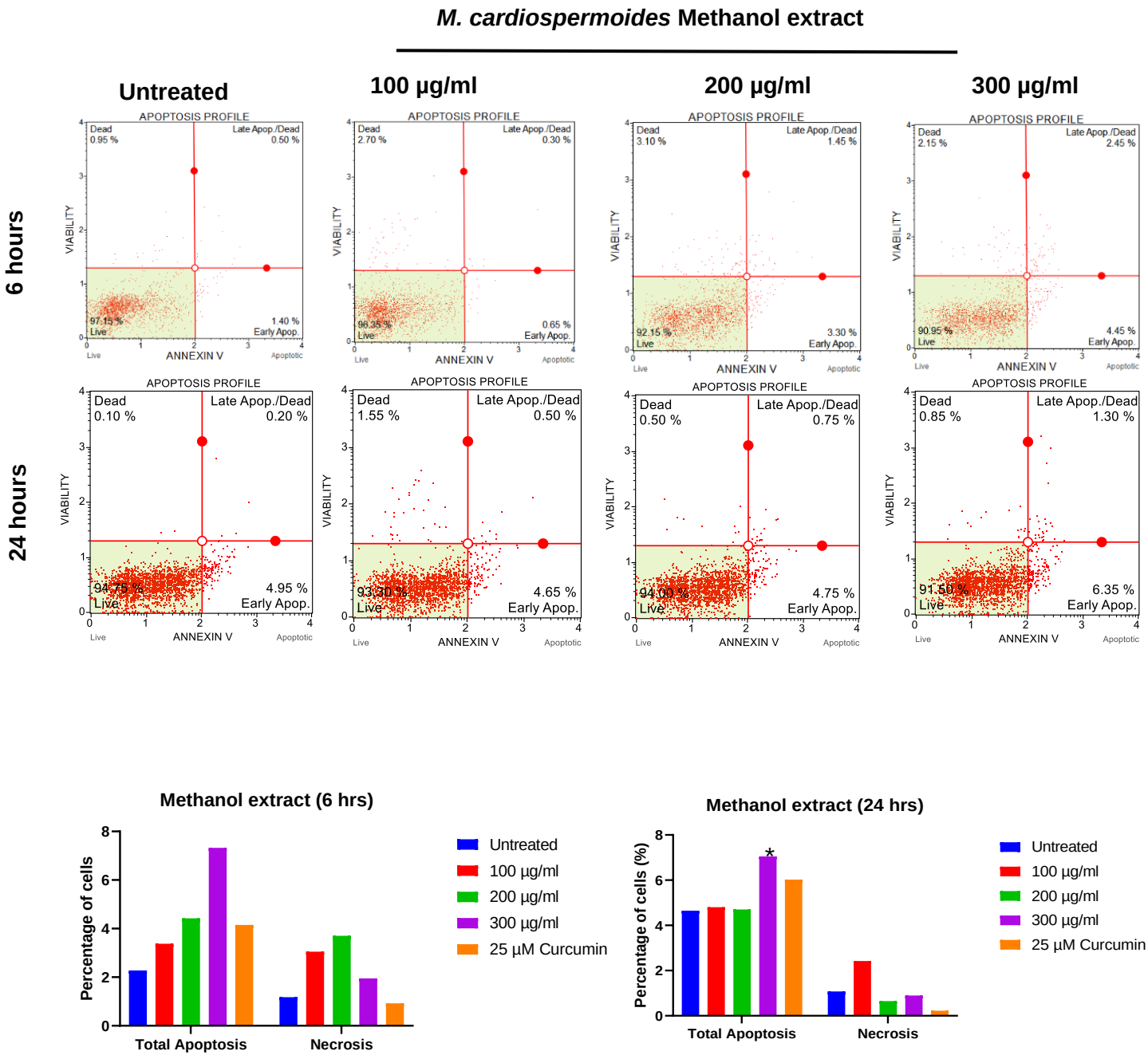


Figure 4.6B: Quantitation of cell death induced by the *M. cardiospermoides* methanol extract in MDA-MB-231 cancer cells. Cells were treated with varying concentrations of either extract or 25 µM of curcumin (positive control), for 6 or 24 hours. The percentage of cells undergoing cell death was quantified using the Annexin V and Dead cell assay kit and analysed using the Muse analyser. Data are expressed as mean ± SD of three independent experiments performed in duplicates, with * $p \leq 0.5$ indicating a significant difference to the untreated control.

4.5. Effect of *Momordica cardiospermoides* crude methanol extract on the migratory abilities of MDA-MB-231 cells.

The migratory ability of tumour cells is one important prerequisite for the successful progression of malignancy. Therefore, the effect of *M. cardiospermoides* crude methanol extract on the migration of MDA-MB-231 cells was assessed using the scratch wound healing assay as described in **section 3.7.1.** As shown in **figure 4.7A,** the migration of MDA-MB-231 cells was inhibited at 6 and 24 hours, following treatment with 150 or 300 µg/ml of the methanol extract, compared to the untreated control. Moreover, treatment with 25 µM of curcumin also inhibited MDA-MB-231 cell migration. Furthermore, a concentration- and time-dependent decrease in wound closure and wound area (**figure 4.7B**) was observed, following treatment with the methanol extract as well as with 25 µM of curcumin.

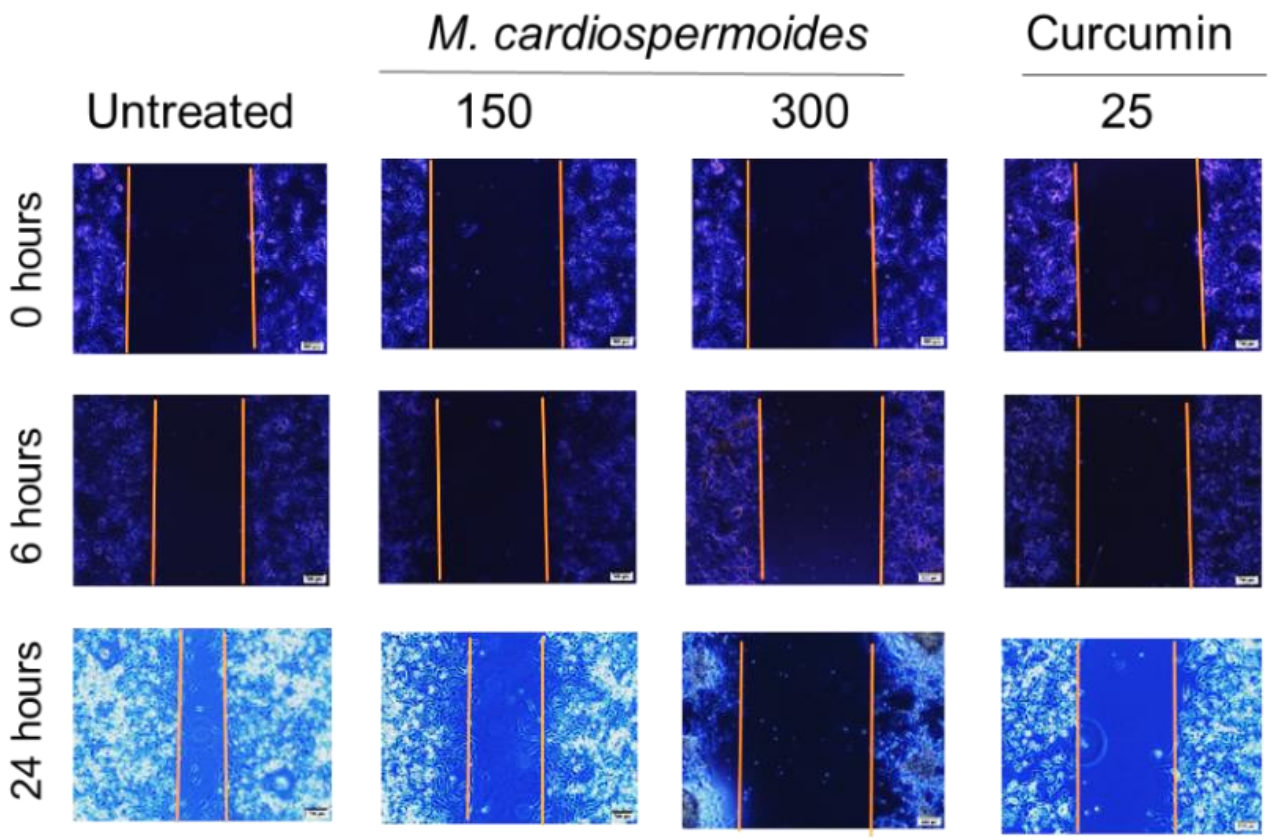


Figure 4.7A: The effect of *M. cardiospermoides* crude methanol extract on the migration of MDA-MB-231 cells. Confluent MDA-MB-231 cell monolayers were wounded and treated with 150 or 300 µg/ml of *M. cardiospermoides* methanol extract or 25 µM curcumin, for 6 or 24 hours. Following each time point, the cells were observed under a phase-contrast inverted light microscope with the 4x objective and captured.

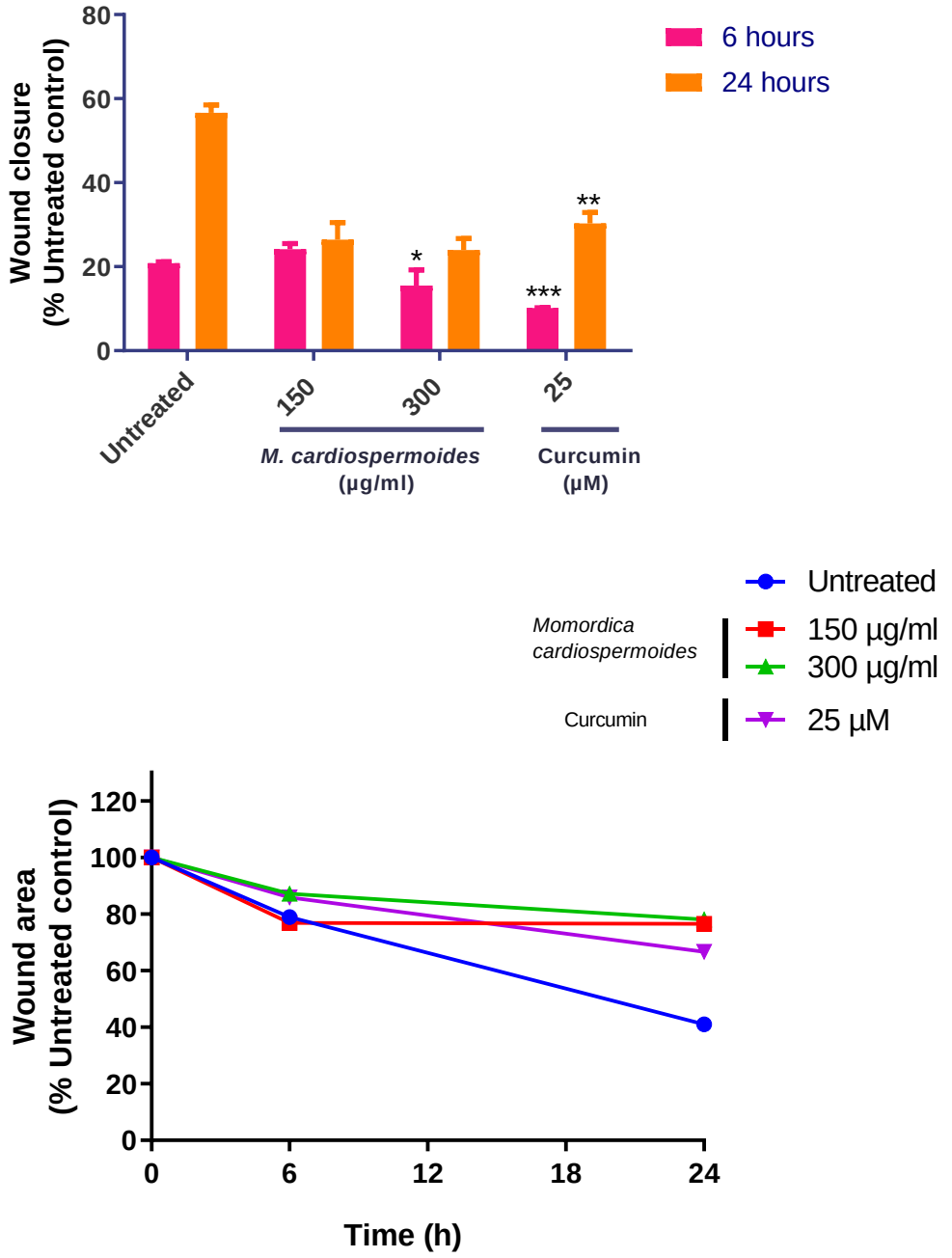


Figure 4.7B: Analysis of the effect of *M. cardiospermoides* methanol extract on MDA-MB-231 cell migration. Wound sizes were measured using the LC-micro software and data were expressed as percentage wound area and wound closure. Individual data points represent the mean ± S.D of three independent experiments, performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01 and ***p ≤ 0.01 indicate significant differences to the untreated control.

4.6. Effect of *Momordica cardiospermoides* methanol extract on the expression levels of MMP-2 and MMP-9

Extracellular matrix remodelling is a complex process that is facilitated by alterations of the ECM by matrix metalloproteinases. Therefore, the effect of *M. cardiospermoides* methanol extract on the expression of matrix metalloproteinase -2 and -9 was assessed using western blotting as described in **section 3.8.1.** As shown in **figure 4.8A,** treatment of MDA-MB-231 cells for 24 hours with 150 or 300 µg/ml of the methanol extract resulted in the downregulation of MMP-2 and MMP-9 protein expression compared to the untreated control. Treatment with 25 µM of curcumin led to a decrease in the protein expression levels of MMP-2 and MMP-9. Furthermore, **figure 4.8B** shows that the downregulation in the expression of MMP-9 was significant ($p \leq 0.001$) and in a concentration-dependent manner. Moreover, a concentration-dependent downregulation in the expression of MMP-2 was also observed, with a significant ($p \leq 0.05$) downregulation observed following treatment with 300 µg/ml of the methanol extract. Treatment with 25 µM of curcumin for 24 hours significantly ($p \leq 0.01$ and $p \leq 0.001$) downregulated MMP-2 and -9 protein expression levels.

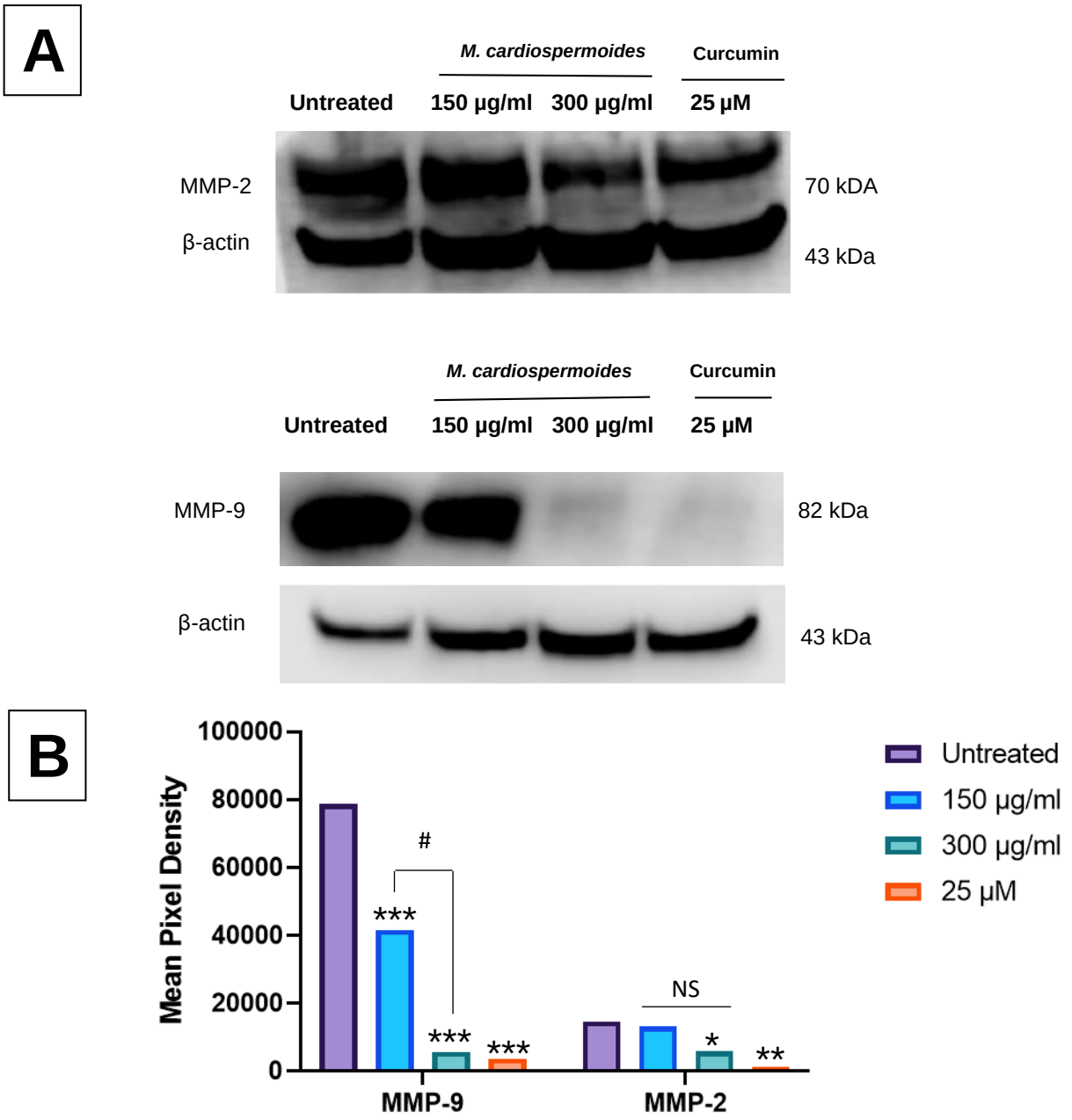


Figure 4.8: The effect of *M. cardiospermoides* methanol extract on the expression of MMP-2 and -9. MDA-MB-231 breast cancer cells were treated with 150 or 300 µg/ml of the methanol extract or with 25 µM of curcumin for 24 hours. Protein samples (30 µg) were resolved on 8–12% SDS-PAGE, electro-blotted onto PVDF membranes, developed and captured using the C-DiGit blot scanner (**A**). Densitometric comparison of the expression of MMP-2 and MMP-9 was done using the Image Studio Digits Version 5.2 software and GraphPad prism (**B**). *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 indicates a significant difference to the untreated control and #p ≤ 0.05 indicates significant difference between treatments.

4.7. Effect of *Momordica cardiospermoides* methanol extract on MDA-MB-231 breast cancer cell adhesion.

Adhesion of cells to the extracellular matrix is essential in secondary tumour formation, as such, the effect of *M. cardiospermoides* crude methanol extract on MDA-MB-231 cell adhesion was assessed using the cell adhesion assay as described in **section 3.7.2.** As seen in **figure 4.9A**, at 24 hours of treatment with 150 or 300 µg/ml of the methanol extract, the number of attached MDA-MB-231 cells decreased (as indicated by cells stained with crystal violet) when compared to the untreated control. Moreover, statistical analysis (**figure 4.9B**) revealed the inhibition of cell adhesion to be significant ($p \leq 0.01$ and $p \leq 0.001$) and in a concentration-dependent manner. Treatment with 25 µM of curcumin for 24 hours also decreased the number of attached MDA-MB-231 cells and significantly ($p \leq 0.01$) inhibited cell adhesion in comparison to the untreated control.

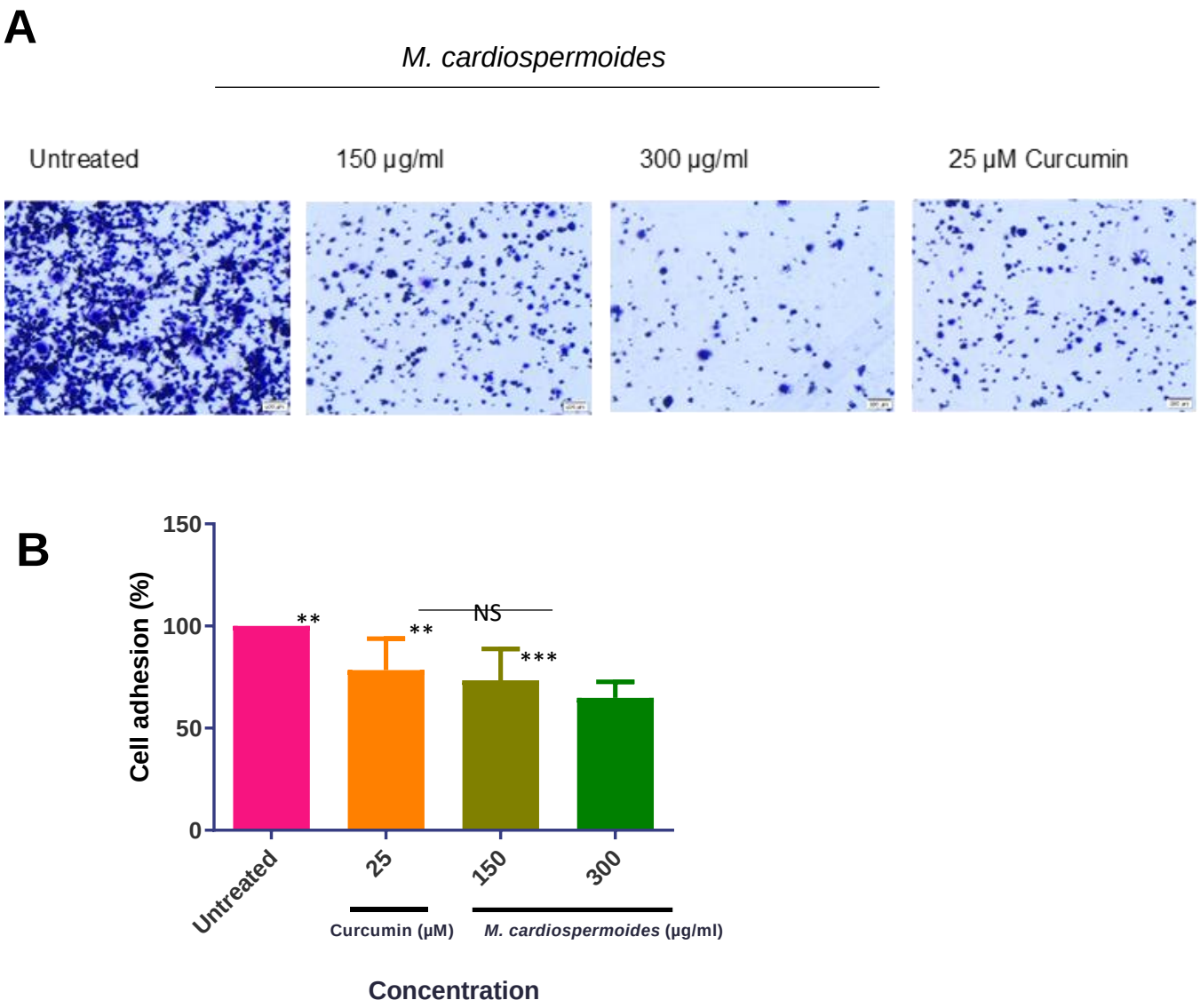
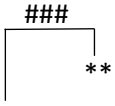


Figure 4.9: The effect of *M. cardiospermoides* crude methanol extract on MDA-MB-231 breast cancer cell adhesion. Cells were treated with varying concentrations of *M. cardiospermoides* crude methanol extract or 25 µM curcumin for 24 hours. The attached cells were stained with crystal violet and captured under 4x objective using a phase-contrast inverted microscope (**A**). The stained cells were then redissolved in DMSO and quantified using the GloMax®-Multi+ Detection System (**B**). Individual data points represent mean ± S.D of three independent experiments, performed in duplicates. **p ≤ 0.01 and ***p ≤ 0.001 indicate significant differences to the untreated control.

4.8. Effect of *Momordica cardiospermoides* methanol extract on the expression levels of angiogenesis proteins.

Anti-angiogenic proteins are essential cancer-fighting agents because they block the formation and growth of blood vessels that support tumour growth. Therefore, the effect of *M. cardiospermoides* methanol extract on the expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) and thrombospondin-1 was assessed as mentioned in **section 3.8.2.** As shown in **Figure 4.10,** treatment of MDA-MB-231 cells for 24 hours with 150 or 300 µg/ml of the methanol extract significantly ($p \leq 0.01$) upregulated the expression of TIMP-1 and thrombospondin-1 in a concentration-dependent manner, in comparison to the untreated control. Furthermore, treatment of MDA-MB-231 cells for 24 hours with 25 µM of curcumin upregulated the expression of TIMP-1 and significantly ($p \leq 0.01$) upregulated the expression of thrombospondin-1, in comparison to the untreated control.



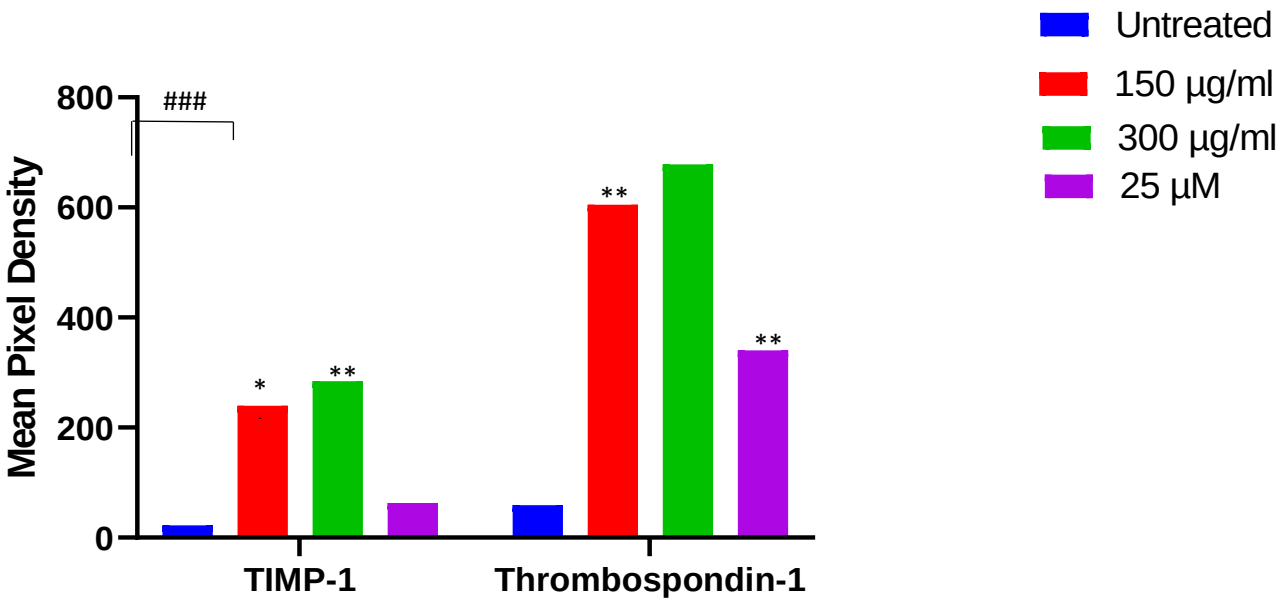


Figure 4.10: The effect of *M. cardiospermoides* methanol extract on the expression levels of angiogenesis factors TIMP-1 and Thrombospondin-1. MDA-MB-231 cells were treated with 150 or 300 µg/ml of the methanol extract or with 25 µM of curcumin for 24 hours. Protein expression was determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised using the C-DiGit blot scanner, and the signal density of each spot representing the proteins of interest was assessed using the Image Studio Digits Version 5.2 software and GraphPad prism. Data show the mean signal density of each representative pair of duplicate spots on the array. **p ≤ 0.01 indicates a significant difference to the untreated control, and ### p ≤ 0.001 indicates a significant difference between treatments.

4.9. Effect of *Momordica cardiospermoides* crude methanol extract on the expression levels of pro-angiogenic proteins

The formation of neo-vessels during angiogenesis is sustained by classical and non-classical pro-angiogenic factors that are also potential targets of anti-angiogenic therapies. In that regard, the effect of *M. cardiospermoides* methanol extract on the expression levels of angiogenic factors was analysed as mentioned in **section 3.8.2.** As shown in **figure 4.11A**, serpin E1 was downregulated in a concentration-dependent manner, and a significant difference to the untreated control was observed following treatment with 300 µg/ml of the extract. Moreover, treatment with the extract significantly ($p \leq 0.01$) upregulated the expression of uPA. Furthermore, treatment with 150 µg/ml significantly ($p \leq 0.01$) upregulated VEGF expression, whereas treatment with 300 µg/ml significantly ($p \leq 0.01$) downregulated VEGF expression, compared to the untreated control. A significant concentration-dependent upregulation of pro-angiogenic factors, IGFBP-2 and -3, following treatment with 150 and 300 µg/ml of the methanol extract was observed **(Figure 4.11B).** However, treatment with 150 µg/ml of the methanol extract resulted in the upregulation of IGFBP-1 expression levels, although not significant. Furthermore, treatment with 25 µM curcumin resulted in the upregulation of IGFBP-3 and the downregulation of IGFBP-2.

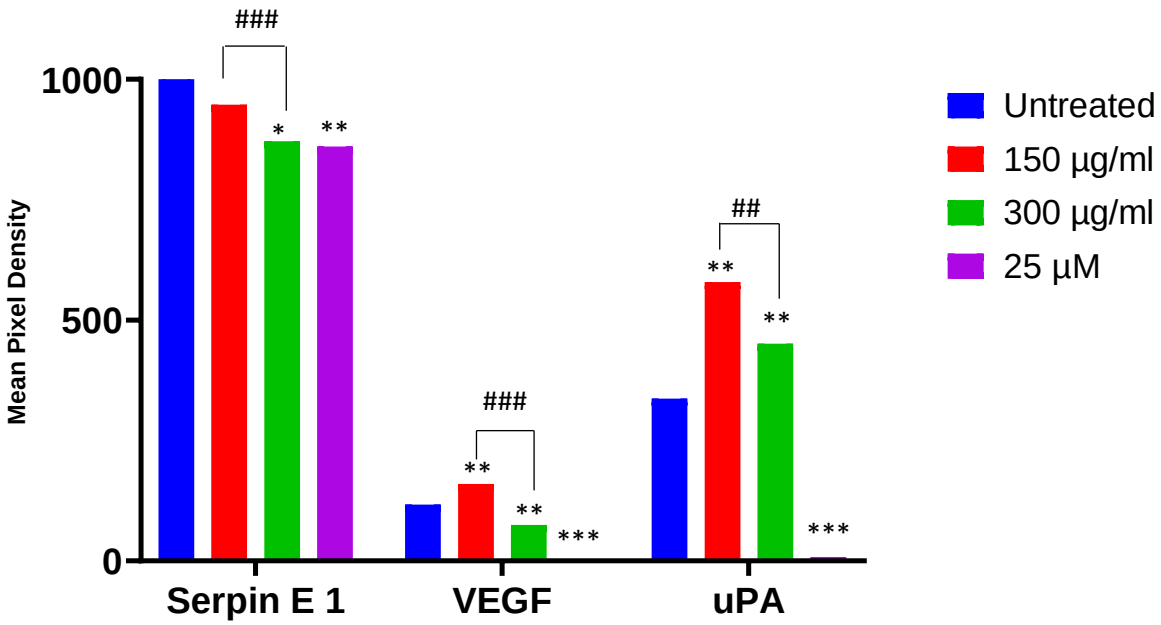


Figure 4.11A: The effect of *M. cardiospermoides* crude methanol extract on the expression levels of angiogenesis factors serpin E1 uPA and VEGF. MDA-MB-231 cells were treated with 150 or 300 µg/ml of the methanol extract or with 25 µM of curcumin for 24 hours. Protein expression was determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised using the C-DiGit blot scanner, and the signal density of each spot representing the proteins of interest was assessed using the Image Studio Digits Version 5.2 software and GraphPad prism (A). Data show the mean signal density of each representative pair of duplicate spots on the array. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 indicate a significant difference to the control and ## p ≤ 0.01, ### p ≤ 0.001 indicates a significant difference between treatments.

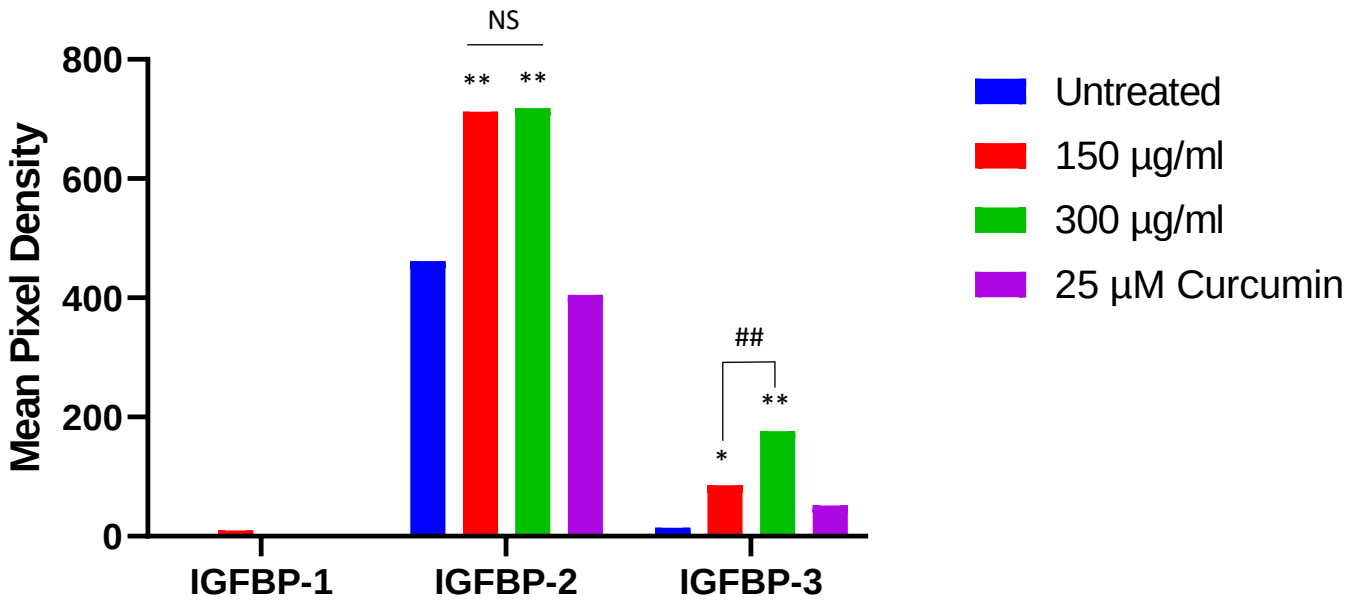


Figure 4.11B: The effect of *M. cardiospermoides* crude methanol extract on the expression levels of angiogenesis factors IGFBP-1, IGFBP-2, and IGFBP-3. MDA-MB-231 cells were treated with 150 or 300 µg/ml of the methanol extract or with 25 µM of curcumin for 24 hours. Protein expression was determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised using the C-DiGit blot scanner, and the signal density of each spot representing the proteins of interest was assessed using the Image Studio Digits Version 5.2 software and GraphPad prism. Data show the mean signal density of each representative pair of duplicate spots on the array. *p ≤ 0.05 and **p ≤ 0.01 indicate a significant difference to the control ##p ≤ 0.01 indicates a significant difference between treatments.

CHAPTER 4

DISCUSSION

As estimated by the International Agency for Research on Cancer (IARC) in the year 2020, on a global scale, 1 in 5 people develop cancer in their lifetime and 1 in 11 women die from the disease (Sung *et al.,* 2021). Breast cancer represents 1 in 4 cancers diagnosed in women worldwide, accounting for 24.5 % (2 261 419) of the new global cases. In South Africa alone, breast cancer is the number 1 ranked diagnosed cancer type and accounts for 14.3 % of all new cancer cases and 8.3 % of all cancer deaths (Ayeni *et al.,* 2020). Metastatic breast cancer poses a greater threat to patients because 90% of all cancer-related mortalities are as a result thereof (Al Deen *et al.,* 2019). Effective treatments of metastatic breast cancer require the inhibition of fundamental metastatic processes and the development of specific preclinical and clinical strategies that do not rely on primary tumour responses. However, conventional clinical drug development strategies generally rely on the demonstration of primary tumour shrinkage, while ignoring the ability to inhibit metastasis (Sampieri and Fodde,2012; Anderson *et al.,* 2019). Thus, there is a continuous attempt at finding novel migrastatics that are not only target specific but also have fewer or no side effects that are known to impair cancer patients' quality of life. Traditional folk remedies from medicinal plants have guided scientists in the search for novel medications for decades, which has led to the discovery of a range of therapeutic properties. There is therefore a growing awareness in connecting phytochemical constituents of medicinal plants with their pharmacological activity. The correlation between phytochemicals and the bioactivity of plants is significant as it aids in the synthesis of compounds with specific activities to treat numerous health ailments and chronic diseases including cancer (Saxena *et al.,* 2013). On this account, this study investigated the anti-metastatic potential of *Momordica cardiospermoides* crude acetone and methanol leaf extracts on MDA-MB-231 breast cancer cells.

Plant extracts are endowed with numerous phytoconstituents, the presence of which is determined by the extractant used, i.e. different extracts of the same plant species may possess different phytoconstituents following extraction with a solvent of a particular polarity (Champakaew *et al.,* 2015). In this study, thin layer chromatography

was employed to determine the fingerprint profiles of *M. cardiospermoides* crude acetone and methanol extracts. The findings revealed that compounds of the acetone extracts were optimally separated in the nonpolar mobile phase (TAE), whereas those of the methanol extract separated best in the polar mobile phase (EMW) **(Figure 4.1).** This suggests that the crude acetone and methanol extracts had more non-polar and polar compounds present, respectively.

Plants extracts or phytochemicals have been studied greatly as they produce desirable physiological activities in humans. These phytochemicals include but are not limited to tannins, flavonoids, terpenoids, saponins, phlabatannins, and coumarins (Kennedy and Wightman, 2011; Saxena *et al.,* 2013). Studies have reported that the aforementioned phytochemicals exhibit a series of biological properties that may influence humans in a health-promoting manner (Barreca *et al.,* 2017; Silva *et al.,* 2020). For instance, flavonoids have been found to possess therapeutic properties such as antioxidant and anti-inflammatory activities, as well as have a positive impact on cancer chemoprotection and chemotherapy (Kikuchi *et al.,* 2019). Qualitative screening of the extracts using standard chemical tests revealed the presence of tannins, flavonoids, terpenoids, saponins, phlabatannins, and coumarins in the methanol and acetone extracts. On the other hand, saponins were found only present in the crude methanol extract (**Table 4.1**). This is because saponins are polar molecules and are therefore better extracted by a polar extractant, which in the case of this study is methanol. The tannin, flavonoid, or phenolic content was quantified and revealed that the highest content of the above-mentioned phytochemical groups was present in the most non-polar extract, the acetone extract, in comparison to the more polar extract, the methanol extract **(figure 4.2.)**. This suggests that the acetone extract was more concentrated with phenolic compounds such as flavonoids and tannins. Moreover, these results also concur that the extractability of the phytochemicals also depends on the extractant used and its polarity (Ahmed *et al.,* 2014), therefore deeming acetone the best extractant in that regard. Quantitative analysis of antioxidant activities of *M. cardiospermoides* crude acetone and methanol extracts revealed that the extracts exhibited an increase in percentage inhibition/DPPH scavenging effect, with an increase in extract concentration **(figure 4.3)**. Furthermore, the acetone extract possessed a higher activity as compared to the methanol extract. These results are in correlation with the quantitative analysis of the extracts (**figure 4.2**) as all the major

phytochemicals found in higher amounts in the acetone extract have antioxidant properties. The upsurge of interest in medicinal plants' antioxidant activity has led to researchers investigating plants further to determine their health-promoting effects *in vitro or* their potential effect to inhibit cancer cell growth and proliferation.

Cancer cells are characterised by their accelerated proliferation rate, which often results in the formation of tumours. Ideally, antineoplastics (cytotoxic drugs) should decelerate tumour formation by inhibiting cell proliferation and viability (Shah and Rawal*,* 2019). Moreover, these antineoplastics should selectively affect the proliferation and viability of cancer cells without showing any toxicity towards surrounding noncancerous cells (Robles-Escajeda *et al.,* 2016; Berning *et al.,* 2019). Therefore, the effects of *M. cardiospermoides* acetone or methanol extracts on MDA-MB-231 cancer cells or HEK-293 cells were assessed. The results (**figure 4.4**) revealed that the methanol extract's cytotoxic activity was selective to cancer cells in comparison to the acetone extract, suggesting that the methanol extract could be a better antineoplastic candidate. According to Manjo and Joris (1995), reduction in cell viability is directly proportional to the induction of cell death, through modes such as apoptosis, necrosis, or both. For that reason, it was paramount to determine the mode of cell death that resulted from the decrease in cell viability.

Cell death can occur in one of two distinct pathways: apoptosis or necrosis. Apoptotic cell death is characterised by condensation of the chromatin, shrinkage of cells, nuclear fragmentation, and externalisation of phosphatidylserine (Fakai *et al.,* 2019). On the other hand, cells undergoing necrosis are characterised by swelling, DNA degradation, plasma membrane dysfunction, and the release of the cytoplasmic contents to the surrounding microenvironment (Lee *et al.,* 2018), which may affect neighbouring cells. The acridine orange/ethidium bromide dual staining assay, which was employed to determine extract-induced cell death, exploits these features to differentiate viable cells from those undergoing different stages of apoptotic cell death or necrosis. As seen in **figure 4.5**, both the acetone and methanol extracts induced morphological and nuclei changes viz. chromatin condensation and loss of membrane function, which are associated with apoptotic cell death. The data, therefore, suggested that the extracts promote MDA-MB-231 cell apoptosis. Although nuclei and cellular morphological changes in the acetone extract-treated cells suggested

apoptosis as the mode of cell death induced by the extract, the Annexin V-FITC/PI data suggested that the acetone extract induced necrotic cell death as well (**figure 4.6A**). This is due to the simultaneous staining of a high percentage of cells with propidium iodide (PI) and annexin V-FITC, because of the loss of membrane function. In correlation with **Figure 4.5**, the results (**figure 4.6B**) also suggested that treatment with the methanol extract induced the externalisation of phosphatidylserine in MDA-MB-231 cells, as indicated by the increase in annexin V-FITC positive cells. Unlike necrosis, apoptosis is an important cell toxicity pathway used to investigate new antitumor agents. As such, the use of the acetone extract was discontinued for proceeding experiments as it proved to be non-selectively cytotoxic and induced necrotic cell death. Furthermore, concentrations 150 µg/ml and 300 µg/ml of the methanol extract were deduced as the best working concentrations for further metastatic assays because they were proven to be non-cytotoxic and induced apoptotic cell death to a minimal degree.

Metastasis is an essential marker of malignant tumours that affects not only the prognosis but also the therapeutic effect and survival time of breast cancer patients (Sun *et al.,* 2019). Cell migration, a major step in the metastatic cascade, plays a key role in both normal and pathological conditions. The study of the cell migration process and its underlying mechanisms is significant in varying fields of research including but not limited to basic biology and pharmaceutical development (Kramer *et al.,* 2013). Consequently, the scratch wound healing assay was utilised to evaluate the effect of the methanol extract on MDA-MB-231 cell migration. The results revealed that the methanol extract significantly suppressed MDA-MB-231 cells migration (**figure 4.7A**). The ability of tumour cells to migrate is partially controlled by proteolytic enzymes. These enzymes represent all classes of proteases, including serine and cysteine proteases. Urokinase plasminogen activator (uPA) initiates a proteolytic cascade that culminates in ECM degradation. It stimulates the production of plasmin, which in turn activates the fibrinolytic pathway and extracellular matrix degradation, leading to enhanced tumour cell migration (Heissig *et al.,* 2016; Kwaan and Lindholm*,* 2019). Serpin E1 is the main regulator of uPA and is also involved in cell migration (Fortenberry 2015). Several studies have reported the up-regulation of serpin E1 expression in many tumour types, validating it as a marker for therapy decision-making in node-negative breast cancer patients (Binder and Mihaly*,* 2008; Pavon *et al.,* 2016;

Zhou *et al.,* 2020). Therefore, the inhibition of the expression of serpin E1 and uPA could serve as an essential strategy for inhibiting cell migration. Based on the observed anti-migrative effects (**figure 4.7A** and **4.7B**), the effect of the methanol extract on the expression of serpin E1 and uPA was assessed. Interestingly, an upregulation in the expression of uPA was observed, coupled with a significant downregulation in the expression of its inhibitor serpin E1 (**figure 4.11**). Therefore, the inhibition of MDA-MB-231 cell migration could be a result of the decrease in serpin E1 expression. Matrix metalloproteinases are major mediators of ECM and basement membrane degradation (i.e. cell invasiveness) and are overexpressed in various cancer tissues (Hofmann *et al.,* 2005; Bonnans *et al.,* 2014). As such, inhibiting their expression may serve as a vital mechanism for inhibiting metastasis. Therefore, the effect of *M. cardiospermoides* methanol extract on MMP-2 and MMP-9 was assessed (**figure 4.8**) and revealed a significant downregulation in their expression. The proteolytic activity of MMPs is specifically regulated by small cysteine-rich proteins known as tissue inhibitors of metalloproteinases (TIMPS). These proteins have been reports to inhibit the proteolytic activity of MMPs by forming 1:1 stoichiometric complexes and regulate the balance of matrix remodelling during ECM degradation (Song *et al.,* 2016). Particularly, TIMP-1 binds and specifically regulates the expression of MMP-9, and has therefore been thought to be crucial for tumour migration and invasion (Chen *et al.,* 2017). As such, the effect of the methanol extract on TIMP-1 expression was assessed (**figure 4.10**) and revealed an upregulation in its expression, inferring the downregulation in MMP-9 to be a result thereof. The data also suggest that the *M. cardiospermoides* methanol extract contains phytochemical that inhibits tumour cell migration and invasion by modulating serpin E1, MMP-2 and -9 and as well as TIMP-1 protein expression.

Cell adhesion is a fundamental phenomenon essential for all multicellular organisms and necessary for cell motility and tumour cell invasiveness (Stuelten *et al.,* 2018; De la Fuente and Lopez*,* 2020). The approach to studying cell adhesion is particularly interesting as it is a relevant phenotype when investigating the effects of novel therapeutic drugs during metastatic progression. Despite numerous studies elucidating the effect that loss of interaction between cells and the ECM may have on the progression of metastatic cancer, the results obtained are drug, compound, or extract dependent (Perez-Sanchez *et al.,* 2019). In this study, the methanol extract

inhibited cell adhesion, as seen by the decrease in the number of cells stained with crystal violet (**figure 4.9 A**). The data, therefore, suggest that the *M. cardiospermoides* methanol extract inhibited MDA-MB-231 cells adhesion, rendering the extract a potential candidate as an anti-metastatic treatment thus far.

The formation of new blood vessels from pre-existing micro-vasculature, also known as angiogenesis, occurs normally during physiological and reparative processes (Nasrollahzadeh *et al.,* 2020). it has therefore been proposed and employed not only as a prognostic indicator in various types of cancer (e.g. breast cancer) but as a target for therapeutic intervention as well (Madu *et al.,* 2020). Essentially, the effect of *M. cardiospermoides* crude methanol extract on the expression of angiogenesis-related factors was investigated. The results (**figure 4.10**) showed an up-regulation in the expression of anti-angiogenic proteins TIMP-1 and thrombospondin-1. Thrombospondin-1 (TSP-1) exerts its inhibitory effect on angiogenesis through its interaction with CD36, a receptor that marks the surface of endothelial cells. The interaction between TSP-1 and CD36 activates a sequence of events that finally results in endothelial cell apoptosis, thereby inhibiting angiogenesis (Zhang *et al.,* 2021). Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a member of a family of secreted proteins that regulate matrix metalloproteinase (MMP) activity by specifically binding to the catalytic domain of the active enzyme. Prior studies have reported inhibition of MMP activity, through upregulation in the expression levels of TIMP-1, to suppresses angiogenesis *in vitro* and *in vivo* (Kim *et al.,* 2018; Zhang *et al.,* 2018; Sanchez-Martin *et al.,* 2019). This is because angiogenesis requires MMPs to digest the extracellular matrix barrier underlying the endothelial cell layers of blood vessels.

Proteins that promote angiogenesis also exist and are known as pro-angiogenic factors or angiogenic factors. These angiogenic factors range from those that activate endothelial cell growth and mobility such as vascular endothelial growth factor (VEGF), to other gene products such as transcription factors that are essential during new vessel formation (Bouvard *et al.,* 2015). In this study, a concentration-dependent downregulation of serpin E1 expression levels, as well as an upregulation of urokinase (uPA) expression levels were observed **(figure 4.11)**. Studies have elucidated the participation of the fibrinolytic system in angiogenesis. The activation of this system depends on the conversion of pro-enzyme plasmogen to plasmin (an enzyme that

degrades blood plasma proteins including fibrin clots) by protein uPA (Gonzalez-Miguel *et al.,* 2016). *Serpin E1* is a protein-coding gene and is the main inhibitor of uPA. Diseases associated with serpin E1 include Plasminogen Activator Inhibitor-1 Deficiency. This inhibitory relationship between uPA and its inhibitor serpin E1 is observed in the results obtained. The findings suggested that *M. cardiospermoides* crude methanol extract downregulated the expression levels of inhibitor serpin E1, therefore leading to the upregulation of uPA. Moreover, the paradox of uPA and its inhibitor serpin E1 producing similar effects (pro-angiogenesis) is solved by the identification of serpin E1 activated signalling pathways independent of uPA (Binder and Mihaly 2008). **Figure 4.11** also showed an upregulation of vascular endothelial growth factor (VEGF). Preclinical models have proposed that VEGF inhibition may regress existing tumour vessels, resulting in a reduction in tumour volume and weight, and although not acquired in the current study, proper regulation of the expression of VEGF may have significant positive clinical implications.

An upregulation of IGFBP-1, IGFBP-2, and IGFBP-3 expression was observed **(figure 4.11B)**. IGFBP-1 is locally expressed by blood vessels and has been reported to have an influence on cellular processes including migration and enhancement of tube formation. Upregulation of the second abundantly expressed IGFBP in the circulation, IGFBP-2, is a signature of many tumours and has been heavily implicated in tumour angiogenesis. Many studies have reported the upregulation of IGFBP-2 to result in the upregulation of VEGF mRNA transcription and upregulation of NF-κB through the activation of integrin-linked kinase pathways (Yao *et al.,* 2016; Slater *et al.,* 2019; Li *et al.,* 2020). That said, the current study infers that the upregulation of the VEGF protein observed in **figure 4.11A** is also a result of the upregulation of IGFBP-2. On the other hand, IGFBP-3, which is abundantly produced in the liver, has been reported to possess both anti-angiogenic and angiogenic activities (Haywood *et al.,* 2019). It has been reported to induce the expression of numerous angiogenesis-related genes including MMP-2, MMP-9, and VEGF, both *in vitro* and *in vivo*, in HUVECs (Slater *et al.,* 2019). However, there is equally significant evidence for IGFBP-3 as an anti-angiogenic factor. IGFBP-3 has been reported to inhibit angiogenesis in both non-small lung cancer and squamous carcinoma of the neck and head, as well as inhibit endothelial cell-to-ECM adhesion, which is essential in angiogenesis (Fang *et al.,* 2014). A wide variety of factors may initiate the cascade of events leading to

angiogenesis, and as seen in the current study, these factors may work in synergism to produce the same (such as uPA and serpin E1 shown in **figure 4.11A**) results. An antagonistic relationship between these factors is expected to exist as well, regardless of their anti-angiogenic or angiogenic nature. In physiological conditions, the overall lack of angiogenesis results from the balance of a complex multifactorial system constituted by inhibitors and stimulators of angiogenesis. The dominant theory is that an imbalance between anti-angiogenic and angiogenic proteins may lead to a switch toward an angiogenetic phenotype. On account of the multitude of angiogenetic signals triggered by tumour cells, it is unlikely that a straightforward inhibition of angiogenic stimuli will be effective as an approach to anticancer therapy (Tonini *et al.,* 2003; Zachary and Morgan*,* 2011)..

In conclusion, the current study demonstrated the potential of *M. cardiospermoides* crude methanol extract as an effective anti-metastatic agent.

CHAPTER 5

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