INVESTIGATION OF THE ANTI-INFLAMMATORY AND ANTI-INVASIVENESS EFFECTS OF *MOMORDICA BALSAMINA* LEAF METHANOL EXTRACT USING TRIPLE-NEGATIVE BREAST CANCER CELL LINE MDA-MB-231.

by

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at the

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DECLARATION

I, Tshwarelo Evidence Mohale, hereby declare that the study titled **Investigation of the anti-inflammatory and anti-invasiveness effects of *Momordica balsamina* leaf methanol extract using triple-negative breast cancer cell line MDA-MB-231** is my own work in design and execution. This dissertation is submitted to the University of Limpopo for the fulfilment of a Master of Science in Biochemistry and has not been previously submitted by me at this or any other institution. All sources used have been indicated and duly acknowledged.

Signature:  Date: 26 May 2023

DEDICATION

I dedicate this work to my daughter, **Molebogeng Destiny Mohale** and my mother, **Motlalahohle Johanna Mohale**.

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LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
Akt	Ak strain transforming
ANGPTL4	Angiopoietin-like 4
ATCC	American type culture collection
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
bFGF	Basic fibroblast growth factor
$CD8^+$	Class of differentiation 8^+
CO_2	Carbon dioxide
COX	Cyclooxygenase
CSC	Cancer stem cell
CSF	Colony stimulating factor
CTC	Circulating tumour cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTC	Disseminated tumour cells
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
Gp130	Glycoprotein 130

HER-2	Human epidermal growth factor
HIF	Hypoxia-induced factor
HR	Hormone receptor
IL	Interleukin
IL-6Rα	Interleukin-6-specific binding receptor alpha
JAK	Janus kinase
Lox	Lipoxygenase
M. balsamina	*Momordica balsamina*
MAPK	Mitogen associated protein kinase
MBE	Basement membrane extract
MET	Mesenchymal-epithelial transition
MMC	Mitomycin C
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane-type matrix metalloproteinase
Myc	Master regulator of cell cycle entry and proliferation metabolism
N-cadherin	Neural cadherin
NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
PBS	Phosphate buffered saline
PI	Propidium iodide
PI3K	Phosphoinositol-3-Kinase
PS	Phosphatidylserine

PVDF	Polyvinylidene difluoride
Ras	Rat sarcoma
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitors of matrix metalloproteinase
TME	Tumour microenvironment
TNBC	Triple-negative breast cancer
Twist	Twist-related protein
TYK	Tyrosine-protein kinase
VEGF	Vascular endothelial growth factor
WHO	World health organization
Zeb	Zinc-finger E-box binding homeobox

ABSTRACT

Cancer is the world's second leading cause of death, with current treatments accompanied by a myriad of undesirable side effects. Medicinal plants contain chemoprotective and more tolerable phytochemicals for cancer treatment, making them a better alternative to current cancer treatments. Thus, many indigenous plants have yet to be investigated for anticancer properties. In this study, the *Momordica balsamina leaf methanol extract* was evaluated for its potential anti-inflammatory and anti-invasiveness effects in interleukin-6-activated triple-negative MDA-MB-231 breast cancer cells. The dried ground leaves of *M. balsamina* were subjected to maceration in absolute methanol to obtain a crude *M. balsamina* leaf methanol extract. The Muse® cell count and viability assay was used to assess the effect of the *M. balsamina* leaf methanol extract on the viability of breast MDA-MB-231 and the kidney HEK-293 cells. The methanol extract induced a significant decrease in MDA-MB-231 and HEK-293 cell viability at 150 µg/mL and 125 µg/mL, respectively. The annexin-V-FLUOS assay was performed to determine the mode of cell death induced by the extract and to further confirm lack of significant cytotoxicity in MDA-MB-231 cells exposed to 75 or 100 µg/mL of the extract. The findings revealed that the reduction in extract-treated MDA-MB-231 cell viability was associated with a non-significant induction of apoptotic cell death. The assessment of the effect of the methanol extract on nitric oxide production using nitric oxide assay revealed that the extract induced nitric oxide production in IL-6-activated MDA-MB-231 cells. Wound healing and transwell cell invasion assays were employed to evaluate the effect of the methanol extract on the migration and invasiveness of IL-6-activated MDA-MB-231 cells, respectively. The results revealed that the methanol extract significantly inhibited the IL-6-induced migratory and invasive potential of IL-6-activated MDA-MB-231 cells. Western blot analysis showed that the methanol extract decreased MMP-2, MMP-9 and vimentin protein expression and increased TIMP-3 protein expression levels in IL-6-activated MDA-MB-231 cells. In addition, gelatin-zymography also showed that the methanol extract significantly decreased the proteolytic activity of MMP-2 and MMP-9 in IL-6-activated MDA-MB-231 cells. Furthermore, an increase in *Bax* and decrease in *Bcl-2*, *JAK2*, *STAT3, MMP-2 and MMP-9* mRNA expression, was observed in extract-treated IL-6-activated MDA-MB-231 cells as determined by quantitative reverse transcriptase

polymerase chain reaction (qRT-PCR). The adhesiveness of IL-6-activated MDA-MB-231 cells to extracellular matrix (ECM) proteins was assessed using the cell adhesion assay and CHEMICON® ECM-cell adhesion array kit, respectively. The results showed that the methanol extract significantly inhibited the IL-6-induced MDA-MB-231 cell adhesiveness. This could be associated with the inhibition of the attachment of IL-6-activated MDA-MB-231 cells to collagen I, II, IV, laminin, tenascin, fibronectin, and vitronectin. In conclusion, the *M. balsamina* leaf methanol extract inhibited the IL-6-induced MDA-MB-231 cell invasiveness, migration, and adhesiveness through the modulation of the IL-6/JAK2/STAT3 pathway.

CHAPTER ONE

1. Introduction

Breast cancer is the most frequent type of malignancy and the primary cause of cancer deaths among women globally (AlGhalban *et al*., 2021). It is considered the world's most common health problem among various populations due to its high recurrence rates (Yari *et al*., 2014). Of the 19.3 million new cancer cases estimated in 2020, 2.3 million were attributed to breast cancer making it the 1st leading form of diagnosed cancer globally with approximately 684 996 deaths (Sung *et al*., 2021). Breast cancer is diverse and classified into three main subgroups for better detection and treatment viz, human epidermal growth receptor-2 (HER2) positive, hormone receptor (HR) positive and triple-negative breast cancer (TNBC) (Gu *et al*., 2016; Yin *et al*., 2020). Triple-negative breast cancer is the most malignant and least treatable breast cancer subtype as well as associated with a dismal prognosis, high metastatic and recurrence rates (Xie *et al*., 2019). Furthermore, TNBC is characterised by a negative expression of receptors for oestrogen (ER), progesterone (PR), and HER2 (Lee *et al*., 2019; Nordin *et al*., 2021). Due to the lack of expression of hormone receptors, TNBC is not subjective to hormonal treatment leaving sequential single-agent chemotherapy as the only viable treatment for TNBC (Bardia *et al*., 2019; Yin *et al*., 2020). However, chemotherapy does not provide adequate therapeutic effects due to cancer cell drug resistance (AlGhalban *et al*., 2021) making the management of TNBC limited due to inadequate treatment options. Moreover, TNBC is the only major subgroup for which no specific targeted treatment has received approval from the United States Food and Drug Administration (Bartholomeusz *et al*., 2012; Gu *et al*., 2016).

Chronic inflammation promotes tumour development and progression as well as metastasis and drug resistance (Kumari *et al*., 2016; Lan *et al*., 2021). Breast cancer associated with chronic inflammation through the overexpression of IL-6 and signal transducer and activator of transcription 3 (STAT3) was reported in more than 50% of breast cancers (Lee *et al*., 2019; Masjedi *et al*., 2018). The tumour and/or stromal cells maintain an inflammatory tumour microenvironment (TME) by secreting pro-inflammatory cytokines and chemokines, thereby promoting epithelial-mesenchymal transition (EMT) which drives tumour progression and metastasis (Kumari *et al*.,

2016). Pro-inflammatory cytokines stimulate tumour cell growth by activating nuclear factor kappa B (NF-κB) and STAT3 pathways, which increase the expression of cytokines, chemokines, and anti-apoptotic chemokines (Kumari *et al*., 2016; Liubomirski *et al*., 2019). The NF-κB and STAT3 pathways phosphorylate tumour suppressor genes resulting in increased tumour cell proliferation and progression. Nuclear factor kappa B and STAT3 activation upregulate anti-apoptotic and pro-angiogenic mediators such as MMP-9, which enhances tumour invasion and migration by promoting angiogenesis, EMT and extracellular matrix (ECM) degradation (Kumari *et al*., 2016; Liubomirski *et al*., 2019; Scheau *et al*., 2019). Increased motility of tumour cells and compromised ECM promote primary tumour cell dissemination to distant organs through the lymphatic and/or blood systems and account for more than 90% of breast cancer-related deaths (Medeiros and Allan, 2019; Tang *et al*., 2021). Therefore, there is a need to screen and develop agents that are safe and efficiently targeted for TNBC as well as breast cancer metastasis that is fuelled by inflammation by targeting the IL-6/STAT3 pathway.

According to the World Health Organization (WHO), traditional medicine is used by over 80% of the world's population in developing countries (Palhares *et al*., 2015; Tesfahuneygn and Gebreegziabher, 2019). Medicinal plants are a vital source of new medicine globally, accounting for 25% of all prescribed medications in developed countries (Chen *et al*., 2016). Moreover, there is an increasing demand for traditional medicine as they are a cheap alternative source for primary health care (Mahomoodally, 2013).

Momordica balsamina L. also known as balsam apple, is a perennial climber of the family of Cucurbitaceae, native to all South African provinces except the Western Cape (Mshelia *et al*., 2017; Souda *et al*., 2018). It exhibits multiple medicinal effects including anti-cancer and anti-inflammatory, antiviral, and hypoglycaemic properties attributed to the availability of flavonoids, alkaloids, saponins, and cardiac glycosides in all parts of the plant (Thakur *et al*., 2011). Methanolic leaf extract of *M. balsamina* was reported to possess polyphenols such as flavonoid glycosides and phenolic acids and to decrease HT-29 colon cancer cell viability (Venter *et al*., 2021). Polyphenols are known to have antioxidant, anti-proliferative and anti-inflammatory effects. Furthermore, the *M. balsamina* leaf acetone extract has been reported to exert inhibitory effects on the invasiveness and migratory activities of HT-29 colorectal

cancer cells (Serala *et al*., 2021). Thus, the study aimed to investigate *M. balsamina* as a potential source of compounds or possible treatment for metastatic TNBC fuelled by inflammation.

1.1. Aim of the study

To investigate the potential anti-inflammatory and anti-invasiveness effects of *Momordica balsamina* leaf methanol extract on triple-negative breast cancer cell line MDA-MB-231.

1.2. Objectives

The objectives of the study were to:

i. investigate the effect of *M. balsamina* leaf methanol extract on the viability of MDA-MB-231 and human embryonic kidney (HEK-293) cells using the MUSE cell count and viability assay.
ii. assess the mode of cell death induced by the *M. balsamina* leaf methanol extract in MDA-MB-231 cells using Annexin-V-FLUOS staining assay.
iii. investigate the effect of *M. balsamina* leaf methanol extract on the production of intracellular nitric oxide in IL-6-activated MDA-MB-231 cells.
iv. assess the effect of the *M. balsamina* leaf methanol extract on the invasiveness of IL-6-activated MDA-MB-231 cells using the cell invasion assay.
v. assess the effect of the *M. balsamina* leaf methanol extract on the migration of IL-6-activated MDA-MB-231 cells using the scratch assay.
vi. determine the effect of the *M. balsamina* leaf methanol extract on the MMP-2 and MMP-9 activity IL-6-activated MDA-MB-231 cells using gelatin-zymography assay.
vii. evaluate the effect of *M. balsamina* leaf methanol extract on the mRNA expression of *Bcl-2, Bax, MMP-2, MMP-9, JAK2* and *STAT3* in IL-6-activated MDA-MB-231 cells using quantitative Real-Time PCR.
viii. evaluate the effect of *M. balsamina* leaf methanol extract on the protein expression levels of MMP-2, MMP-9, TIMP-3, and Vimentin in IL-6-activated MDA-MB-231 cells using Western blotting.
ix. assess the effect of *M. balsamina* leaf methanol extract on the adhesiveness of IL-6-activated MDA-MB-231 cells using cell adhesion assay.

x. assess the effect of *M. balsamina* leaf methanol extract on the attachment of IL-6-activated MDA-MB-231 cells to ECM proteins using CHEMICON® ECM Cell Adhesion Array Kit.

CHAPTER TWO

2. Literature Review

2.1. Cancer

Cancer is one of the major contributors to increased morbidity and mortality worldwide, with its enormous incidence rates continuously increasing yearly. In South Africa, an annual 3-4% increase in new cancer cases was reported (Sung *et al*., 2021). In 2020, 1.4 million cancer cases were estimated with mortality rates (7.2%) being higher than the incidence rates (5.7%) in Africa, indicating the fatality of cancer in this region. In 2018, following lung cancer, breast cancer was the second leading form of frequently diagnosed cancer worldwide, however; in 2020 with 2.3 million new cancer cases and 684 996 deaths, breast cancer was reported to be the most diagnosed form of cancer and the fifth leading cause of cancer mortality globally (Bray *et al*., 2018; Sung *et al*., 2021).

Triple-negative breast cancer typically affects younger women accounting for 15-20% of all diagnosed breast cancers and 25% of breast cancer-related deaths, with median overall survival of 13 months (Shan *et al*., 2019; Sinka *et al*., 2018; Yin *et al*., 2020). It is linked to a dismal prognosis, high metastatic rates, higher recurrence rates, and it is the least treatable due to regular drug resistance (Xie *et al*., 2019). TNBC is highly invasive, and at the initial stages of development, the lymphatic vessels and reflux veins in the breasts provide a highway for metastatic progression, predominantly to the brain, lungs, and liver (Li *et al*., 2017; Yin *et al*., 2020). Furthermore, it is associated with early relapse with a 25% chance of recurrence following treatment and about 75% chance of death within 3 months of recurrence (Yin *et al*., 2020).

2.2. Cancer development and inflammation

Cancer development is multifactorial, with epigenetic alterations, secretion of pro-inflammatory cytokines and chemokines that cause the recruitment of immune cells and stimulate the inflammatory process, being some of the contributing factors (Crusz and Balkwill, 2015; Munn, 2017). The recruited immune cells secrete more pro-inflammatory mediators in the TME creating a favourable inflammatory microenvironment for the development of cancer (Munn, 2017). The continuous growth of tumour cells can inflict physical damage to the surrounding normal tissue

resulting in the release of damage-associated molecular patterns (DAMPs). The release of DAMPs activates granulocytes leading to the secretion of pro-inflammatory mediators and reactive oxygen species (ROS) in response to tumour growth (Lan *et al*., 2021; Sirotković-Skerlev *et al*., 2012). Furthermore, the growth also causes hypoxia by compressing the lymphatic and blood vessels thereby inducing angiogenic growth factors and recruiting more blood vessels for nutrient and oxygen supply (Lan *et al*., 2021; Munn, 2017). Therefore, cancer can also induce inflammation as oncogenic transformations in malignant cells leads to inflammatory pathways in the cells (Crusz and Balkwill, 2015).

Inflammation is a biological response to infectious agents, tissue damage, or cellular stress that activates repair mechanisms by recruiting immune cells to contain the pathogenic agents and restrict further tissue damage (Landskron *et al*., 2014; Munn, 2017). The main recruited cells to the infectious site are macrophages and neutrophils, which then produce ROS and reactive nitrogen species (RNS), cytokines and chemokines to alleviate the infectious agents and prevent tissue damage (Lan *et al*., 2021; Piotrowski *et al*., 2020; Qian, 2017). Acute inflammatory response immunosurveillance inhibits tumorigenesis by inducing an immune response against transformed cells (Sullivan., 2011). However, unregulated inflammation can become chronic causing malignant cell transformation in the surrounding tissue (Landskron *et al*., 2014).

Chronic inflammatory response induces tumourigenesis and favours all stages of tumour development which are initiation, promotion, and progression (Piotrowski *et al*., 2020). About 25% of all cancer cases are instigated by inflammation, which is the 7th hallmark of cancer (Lan *et al*., 2021; Multhoff *et al*., 2012). Inflammation leads to the activation of various pro-inflammatory transcription factors such as STAT3, NF-κB, and hypoxia-inducible factor (HIF-1α) within the tumour cells. These activated transcription factors upregulate the expression of chemokines and cytokines that maintain a network of inflammatory responses within the TME (Crusz and Balkwill, 2015).

A significant promoter and key mediator of the inflammatory response is NF-κB, a transcription factor that regulates inflammation through inflammatory mediators (Multhoff *et al*., 2012). Nuclear factor kappa B orchestrates the expression of ROS,

RNS, cytokines (IL-1. IL-6, and TNF-α), chemokines (IL-8), and growth factors, all of which play an essential role in the inflammatory TME (Sonnessa *et al*., 2020). The activation of cytokine receptors and intracellular signalling by NF-κB accelerates tumour progression (Esquivel-Velázquez *et al*., 2015). Signal transducer and activator of transcription 3 overexpression has been linked to TNBC and enhances tumour-promoting inflammation by upregulating the expression of pro-inflammatory cytokines, growth factors, and pro-angiogenic factors, all of which enhance tumour growth and progression. In addition, the binding of STAT3 mediators to their corresponding receptors results in the hyperactivation of STAT3 resulting in the suppression of anti-tumour immunity (Johnson *et al*., 2018; Multhoff *et al*., 2012).

2.2.1. Cytokines

Cytokines are low-molecular weight secretory proteins that regulate intracellular communication in the immune system as well as pathological conditions such as cancer (Bonati and Tang, 2021; Esquivel-Velázquez *et al*., 2015). They are subtyped into distinct groups namely interleukins, chemokines, interferons, tumour necrosis factors, and colony-stimulating factors (CSF) (Bonati and Tang, 2021). Cytokines are highly inducible and elicit their function by binding to their respective receptors. The cytokine-receptor interaction results in the downstream activation of signalling transcription factors such as STAT3 and NF-κB (Adekoya and Richardson, 2020). Upon secretion, cytokines act in either an autocrine or paracrine manner to mediate communication between the tumour and host cells (immune, endothelial, and /or stromal cells), this activates signalling pathways that regulate tumour growth. Cytokines primarily regulate cell division, migration, and apoptosis (Crusz and Balkwill, 2015; Sağlam *et al*., 2015). Additionally, cytokines exhibit pleiotropic effects on the tumour and host cells, to mediate various cancer hallmarks including EMT and enhancement of metastasis, which sustain the progression of cancerous cells (Crusz and Balkwill, 2015).

2.2.2. Interleukin-6

Interleukin-6 is a soluble, multifunctional pleiotropic cytokine responsible for a variety of biological processes within and outside the immune system. It is the major mediator of acute and chronic inflammation and exhibits both pro-inflammatory and anti-inflammatory effects (Fujihara *et al*., 2020; Rose-John, 2020). IL-6 is highly regulated and produced by different immune cells such as macrophages, endothelial cells,

fibroblasts, T and B-cells. The dysregulation of IL-6 expression is associated with the onset and maintenance of various inflammatory diseases such as rheumatoid arthritis and cancer (Fujihara *et al*., 2020). Interleukin-6 is a primary regulator of several types of cancers including breast cancer. This is achieved by modulating genes responsible for the progression of the cell cycle and anti-apoptotic pathways which in turn promote the survival, progression, and migration of DNA damaged cancerous cells (Rose-John, 2020; Sirotković-Skerlev *et al*., 2012).

Interleukin-6 exhibits biological function by binding to its receptors using two different IL-6 signalling pathways namely the classical- and trans- signalling pathways (Johnson *et al*., 2018; Razidlo *et al*., 2018). The IL-6 receptor complex is made up of IL-6-specific binding receptor α (IL-6Rα) and signal transducing coreceptor glycoprotein-130 (gp130), whereby the IL-6 receptor exists in both transmembrane and soluble forms (Razidlo *et al*., 2018; Reeh *et al*., 2019). The soluble IL-6Rα (sIL-6Rα) is generated from proteolytic cleavage of the IL-6Rα by zinc-dependent metalloproteinases, a disintegrin and metalloprotease 17 (ADAM17) or alternative splicing of the IL-6Rα mRNA. The classical signalling pathway is denoted by binding of IL-6 to the membrane bound IL-6Rα (mIL-6Rα) while the trans-signalling pathway is denoted by the binding of the IL-6 to the sIL-6Rα. Classical signalling occurs during acute-phase inflammatory response and induces anti-inflammatory effects and is limited to only hepatocytes and leucocytes as they are the only cells expressing mIL-6Rα, while the *trans*-signalling dominates in the tumour microenvironment, inducing pro-inflammatory effects and predominant in various cells that express the gp130 even without IL-6Rα expression (Kumari *et al*., 2016; Reeh *et al*., 2019). Generation of sIL-6Rα increases IL-6 spectrum on cells as all cells express the gp130 with only a few expressing the mIL-6R, and the IL-6/sIL-6Rα complex can bind with the co-receptor gp130 on cells without the IL-6R (Schumacher and Rose-John, 2022).

The IL-6 signalling occurs when IL-6 binds to its specific receptor either through the classical- or trans-signalling pathway. The IL-6/IL-6Rα binary complex then binds to the signal-transducing co-receptor gp130, leading to its dimerization and activation of its cytoplasmic tyrosine kinases which are responsible for intracellular signalling by activating and phosphorylating various transcription factors (Johnson *et al*., 2018; Li *et al*., 2020; Sun *et al*., 2014). The IL-6/IL-6Rα/gp130 heterohexameric complex initiates intracellular signalling by phosphorylating and activating members of the

Janus kinase (JAK) family namely JAK1, JAK2, and tyrosine-protein kinase 2 (TYK2) (Jin, 2020; Xie *et al*., 2019). This results in the subsequent activation of various pathways including the JAK/STAT3, phosphoinositol-3-kinase/Akt (PI3K/Akt), and Ras/mitogen associated protein kinase (Ras/MAPK) pathways (**Figure 2.1**) (Razidlo *et al*., 2018; Sun *et al*., 2014).

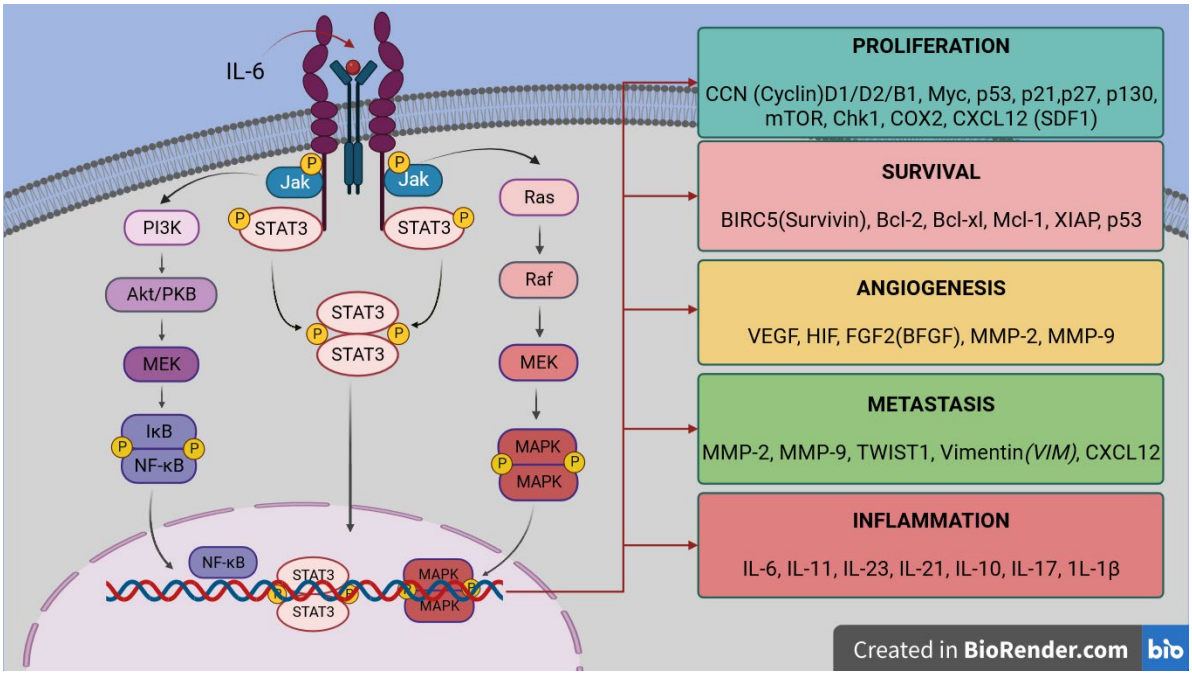


Figure 2.1: A schematic representation of the role of IL-6 signalling in the development of cancer. The binding of IL-6 to its receptor leads activation of various signalling pathways which subsequently leads in activation of transcription factors such as STAT3, NF-κB, and MAPK. These transcription factors target genes that results in expression molecules maintain cancer cell proliferation and survival, and those that drive angiogenesis, metastasis, and inflammation. The figure was adapted from Masjedi *et al*. (2018), and created using BioRender.com.

2.2.3. IL-6/JAK2/STAT3 pathway

Interleukin-6 and STAT3 are constitutively overexpressed and activated in triple-negative breast cancer and they mediate tumour cell survival, progression, invasion, metastasis, immunosuppression, and poor patient outcome (Banerjee and Resat, 2016; Johnson *et al*., 2018; Qin *et al*., 2019). Upon phosphorylation by the IL-6/IL-6R/gp130 complex, the phosphorylated JAK2 (pJAK2) activates the STAT3 transcription factor via phosphorylation, and the phosphorylated STAT3 (pSTAT3) then dimerizes and translocates from the cytoplasm to the nucleus where it mediates

the transcriptional expression of STAT3 target genes *(Razidlo et al., 2018; Reeh et al*., 2019). The STAT3 target genes include genes that play a role in apoptosis (*Bcl-2, Bax and Survivin*), cell division cycle (*Cyclin D1 and c-Myc*), metastasis (*MMP-2, MMP-9, vimentin, and Twist*), angiogenesis (*VEGF, HIF-1α*) and immune evasion (*IL-6, IL-10, and TGF-β*) (Banerjee and Resat, 2016; Jin, 2020; Qin *et al*., 2019). The transcriptional activation of IL6/JAK2/STAT3 and the above-mentioned genes drive inflammatory-mediated tumour cell progression, metastasis, and immune suppression in TNBC.

2.3. Metastasis

Metastasis is a multistep, organ-selective process that begins with tumour cell dissemination from primary lesions and ends with secondary colonisation of tumour cells in distant sites (Majidpoor and Mortezaee, 2021; Medeiros and Allan, 2019). It accounts for more than 90% of breast cancer-related deaths, with the dissemination occurring through the lymphatic and/or haematogenous system (Medeiros and Allan, 2019; Tang *et al*., 2021). For a successful metastatic cascade, tumour cells must detach from the primary lesion, intravasate into the lymphatic and/or haematogenous system, evade immune surveillance and anoikis, extravasate into distant capillary beds, invade and colonise at the secondary site (**Figure 2.2**) (Majidpoor and Mortezaee, 2021; Seyfried and Huysentruyt, 2013).

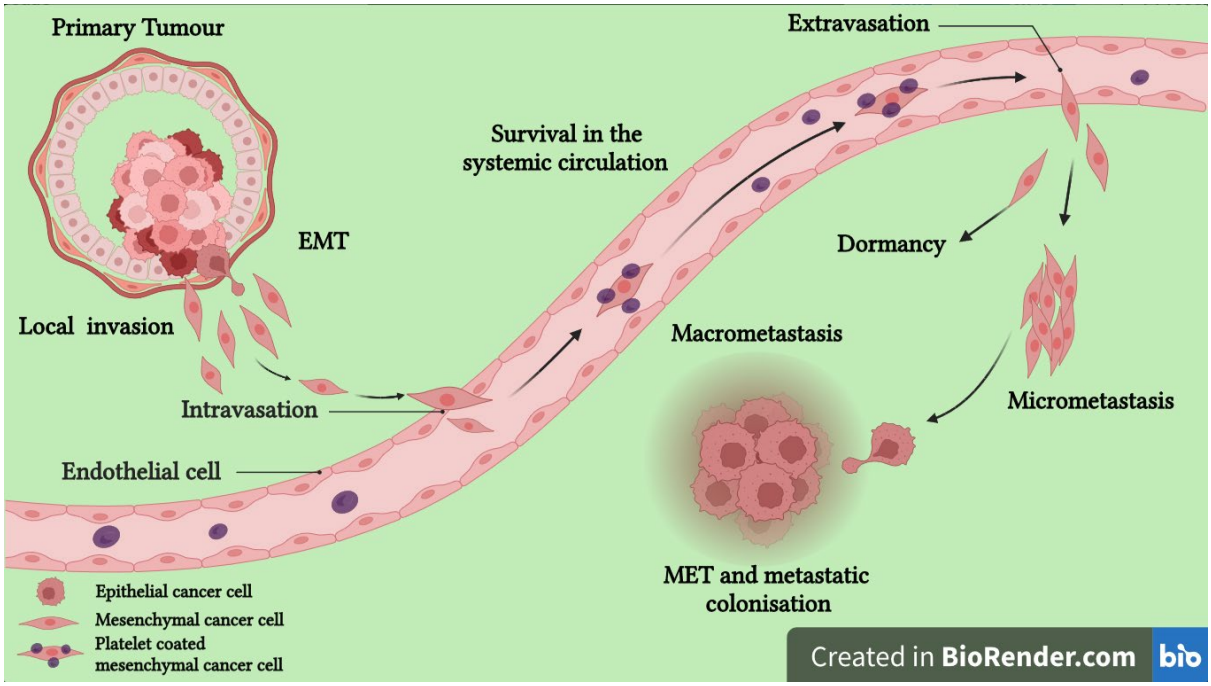


Figure 2.2: The Metastatic cascade. Cancer cells undergo morphological transformation through epithelial-to-mesenchymal transition (EMT) process to invade the neighbouring tissue parenchyma and then intravasate into the blood circulatory system. In the circulation, they associate with the immune cells such as platelets for survival, resist anoikis, and then attach to the capillary bed of the blood vessels and extravasate the circulatory system to the target site where they undergo MET and form colonies that develop into clinically detectable macro-metastasis. The figure was adapted from Datta *et al.*, (2021), and created using BioRender.com.

2.3.1 Epithelial-mesenchymal transition and tumour cell invasion

For a local invasion to occur the stationary epithelial tumour cells must acquire migratory and invasive characteristics to disseminate from the primary tumour and invade neighbouring tissue parenchyma (Majidpoor and Mortezaee, 2021; Yeung and Yang, 2017). The EMT is a critical event in promoting stationary epithelial tumour cells with migratory, invasive, and subsequently metastatic potential. Epithelial-to-mesenchymal transition is a biochemical transition process that enables the partial loss of epithelial features and partial acquisition of mesenchymal phenotype in tumour cells (Lambert *et al.*, 2017; Scheau *et al.*, 2019; Yeung and Yang, 2017). The partial transition to mesenchymal phenotype is induced by various signalling pathways including IL6/STAT3, TGF-β, Wnt, and Notch. Additionally, the secretion of cytokines, chemokines, and growth factors by stromal and immune cells in the TME promote

EMT by activating the expression of EMT-inducing transcription factors such as zinc-finger E-box binding homeobox factors (Zeb1 and Zeb2), Snail zinc-finger family (Snail1 and Snail2), and the basic helix–loop–helix transcription factors (Twist1, Twist2) (Dongre and Weinberg, 2019; Yeung and Yang, 2017).

These transcription factors promote EMT through repression of epithelial markers including E-cadherin, β-catenin, cytokeratin, and laminin and upregulation of mesenchymal markers such as N-cadherin, vimentin, and fibronectin (Cao *et al*., 2015; Lambert *et al*., 2017; Scheau *et al*., 2019). This disrupts the cell-cell junctions and basement membrane as well as reorganises the ECM. E-cadherin repression results in the loss of cell-ECM adhesion, subsequently altering the apical-substrate polarity of tumour cells to apical-posterior polarity that initiates cell migration and enhances tumour invasiveness (Guo *et al*., 2020; Tsuji and Plock, 2017). When epithelial tumour cells detach, they lose matrix-mediated survival signals and enter a programmed mode of cell death known as anoikis; however, cell detachment through EMT causes anoikis resistance in tumour cells, allowing them to metastasize (Cao *et al*., 2016). Furthermore, Snail1 and Zeb2 promote the expression of matrix metalloproteinases (MMPs) that facilitate the degradation of the basement membrane and ECM, thereby promoting cancer cell invasiveness (Dongre and Weinberg, 2019).

Matrix metalloproteinases are zinc-dependent and calcium-containing proteolytic endopeptidases that are produced and secreted in the cell surface or extracellular matrix as pro-enzymes (Scheau *et al*., 2019; Zhang *et al*., 2020). They facilitate the proteolytic degradation of numerous protein substrates upon activation by serine proteases or other MMPs, leading to structural changes in the basement membrane and ECM (Jiang and Li, 2021; O'Sullivan *et al*., 2014). They are expressed in lower levels during physiological processes and facilitate embryogenesis, wound healing, and angiogenesis (Cabral-Pacheco *et al*., 2020).

Matrix metalloproteinases are classified into subtypes based on their target substrates on the ECM components and sequence homology. The different subtypes include collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), matrilysins (MMP-7 and -26), membrane-type MMPs (MT-MMPs) (MMP-14, -15, -16, -17, -24, and -25), stromelysins (MMP-3, -10, and -11), and unclassified others (MMP-4, -5, -6,

-12, MMP-19, -20, -21, -22, -23, -27, and MMP-28) (Abdel-Hamid and Abass, 2021; Cabral-Pacheco *et al*., 2020).

Amongst the MMPs, gelatinases are the primary promoters of local invasion and distant metastasis, and their overexpression has been reported in aggressive cancers including breast cancer (Laronha and Caldeira, 2020; Scheau *et al*., 2019). Gelatinases have a specific domain at the N-terminus of the catalytic domain that comprises three fibronectin type II tandem repeats that enhance collagen, gelatin, and laminin binding. This allows gelatinases to proteolytically degrade gelatin, laminin, and type-IV, -V, -VIII, -X, -XI, and -XIV collagen (Cui *et al*., 2017; Laronha and Caldeira, 2020). MMP-2 (gelatinase A, 72 kDa collagenase type-IV) and MMP-9 (gelatinase B, 92 kDa collagenase type-IV) restructure the basement membrane and ECM by facilitating the digestion of gelatin and collagen type-IV, permitting the invasive tumour cells to invade the adjacent tissues and metastasize to distant sites (Jiang and Li, 2021; Yari *et al*., 2014).

The activity of MMPs is endogenously regulated through inhibition by tissue inhibitors of matrix metalloproteinases (TIMPs). There are four types of TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4), which differ in tissue-specific expression and their efficacy to inhibit different MMPs (Hrabia *et al*., 2019; O'Sullivan *et al*., 2014). Amongst the four TIMPs, TIMP-1 and TIMP-2 are the most potent gelatinase inhibitors, with a high affinity for MMP-9 and MMP-2, respectively (Das *et al*., 2021; O'Sullivan *et al*., 2014). TIMP-1 inhibits MMP-3, -7, and -9 activity, whereas TIMP-4 inhibits the activity of MMP-2 by competing for binding against TIMP-2. (Hrabia *et al*., 2019; Jackson *et al*., 2017). TIMP-3 exerts its inhibitory activity to a wide range of MMPs including MMP-1, -2, -3, -9, -13, and all MT-MMPs (Cabral-Pacheco *et al*., 2020; Groblewska *et al*., 2012). The balance between MMPs and TIMPs determines the extent of ECM degradation and therefore regulates the access of invasive tumour cells to the blood and lymphatic vessels (Cui *et al*., 2017).

The proteolytic activity of MMP-2 and MMP-9 on the basement membrane and ECM results in the release of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiogenin (Deryugina and Quigley, 2015; Park and Shin, 2020). The expression of these angiogenic factors results in the formation of structurally and functionally defective angiogenic vessels in

the TME, which are disorganised, dilated and highly permeable (Viallard and Larrivée, 2017). This enables a sufficient supply of oxygen and nutrients to the tumour cells while the permeability of the vasculature also allows for active intravasation and dissemination to distant sites.

2.3.2. Intravasation

Intravasation is a process whereby invasive tumour cells penetrate the endothelial walls of the blood vessels (haematogenous intravasation) or lymphatic vessels (lymphatic intravasation) to enter the circulatory system (Sznurkowska and Aceto, 2021; Valastyan and Weinberg, 2011). Circulating tumour cells (CTC) are metastatic precursors, which can be found as either single-cell, clusters of cells, or bound to immune or stromal cells (Szczerba *et al*., 2019). Intravasation is dependent on angiogenesis through VEGF activity that increases the permeability of vasculature in the TME and the formation of invadopodia by tumour cells (Marciel and Hoffmann, 2017; Zavyalova *et al*., 2019). Invadopodia is a specialised invasive F-actin-rich surface protrusion with a high concentration of MMPs that stimulates tumour cell invasion and penetration into the vasculature (Meirson and Gil-Henn, 2018; Yadav *et al*., 2014).

2.3.3. Survival in circulation

In circulation, the CTC must survive harsh conditions such as increased shear forces, and the immune system. The CTC express tissue factors on their surfaces which triggers platelet attachment to the CTC clusters in circulation. CTCs can also bind to neutrophils resulting in CTC heteroaggregates (Fares *et al*., 2020; Lambert *et al*., 2017), which increases the survival of CTC by inducing immunosuppression through inhibition of cytotoxic CD8+ T cell response and Natural Killer (NK) recognition (Lambert *et al*., 2017; Massagué and Obenauf, 2016). Furthermore, the coating of CTCs by platelets provides a protective shield that hinders the immune elimination of CTC through the recognition by NK cells and immune cells. In addition, the shield also provides structural support for CTC to withstand the physical stress in circulation. As the CTC migrate in the circulation, they become arrested at the capillary beds of small vasculature and extravasate into the adjacent tissue parenchyma (Fares *et al*., 2020).

2.3.4. Extravasation

The extravasation process involves the arrest or adhesion of the CTC at the capillary beds of the blood vessels and their escape from the vessels to invade the neighbouring matrix through transendothelial migration (Chen *et al*., 2013; Strilic and Offermanns, 2017). Upon entrapment, the CTCs can either proliferate and grow within the lumina of the vessel forming an embolus that results in microvascular rupture gaining direct access to adjacent tissue parenchyma or extravasates through transendothelial migration (Lambert *et al*., 2017; Massagué and Obenauf, 2016). Tumour cells primarily use transendothelial migration, a process in which tumour cells transverse between endothelial walls to extravasate. It requires inter-endothelial cell-cell junctions' disruption and cellular rearrangements facilitated by the activity of MMPs and VEGF. Tumour cells secrete TGF-β which upregulates the expression of the protein angiopoietin-like 4 (ANGPTL4) which promotes tumour cell extravasation through the inhibition of vascular endothelial tight and adherens junctions (Massagué and Obenauf, 2016; Strilic and Offermanns, 2017). Following vasculature extravasation, CTCs must initiate distant metastasis colonisation (Yeung and Yang, 2017).

2.2.5. Colonisation

Colonisation is the process whereby the disseminated tumour cells (DTC) survive and proliferate at the infiltrated secondary site to form micrometastases and/or macrometastases (Maeshiro *et al*., 2021; Su *et al*., 2015). The DTC undergo a mesenchymal-to-epithelial transition (MET) to reverse the mesenchymal characteristics and reacquire their epithelial cellular features. This is because the secondary site lacks stromal cells, ECM constituents, and growth factors that promote DTC growth, making it less favourable for tumour growth (Lambert *et al*., 2017). Furthermore, the DTC must acquire immune evasion to avoid elimination from tissue parenchyma or enter the dormancy stage as either single cells or as clusters for a period of months to decades (Lambert *et al*., 2017; Massagué and Obenauf, 2016). However, tumour cells tend to secrete TGF-β which recruits stromal cells to the secondary site, where they will secrete IL-6 and ECM mediators which will then activate and amplify the growth and survival pathways, rendering DTC at the secondary site tumour cell initiating abilities (Massagué and Obenauf, 2016). They then grow and form potentially life-threatening clinically detectable macro-metastasis (Datta *et al*., 2021).

2.3.6. Angiogenesis

Angiogenesis is a process that involves the generation of new blood vessels from pre-existing blood vessels and is essential for tumour growth and metastasis (Yadav *et al*., 2014). The continuous growth of tumours in the secondary site leads to oxygen and nutrient gradient, which limits the proliferative abilities of tumour cells. The hypoxic conditions lead to an angiogenic switch through the stabilisation of a pro-angiogenic transcription factor, hypoxia-inducible factor (HIF) (Muz *et al*., 2015). The induced expression of HIF-1α and HIF-2α leads to the upregulation of pro-angiogenic mediators such as VEGF, bFGF, angiopoietin-1 and angiopoietin-2 (Krock *et al*., 2011; Lugano *et al*., 2020). VEGF the primary mediator of angiogenesis, channels the formation of blood vessels through stimulation of VEGF-receptor 2 (VEGF-R2) (Wang *et al*., 2020). HIF-1α and HIF-2α induce the expression of MMP-2 and MMP-9, whose basement membrane and ECM degradation leads to more release of VEGF from the matrix and sprouting of approximate blood vessels (Lugano *et al*., 2020; Muz *et al*., 2015). Permeability is a notable feature of VEGF-induced angiogenic blood vessels; however, the resulting blood vessels are morphologically and functionally defective due to overexpression and dysregulation of pro-angiogenic mediators during hypoxia. Despite this, the blood vessels appear to be functional enough to provide not only nutrients and oxygen to a growing tumour (both primary and secondary sites), but also channels for metastatic dissemination of the detached and invasive tumour cells (Deryugina and Quigley, 2015; Lugano *et al*., 2020).

Solid tumours are associated with hypoxia, which limits nitric oxide (NO) generation and consequently leads to persistently lower levels of NO (hyponitroxia). The relatively low intratumoral levels of NO induced by hypoxia are at optimal concentrations for tumour progression (Huang *et al*., 2017; Oronsky *et al*., 2014). Low levels of NO promote angiogenesis and metastasis through the induction of SNAIL and VEGF with the repression of anti-angiogenic Thrombospondin 1 (Khan *et al*., 2020; Oronsky *et al*., 2014). In contrast, either tumour development is inhibited, or apoptotic cell death is induced upon a slight increase or decrease in NO from the optimal intratumoral NO concentrations (Huang *et al*., 2017; Oronsky *et al*., 2014). Elevated NO reacts with ROS to generate RNS such as nitrogen trioxide (NO_3) and peroxynitrite ($ONOO^-$), which exert their cytotoxic effects through signalling pathways that result in upregulation of p53, the release of cytochrome C from mitochondria, degradation of

anti-apoptotic molecules. Increased $ONOO^-$ levels, inhibit the catalytic activity of MMP-2 (Jacob-Ferreira *et al*., 2013). Furthermore, elevated NO attenuates angiogenesis, invasion, and metastasis via the downregulation of N-cadherin, MMPs, and SNAIL with the upregulation of E-cadherin (Huang *et al*., 2017; Khan *et al., 2020;* Oronsky *et al*., 2014). The bimodal behaviour of NO provides a therapeutic opportunity to target cancer treatment through upregulation or downregulation of intratumoral NO concentrations to repress the metastatic potential of cancer cells and induce apoptosis.

2.4. Medicinal plants

Despite the advancement of the current cancer treatment, the demand for natural medicine is continuously increasing due to the adverse side effects, poor specificity, and resistance towards synthetic drugs (Semwal *et al*., 2019). With chemotherapy as the only viable treatment for TNBC, it has been shown to enhance the formation of cancer stem cells (CSC) capable of initiating new cancer cells. In addition to causing therapeutic resistance, breast CSC contributes greatly to the recurrence, progression, and metastasis of cancer cells (El-Sahli *et al*., 2021). Chemotherapy is also associated with cardiac, renal, and pulmonary toxicity. This toxicity poses a challenge in cancer treatment; however, plants are reservoirs of natural bioactive compounds that may have chemoprotective effects against cancer and can contribute greatly to the development of new cancer treatment drugs/ therapy (Bardia *et al*., 2019; Desai *et al*., 2008). About 90% of the African population depends on traditional medicine and they use plants to treat various acute and chronic diseases, however, their treatment lacks scientific backing (Mbele *et al*., 2017). Currently, natural products account for approximately 60% of cancer drugs, these include vinca alkaloids (vinblastine and vincristine) which were isolated from *Catharanthus roseus* and taxanes (Taxol) from *Taxus brevifolia*. Taxol is used in the treatment of breast, lung, and ovarian cancer (Desai *et al*., 2008; Mbele *et al*., 2017; Semwal *et al*., 2019). Combrestatins are a class of compounds isolated from the South African plant *Combretum caffrum* that have been shown in clinical trials to have anti-cancer properties (Berrington and Lall, 2012; Cragg and Newman, 2005; Karatoprak *et al*., 2020).

The anticancer and chemoprotective properties of plants are attributed to their bioactive phytochemical compounds such as alkaloids, flavonoids, phenols, saponins, tannins, terpenoids, and steroids. Because they contain anti-cancer phytochemical

compounds medicinal plants have become the primary source of compounds for development of cancer treatment (Kooti *et al*., 2017; Simo Richard *et al*., 2015). As such, the search for different medicinal plants that may contain anticancer compounds that can inhibit cancer initiation and progression continues (Raina *et al*., 2014). Although the isolation of phytochemicals used as single compounds for disease management has been used as an alternative treatment, the reliance on herbal concoctions prepared from plants, such as *Momordica balsamina*, suggests that there may be advantages in using plants' crude and/or standardised extracts instead of single compounds as the extracts may have synergistic effects and have multiple therapeutic effects (Mabasa *et al*., 2021; Mahomoodally, 2013; Olalere and Gan, 2020). As such, there is an urge to scientifically validate the biological effects that the extracts may have.

2.4.1. *Momordica balsamina* Linn.

Figure 2.3: *Momordica balsamina* plant.

The plant *M. balsamina* L., commonly known as African pumpkin or cucumber, or southern balsam apple or pear, is an important medicinal plant belonging to the Cucurbitaceae family that exhibits significant nutritional and medicinal properties (**Figure 2.3**) (Chinthan *et al*., 2022; Kadiri Okai Jonathan *et al*., 2020). It is a perennial climber with tendrils that grows up to 1.5 – 3.0 m tall and is native to South Africa and tropical Africa (Chinthan *et al*., 2022). *Momordica balsamina* is an edible vegetable known for its bitter taste that is attributed to the phytochemicals such as cucurbitacin and alkaloids. In addition to the nutritional value, the plant was also reported to have a substantial number of phytochemicals such as flavonoids, glycosides, terpenes, resins, tannins, and saponins which account for the plant's medicinal value (Ajji *et al*., 2017; Mabasa *et al*., 2021; Souda *et al*., 2018). Furthermore, studies from our laboratory, revealed that the leaf extracts of *M. balsamina* exhibit anti-migratory and anti-invasive activities against colon cancer cells (HT-29) (Serala *et al*., 2021), pro-apoptotic effects against lung cancer cell line (A549) (Mudalahothe, 2018). However, it is unknown whether the *M. balsamina* extract has anti-inflammatory and anti-invasive effects against the MDA-MB-231 breast cancer cell line.

The major compounds isolated from the aqueous leaves extract of *M. balsamina* include cucurbitane-type triterpenoids and flavonol glycosides (Ramalhete *et al*., 2022; Serala *et al*., 2021; Venter *et al*., 2021). Several studies have reported the anticancer, antidiabetic, antioxidant, antiviral, hepatoprotective, and anti-inflammatory activities of the major compounds isolated from the plant and the plant's extracts (Ramalhete *et al*., 2022). Akula and Odhav, 2008, reported the anti-inflammatory effects of the methanolic leaf extract of *M. balsamina* through inhibition of the activity of 5-lipoxygenase (5-LOX) of the lipoxygenase pathway. They further attributed the extract's anti-inflammatory activity to the presence of free radical scavenging phenolic compounds (Akula and Odhav, 2008). Additionally, Ndhlala *et al*., 2011, reported the anti-inflammatory effects of *M. balsamina* water extract through inhibition of COX-1 and COX-2 enzymes. In relation to COX-1, the inhibitory effect was more selective and potent on COX-2 with more than 70% inhibition, thereby limiting the chances of gastric irritation and ulceration which are associated with COX-1 inhibition (Ndhlala *et al*., 2011). It is worth noting that COX-2 and 5-LOX are key enzymes in the arachidonic acid cascade involved in the lipoxygenase pathway and their overexpression is associated with cancer progression and inflammation, thus, inhibiting their activity

provides an opportunity for anti-cancer and anti-inflammatory activity (Charlier and Michaux, 2003). Interestingly, the *M. balsamina* extracts have been reported to target both enzymes. Despite the few available reports about the anti-inflammatory activities of *M. balsamina,* the plant's anti-inflammatory activity and link to cancer remain elusive, necessitating further research.

CHAPTER THREE

3. Methodology

3.1. Plant collection and extraction

Momordica balsamina plant leaves were collected from Mankweng (23°53047.7" S 29°43044.1" E), in the Limpopo province, South Africa. The voucher specimen (UNIN121046) was deposited at the Larry Leach Herbarium (UNIN) at the university of Limpopo. The leaves were air-dried and ground into a fine powder using a blender. The ground leaves were then extracted by maceration at a ratio of 1:10 (w/v) using methanol for 3 days, in a shaker at 10 ×g for frequent agitation at 25°C. Following extraction, the extract was then filtered with Munktell 3HW 90 MM filter paper (Ahlstrom, Germany), then the filtrates were air-dried under an industrial fan. The dried filtrate was then dissolved in dimethyl sulfoxide (DMSO) (>99.9%) to prepare stock extract of 100 mg/mL of methanol extract, aliquoted, and stored at -20°C until use.

3.2. Cell culture and treatment

The MDA-MB-231 (ATCC® CRM-HTB-26™) breast cancer cells and HEK-293 (ATCC® CRL-1573) kidney cells were purchased from the American Type Culture Collections (ATCC). MDA-MB-231 and HEK-293 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) antibiotic solution containing Penicillin and Streptomycin. The cells were maintained in a humidified incubator with 5% CO_2 at 37°C. The cells were treated with various concentrations of filter-sterilised *M. balsamina* leaf methanol extract diluted in culture media or 0.15% DMSO (vehicle control) or with curcumin as a positive control for cell viability and apoptosis assays.

3.3. Cell Count and Viability assay

The effect of the *M. balsamina* leaf methanol extract on MDA-MB-231 breast cancer cells and an *in vitro* toxicology model HEK-293 cell viability was assessed using the Muse® Count and Viability kit, following the manufacturer's instructions (Luminex Corporation, USA). The Muse® Count and Viability reagent is a mixture of two DNA intercalating fluorescent dyes with one dye being a membrane-permeant that stains all nucleated cells to distinguish between nucleated cells and debris. The second dye is not membrane-permeable and only stains cells whose membrane integrity has been lost. This then gives information on the total cell concentration and viability. Cells

(2×10^4 cells/well) were seeded in a 48-well cell culture plate and incubated overnight for attachment. The cells were then treated with 0−150 µg/mL of *M. balsamina* leaf methanol extract or 20 µM of curcumin and incubated for 24 hours. Following treatment, the supernatant was collected, and the cells were washed with 1× PBS (150 µL), detached with 100 µL trypsin and centrifuged at 10 × *g* at 25°C for 5 minutes. The pelleted cells were resuspended in 25 µL of 1× PBS followed by the addition of 225 µL of Muse® cell Count and Viability reagent as well as incubated for 5 minutes in the dark. The samples were then analysed using Muse™ cell analyser (Merck-Millipore, Germany).

3.4. Apoptosis Assay

The mode of cell death induced by the *M. balsamina* leaf methanol extract in the MDA-MB-231 cells was analysed using the Annexin-V-FLUOS staining kit, following the manufacturer's instructions (Roche, Germany). The Annexin-V-FLUOS staining kit uses Annexin-V and Propidium iodide (PI) dual staining to quantitatively distinguish between live and necrotic cells as well as cells undergoing early and late apoptosis. Phosphatidylserine (PS) is a cell cycle signalling membrane phospholipid that is normally found on the inner side of the viable cell's membrane that translocates to the outer membrane during apoptosis. Phosphatidylserine translocation is an early morphological hallmark of apoptosis that is recognised by its binding to fluorescent-labelled annexin V. Furthermore, the addition of DNA binding propidium iodide (PI) allows for the recognition of necrotic and late apoptotic cells as PI can only permeate cells that have lost membrane function (Sathiya Kamatchi *et al*., 2020). This assay allows for the determination of four different populations represented in four different quadrants: live cells (lower left quadrant-annexin V(−)/PI (−)), necrotic cells (upper left quadrant-annexin V(-)/PI(+)), early apoptotic cells (lower right quadrant-annexin V(+)/PI(−)) and late apoptotic cells (upper right quadrant-annexin V(+)/PI(+)). Cells (2×10^4 cells/well) were seeded in a 48-well cell culture plate and incubated overnight to attach. Cells were then treated with 50 or 100 µg/mL *M. balsamina* leaf methanol or 20 µM of curcumin and incubated for 24 hours. Following treatment, the media was harvested, and the cells were washed with 1× PBS, detached with 100 µL trypsin and centrifuged at 10 × *g* for 5 minutes. The supernatant was discarded, and the pelleted cells were washed with 1× PBS and centrifuged at 10 ×*g* for 5 minutes. The pelleted cells were resuspended in 100 µL of Annexin-V-FLUOS labelling solution and

incubated for 15 minutes at room temperature. The samples were then analysed using the Muse™ cell analyser (Merck-Millipore, Germany).

3.5. Nitric oxide assay

The effect of the *M. balsamina* leaf methanol extract on intracellular nitric oxide production was assessed using the Muse® nitric oxide kit, following the manufacturer's instructions (Luminex Corporation, USA). The Muse® nitric oxide kit uses a membrane permeable nitric oxide reagent DAX-J2 Orange, which emits a highly fluorescent product upon NO oxidation, as well as a dead cell marker (7-AAD) to detect both nitric oxide activity and cell death. Cells (2×10^4 cells/well) were seeded in a 24-well plate overnight to attach. The cells were then treated with 50 ng/mL of IL-6 or a combination of 50 ng/mL of IL-6 with methanol extract (75 or 100 µg/mL) for 24 hours. Following treatment, cells were harvested using trypsin and centrifuged at 10 × *g* for 5 minutes. The pelleted cells were resuspended in 10 µL of 1X assay buffer, 100 µL of Muse® nitric oxide reagent working solution was added to the cells and they were incubated for 30 minutes at 37°C with 5% CO_2. Following incubation 90 µL of 7-AAD working solution was added to the cells, thoroughly mixed, and incubated for 5 minutes in the dark. The samples were then analysed using the Muse™ cell analyser (Merck-Millipore, Germany).

3.6. Transwell invasion assay

The cell invasion assay was performed using the Boyden chamber assay. The assay is based on the ability of cells to produce active MMPs that degrade the Matrigel matrix [Basement Membrane Extract (BME)] and invade the lower chamber in response to a chemoattractant. Cells (2×10^4 cells/well) were seeded in a 48-well plate overnight to attach. The cells were then treated with 50 ng/mL of IL-6 or a combination of 50 ng/mL of IL-6 with methanol extract (75 or 100 µg/mL) for 24 hours. The BME was thawed at 4°C overnight, then 100 µL was added into the inserts and allowed to solidify at 37°C for 1 hour. Following treatment, treatment media was harvested, and cells were trypsinised, centrifuged at 10 × *g* for 5 minutes, resuspended in 200 µL serum-free DMEM and placed in the top chamber. Then 600 µL of DMEM supplemented with 10% FBS (chemoattractant) was added in the bottom chamber. Cells were incubated and allowed to invade for 3 hours at 37°C. The invaded cells were fixed with 4% (w/v) paraformaldehyde for 30 minutes, followed by staining with 0.5% (w/v) crystal violet for 10 minutes. The invaded cells were then photographed under 4X magnification

using Olympus CKX53 inverted microscope (Olympus, Japan), quantified using ImageJ Version 1.53s Software and the data expressed as fold change of cell invasion relative to untreated control based on equation shown below (1).

$$Cell\ invasion\ (Fold\ change) = \frac{Crystal\ violet\ intensity\ of\ invaded\ cells}{Crystal\ violet\ intensity\ of\ untreated\ cells} \quad (1)$$

3.7. Cell migration assay

The wound healing cell migration assay was used to assess the potential anti-migratory activities of the *M. balsamina* leaf methanol extract in IL-6-induced migration of MDA-MB-231 cells. This assay measures the two-dimensional collective cell migration and wound healing, whereby a wound is created by scratching a confluent cell monolayer, creating a cell-free area which induces cells to migrate into the gap to heal the wound (Jonkman *et al*., 2014). To distinguish migrating cells from proliferating cells the cells were pre-treated with mitomycin C (MMC) prior to the wound-healing assay. Cells (8×10^4 cells/well) were seeded in a 24-well plate overnight for attachment, the spent media was discarded the following day and the cells were pre-treated with media containing 10 µg/mL MMC for 2 hours at 37°C and 5% CO_2 to inhibit cell proliferation. Following pre-treatment, the MMC-containing media was removed, and the cells were washed once with 1× PBS. Then 400 µL of 1× PBS was added, and a wound was created using a sterile 200 µL pipette tip, PBS containing cell debris was discarded. The cells were washed twice with 1× PBS, followed by treatment with 50 ng/mL IL-6 or a combination of 50 ng/mL of IL-6 with methanol extract (75 or 100 µg/mL) for 24 hours. The wound area and closure were monitored, and images captured at 0, 6, and 24 hours of incubation under 10x magnification using Olympus CKX53 inverted microscope (Olympus, Japan). The wound sizes were measured using LCmicro software (Version 2.2) and used to quantify the percentage (%) of wound area and closure using equations (2) and (3), respectively.

$$Wound\ area\ (\%) = \frac{Wound\ size\ (T_n)}{Wound\ size\ (T_0)} \times 100 \quad (2)$$

$$Wound\ closure\ (\%) = \frac{Wound\ size\ (T_0) - Wound\ size\ (T_n)}{Wound\ size\ (T_{0)}} \times 100 \quad (3)$$

3.8. Gelatin-Zymography Assay

The gelatin-zymography assay was used to assess the effect of *M. balsamina* leaf methanol extract on the enzymatic activity of matrix metalloproteinases (MMPs). This assay separates proteins based on their molecular weight using SDS-PAGE and quantifies the proteolytic activity of gelatinase A (MMP-2) and gelatinase B (MMP-9) based on their ability to degrade gelatin incorporated in the polyacrylamide gel (Raykin *et al*., 2017). MDA-MB-231 cells were seeded in a 25 cm^2 cell culture flask overnight to attach (80% confluency), then treated with 50 ng/mL of IL-6 or a combination of 50 ng/mL of IL-6 with 75 or 100 µg/mL *M. balsamina* leaf methanol extract in a serum-free DMEM for 24 hours. Following treatment, the conditioned media was collected, and the total protein content was quantified using the BCA protein assay kit (Thermo Fisher Scientific, USA). The protein was mixed with non-reducing sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 1% (v/v) SDS, and 0.01% (w/v) Bromophenol blue]. The samples (10 µg/µL) were electrophoresed using the Novex™ 10% Zymogram Plus (Gelatin) gel (Thermo Fisher Scientific, USA) at 100 V for 60 minutes at 4°C. Following electrophoresis, the gels were washed for 40 minutes at room temperature on a rocking shaker with 2.5% (v/v) Triton X-100 to remove the SDS and renature the gelatinases (MMP-2 and -9). The gels were then incubated overnight at 37°C in a developing buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM $CaCl_2$], to allow for the gelatinolytic activity to occur. Following incubation, the gels were stained for an hour on a rocking shaker with Coomassie Blue R-250 stain, then destained for 40 minutes with destaining solution [50% (v/v) methanol, 10% (v/v) glacial acetic acid], and then imaged. The densitometric analysis of the bands was done using ImageJ Version 1.53s Software, the relative change in MMPs activity was determined using equation (4).

$$MMP\ activity = \frac{Mean\ intergrated\ density\ of\ band\ of\ treated\ cells}{Mean\ intergrated\ density\ of\ band\ of\ untreated\ cells} \times 100 \quad (4)$$

3.9. Real-time quantitative Reverse Transcriptase Polymerase Chain Reaction assay

Real-time quantitative RT-PCR (qRT-PCR) was performed to assess the effect of *M. balsamina* leaf methanol extract on the relative mRNA expression of the target genes in IL-6-activated MDA-MB-231 breast cancer cells. The total RNA was extracted using RNeasy® Mini Kit (Qiagen, Germany), following the manufacturer's protocol. Briefly, MDA-MB-231 cells were seeded in a 75 cm^2 cell culture flask overnight to reach 80% confluency, then treated with 50 ng/mL of IL-6 or a combination of 50 ng/mL of IL-6 with 75 or 100 µg/mL *M. balsamina* leaf methanol extract for 24 hours. The cells were harvested and pelleted. The cells were lysed by adding buffer RTL (600 µL) and vortexed for 1 minute, then 70% ethanol (600 µL) was added to the tube and mixed by gentle pipetting. About 700 µL of the sample mixture was transferred into a RNeasy Mini spin column placed in a 2 mL collection tube and centrifuged at 8000 × *g* for 15 seconds. This step was repeated until all the sample mixture was finished. Buffer RW1 (700 µL) was added to the RNeasy spin column and centrifuged at 8000 × *g* for 15 seconds. Buffer RPE (500 µL) was added to the RNeasy spin column and centrifuged at 8000 × *g* for 15 seconds. Buffer RPE (500 µL) was added again to the RNeasy spin column and centrifuged at 8000 × *g* for 2 minutes. The RNeasy spin column was placed in a new 1.5 mL collection tube, and 30 µL of RNase free water was added directly to the spin column membrane and centrifuged at 8000 × *g* for 1 minute to elute the RNA. The column was removed and discarded. A nanodrop was used to assess the quality and quantity of the eluted RNA. The complementary DNA (cDNA) was synthesised using the Accuris™ qMax First strand cDNA synthesis Flex Kit was used to generate first strand cDNA (Accuris, USA). Ten microlitres of RNA (410 ng) was mixed with 2 µL oligo-dT primers (10 µM), vortexed, centrifuged, and then heated at 70°C for 2 minutes, and then cooled on ice. In a separate tube, RTase mix consisting of 4 µL 5X RT buffer, 1 µL of High-capacity RT and 3 µL PCR grade water was prepared. Then the 8 µL RTase mix was added to the 12 µL RNA/primer mix tube, gently mixed, incubated at 42°C for 1 hour and denatured at 70°C for 10 minutes. The cDNA was quantified using a nanodrop and stored at -20°C till further use. The synthesised cDNA was subjected to real-time PCR with primers listed in **Table 3.1** using Corbett Rotor-gene 6000 PCR machine (Qiagen, Germany). The reaction mixture of 25 µL contained 4 µL of cDNA (50 ng/µL), 2 µL of forward and reverse primer mix (10 µM), 12.5 µL of 2x SensiMix™ SYBR No-ROX master mix (), and 6.5

µL water. The amplification was carried out with the initial polymerase activation step for 10 minutes at 95°C, followed by 40 thermal cycles. The expression levels of each target gene were normalised using *GAPDH*, as a housekeeping gene, and the relative levels of genes were quantified by using the $2^{-\Delta\Delta Ct}$ method.

3.10. SDS-PAGE and Western blotting

The effect of *M. balsamina* leaf methanol extract on the protein expression of MMP-2 and MMP-9 was assessed using western blotting. MDA-MB-231 cells were seeded in a 75 cm^2 cell culture flask overnight to attach (80% confluency), then treated with 50 ng/mL IL-6 and a combination of 50 ng/mL IL-6 with 75 or 100 µg/mL of *M. balsamina* leaf methanol extract for 24 hours. Following treatment, the cells were harvested by scrapping the attached cells and then centrifuged at 120 × *g* for 10 minutes. The pelleted cells were resuspended in 1× PBS and centrifuged at 100 × *g* for 10 minutes twice. Then the pelleted cells were resuspended in RIPA lysis buffer [10mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate] supplemented with protease inhibitor cocktail, vortexed and incubated for 30 minutes on ice. Then the cells were centrifuged at 20 590 × *g* for 10 minutes at 4°C. The total protein content was quantified using the bicinchoninic acid (BCA) protein assay kit. Proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) at 100 V for 120 minutes. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane at 200 mA for 90 minutes. To prevent non-specific bindings, the membranes were blocked with 3% bovine serum albumin (BSA) in 0.1% Tris-buffered saline-Tween-20 (TBST) for an hour at room temperature and then incubated with primary antibodies in 0.1% TBST-BSA overnight at 4°C. The used primary antibodies were rabbit anti-MMP-2 (1:500), rabbit anti-MMP-9 (1:500), rabbit anti-vimentin (1:1000), mouse anti-β-actin (1:1 000) and mouse anti-TIMP3 (1:1000). The membranes were washed with 0.1% TBST three times at 5-minute intervals and once with 1×TBS for 5 minutes before being incubated in peroxidase labelled secondary antibodies, goat-(anti-mouse and anti-rabbit) (1:20 000) for an hour on a rocking platform at room temperature. Following incubation, the membranes were washed with 0.1% TBST three times at 5 minutes intervals and once with 1×TBST for 5 minutes. The membranes were visualised using the Chemi Reagent Mix: ECL detection system (v/v) and captured using C-Digit Blot Scanner (LI-COR Biosciences, USA). The densitometric analysis of the bands was done using ImageJ Version 1.53s

Software and quantified by normalising protein expression to β-actin. Data was shown as fold change relative to untreated control.

3.11. Cell adhesion assay

The effect of *M. balsamina* leaf methanol extract on the adhesiveness of IL-6-activated MDA-MB-231 cells was assessed using cell attachment assay as previously described Yahayo *et al*., 2013, with minor modifications. This assay assesses the ability of the cells to adhere to the other cells and specific surface such as cell culture plates. Briefly, MDA-MB-231 cells (2×10^4 cells/well) were seeded in a 24-well plate overnight to attach. The cells were then treated with 50 ng/mL of IL-6 or a combination of 50 ng/mL of IL-6 with methanol extract (75 or 100 µg/mL) for 24 hours. Following treatment, the cells were washed with 1× PBS, and detached using 1X trypsin. The detached cells were reconstituted in 1000 µL of DMEM and replated onto a new corning 24-well plate, incubated at 37°C in a humidified environment with 5% CO_2 and allowed to adhere to the surface of the plate for 3 hours. After incubation, the media was aspirated, and the non-adherent cells were washed three times with 1× PBS. The adherent cells were fixed with 200 µL of fixation solution (Abcam, USA) for 30 minutes at room temperature. Following fixation, the cells were stained with cell stain (EMD Millipore, USA) for 5 minutes at room temperature. The cell stain was discarded, the cells were washed 5 times with 1× PBS and allowed to airdry before being photographed. The images were photographed under a 4x objective lens of Olympus CKX53 inverted light microscope (Olympus, Japan). Then, the cell-bound stain was solubilised by adding 250 µL of extraction buffer (EMD Millipore, USA) for 15 minutes on a rocking platform. The absorbance was measured at 560 nm using Multiskan Sky microplate spectrophotometer (Thermo Scientific, USA). The fold change of attached cells relative to untreated control was calculated using equation 5.

$$Cell\ adhesion\ (Fold\ change) = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \qquad (5)$$

3.12. Extracellular matrix protein attachment assay

The effect of *M. balsamina* leaf methanol extract on the attachment of IL-6-activated MDA-MB-231 to extracellular matrix proteins was assessed using the CHEMICON® ECM cell adhesion array kit (Merck, USA). This colorimetric assay evaluates the binding of cells to the specific ECM proteins coated on well strips. Briefly, MDA-MB-231 cells (2×10^6) treated with 50 ng/mL of IL-6 or a combination of 50 ng/mL of IL-6 with 75 or 100 µg/mL *M. balsamina* leaf methanol leaf extract for 24 hours were resuspended in assay buffer. Then 100 µL of the cell suspension was added in the wells of the ECM array plate and incubated for 1 hour at 37°C and 5% CO_2. Following incubation, the assay buffer was discarded from the wells, and the wells were washed 3 times with 200 µL of assay buffer. After washing the cells were stained with 100 µL of cell stain solution for 5 minutes at room temperature on a rocking platform. The stain was discarded, and the wells were washed 5 times with distilled water and allowed to airdry for 3 minutes. Then, the cell-bound stain was solubilised by adding 100 µL of extraction buffer for 10 minutes on a rocking platform. The absorbance was measured at 560 nm using Multiskan Sky microplate spectrophotometer (Thermo Scientific, USA). Percentage of attached cells was calculated using equation 6.

$$cell\ attachment\ (\%) = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ control} \times 100 \qquad (6)$$

3.13. Statistical analysis

The results were expressed as the mean ± SEM of three independent experiments performed in duplicates. Graphical data were analysed using GraphPad Prism Version 8.4.2 Statistical Software, by one-way analysis of variance (ANOVA), followed by Tukey's Multiple Comparisons test. Statistical differences between treatments and the controls were calculated using GraphPad Prism Version 8.4.2 Statistical Software, by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparisons test. Data was considered significant where $p \leq 0.05$.

CHAPTER FOUR

4. Results

4.1. The effect of *Momordica balsamina* leaf methanol extract on the viability of HEK-293 kidney and MDA-MB-231 breast cancer cells.

An important characteristic of an ideal anticancer agent is its ability to selectively interfere with and reduce the viability and/or proliferation of cancer cells while not affecting highly proliferative normal cells (Senggunprai *et al*., 2016; Singh *et al*., 2016). Hence, the effect of *M. balsamina* leaf methanol extract (MBME) on the viability of MDA-MB-231 breast cancer and an *in vitro* toxicology model HEK-293 kidney cells was investigated using Muse® Count and Viability assay. As depicted in **Figure 4.1A**, the MBME at concentrations below 125 µg/mL did not have a notable effect on the viability of MDA-MB-231 cells; however, a non-significant and significant ($p < 0.05$) reduction in cell viability was observed in MDA-MB-231 exposed to 125 µg/mL and 150 µg/mL, respectively, of MBME compared to the untreated control. On the other hand, MBME was seen to reduce HEK-293 cell viability in a concentration-depended manner with a significant ($p < 0.001$) reduction in cell viability at concentrations of 125 µg/mL and above (**Figure 4.1B**). Curcumin was seen to have no effect on the viability of both the cell lines. Based on these findings, the concentrations of MBME (75 and 100 µg/mL) were chosen for further assays as they exhibited no significant toxicity towards the HEK-293 cells.

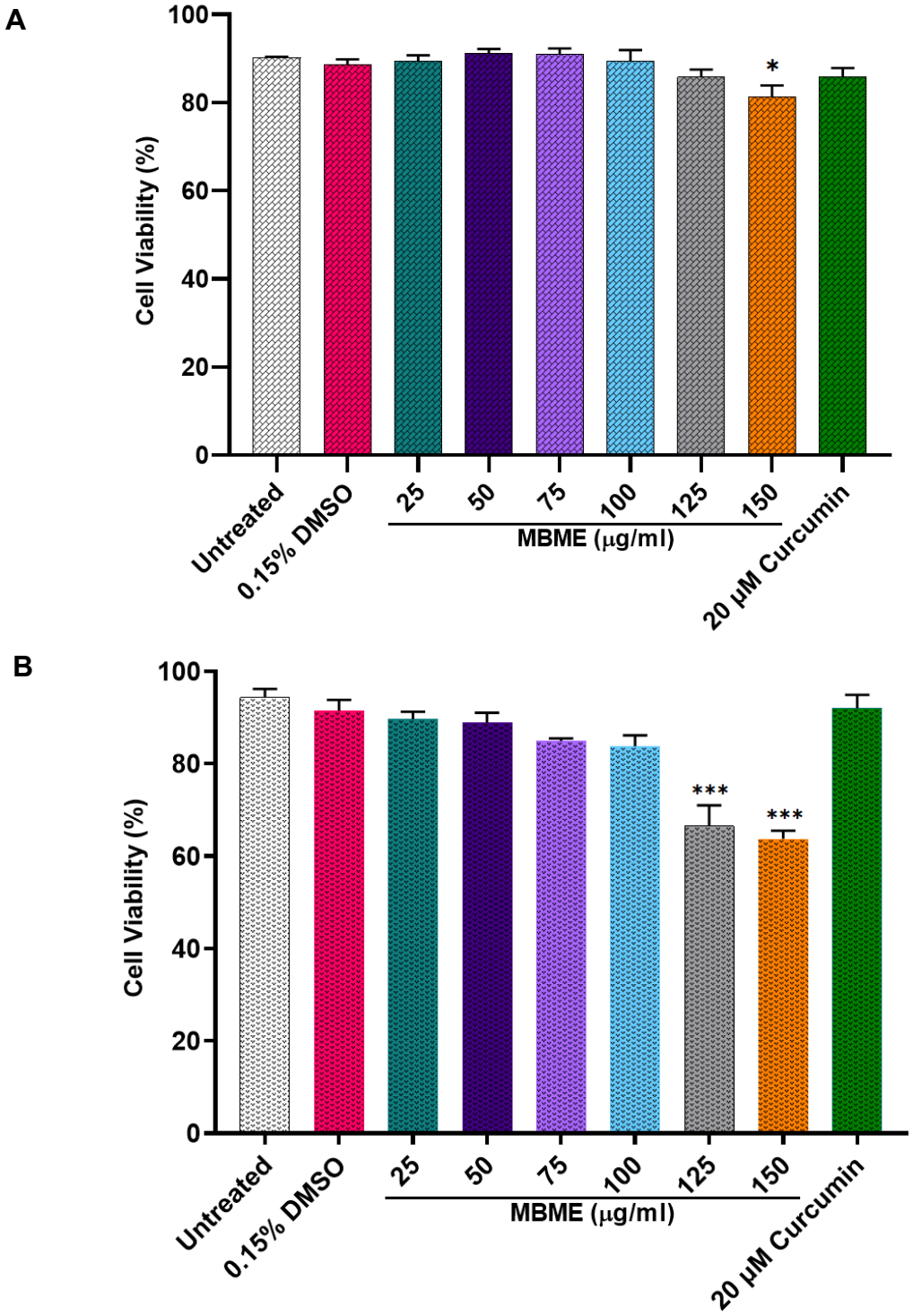


Figure 4.1: The effect of *Momordica balsamina* leaf methanol extract (MBME) on the viability of MDA-MB-231 (A) and HEK-293 cells (B). Cells were treated with varying concentrations of MBME or 20 µM curcumin for 24 hours. The effect of the extract on cell viability was assessed using Muse® Count and viability assay. Each data point is a representative of mean ± SEM of three independent experiments, performed in duplicates. *p < 0.05, ***p < 0.001, indicates significant statistical difference to the untreated control.

4.2. Determination of the mode of cell death induced by treatment of *Momordica balsamina* leaf methanol extract on the MDA-MB-231 cells.

To further confirm the lack of significant cytotoxicity of the MBME on the MDA-MB-231 cells, the potential mode of cell death induced by the MBME in MDA-MB-231 cells was assessed using the Annexin-V-FLUOS kit. As demonstrated in **Figure 4.2** treatment with MBME (50 or 100 µg/mL) resulted in a non-significant concentration-dependent increase in the percentage of MDA-MB-231 cells undergoing apoptosis as compared to the untreated control. Treatment with 20 µM curcumin, however, resulted in a significant ($p < 0.001$) increase in the percentage of MDA-MB-231 cells undergoing apoptosis compared to untreated control.

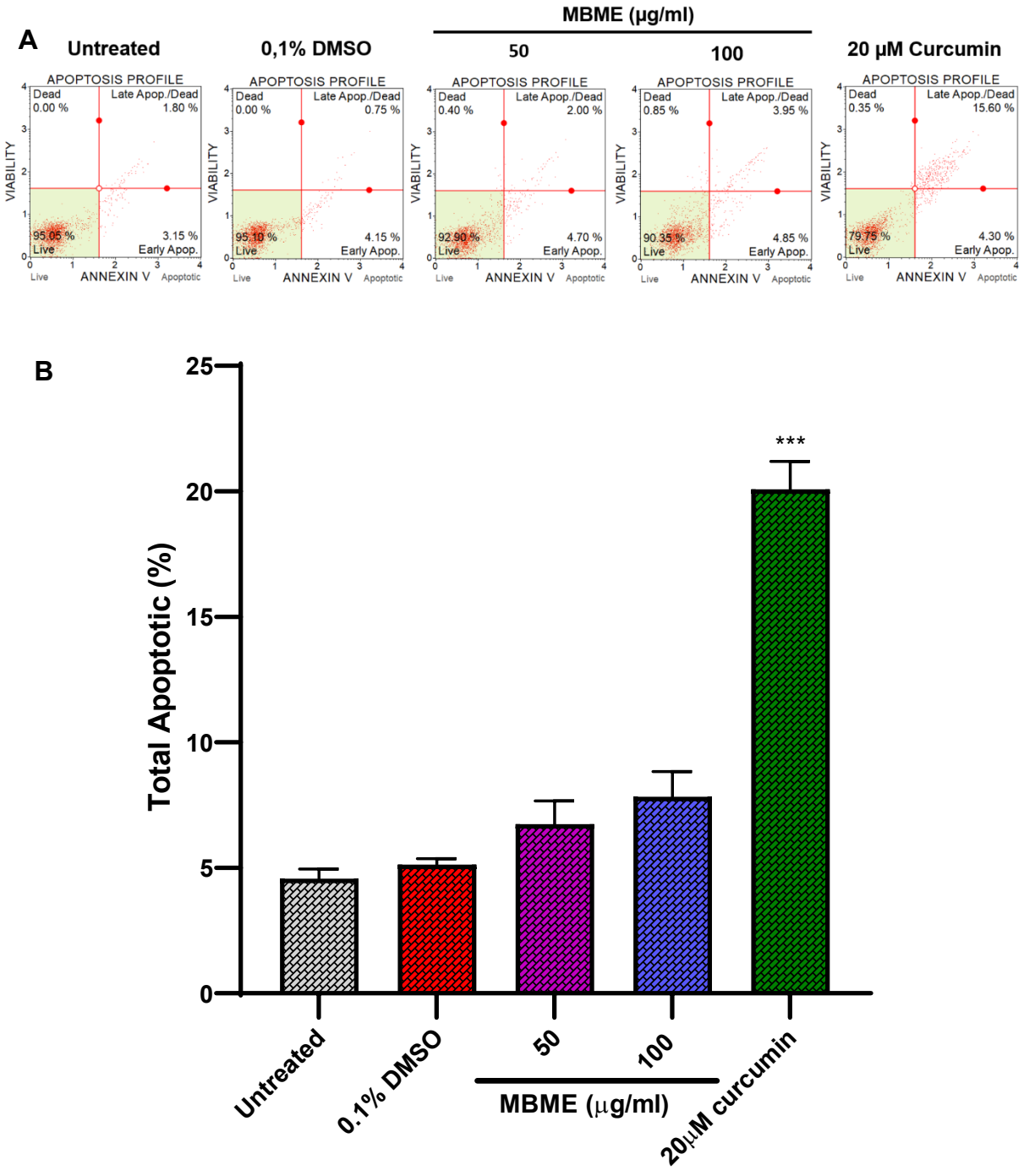


Figure 4.2: The effect of *Momordica balsamina* leaf methanol extract on the induction of apoptosis on the MDA-MB-231 cells. Cells were treated with 50 or 100 µg/mL of MBME, 0.1% DMSO or 20 µM curcumin for 24 hours. The Annexin-V-FLUOS kit was used to determine the percentage of cells undergoing apoptosis. Figure A is a represents the Muse® apoptotic profiles from one of three independent experiments, while Figure B represents the percentage of cells undergoing apoptosis. Each data point is a representative of mean ± SEM of three independent experiments, performed in duplicates. ***$p < 0.001$, indicates significant statistical difference.

4.3. The effect of *Momordica balsamina* leaf methanol extract on the production of intracellular nitric oxide in IL-6-activated MDA-MB-231.

Nitric oxide is a free radical that has been linked to tumour progression and metastatic cascade (Huang *et al*., 2017). The effect of *Momordica balsamina* leaf methanol extract on the intracellular nitric oxide production of IL-6-activated MDA-MB-231 cells was investigated using a Muse® Nitric oxide kit. **Figure 4.3.** shows that activating MDA-MB-231 breast cancer cells with IL-6 (50 ng/mL) resulted in a non-significant increase in intracellular nitric oxide production. At 24-hours of treatment with 75 and 100 µg/mL of MBME, there was a concentration-dependent induction of intracellular nitric oxide production in IL-6-activated MDA-MB-231 cells, with 100 µg/mL of MBME significantly inducing nitric oxide production in comparison to IL-6-activated cells ($p < 0.05$) and untreated cells ($p < 0.01$).

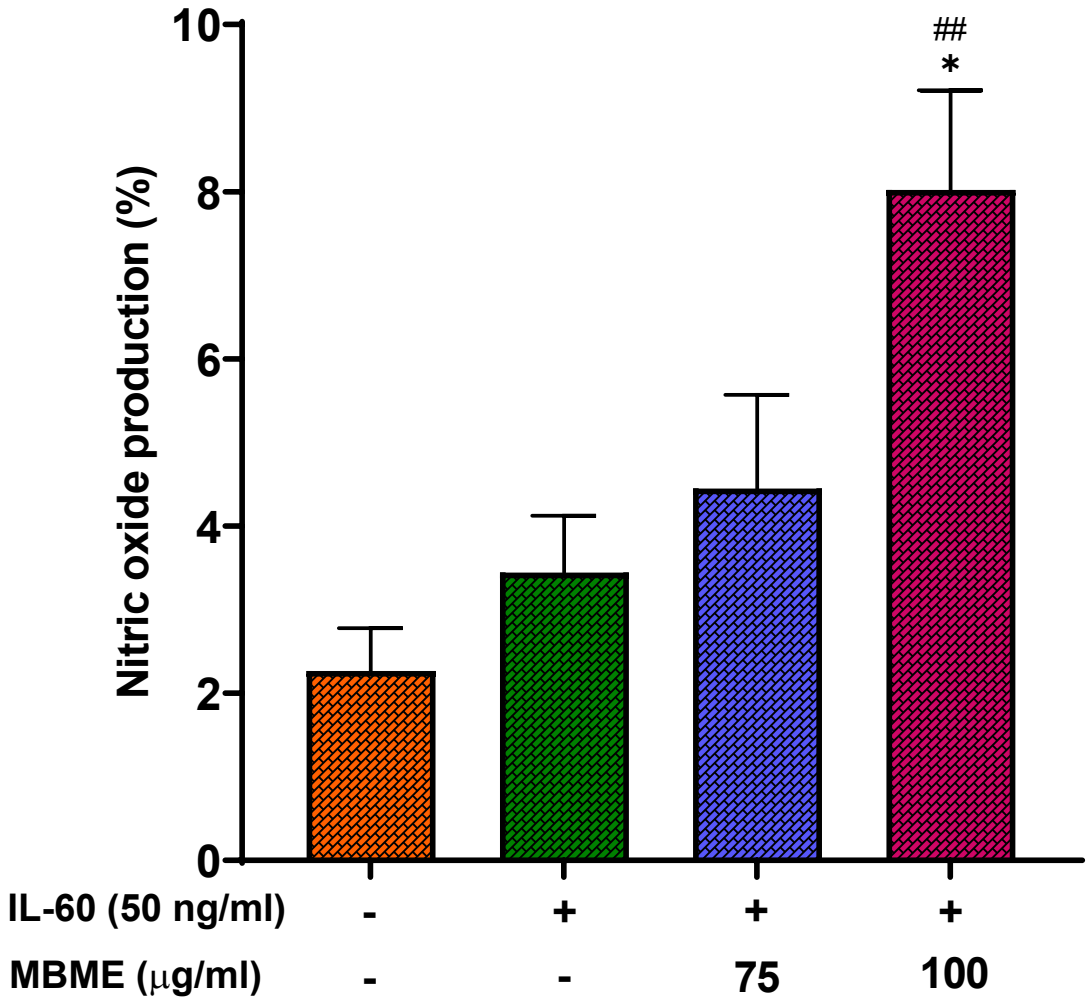


Figure 4.3: The *Momordica balsamina* leaf methanol extract induces intracellular nitric oxide production in IL-6-activated MDA-MB-231 cells. Cells were activated with IL-6 (50 ng/mL), then treated with 75 or 100 µg/mL of MBME for 24 hours. The effect of the MBME on the intracellular production of nitric oxide was assessed using Muse® Nitric oxide kit. Each data point is a representative of mean ± SEM of two independent experiments, performed in duplicates. $^{\#\#}p < 0.01$, indicates statistical difference to the untreated control and $^{*}p < 0.05$, indicates statistical difference to the IL-6-activated cells.

4.4. The effect of *Momordica balsamina* leaf methanol extract on the invasiveness abilities of IL-6-activated MDA-MB-231 cells.

Tumour cells' invasive abilities is attributed to the proteolytic degradation of the ECM and BM by MMPs to provide a passage for migratory cells to disseminate to distant sites (Odenthal *et al*., 2016). The transwell cell invasion assay was used to assess the anti-invasiveness effects of *Momordica balsamina* leaf methanol extract on the IL-6-activated MDA-MB-231 cells. Activation of MDA-MB-231 cells with 50 ng/mL of IL-6 enhanced the invasive abilities of the MDA-MB-231 cells, which can be seen by the intensity of invaded crystal violet-stained cells in **Figure 4.4A**, the treatment with 75 or 100 µg/mL of MBME, however; showed a concentration-dependent inhibition in cell invasiveness. Furthermore, quantitative analysis of invaded cells revealed that there was a significant induction in MDA-MB-231 cell invasiveness after IL-6 activation ($p < 0.01$), followed by a significant concentration-dependent inhibition in IL-6-induced MDA-MB-231 cell invasiveness when treated with 75 µg/mL ($p < 0.01$) or 100 µg/mL ($p < 0.001$) of MBME after 24 hours (**Figure 4.4B**).

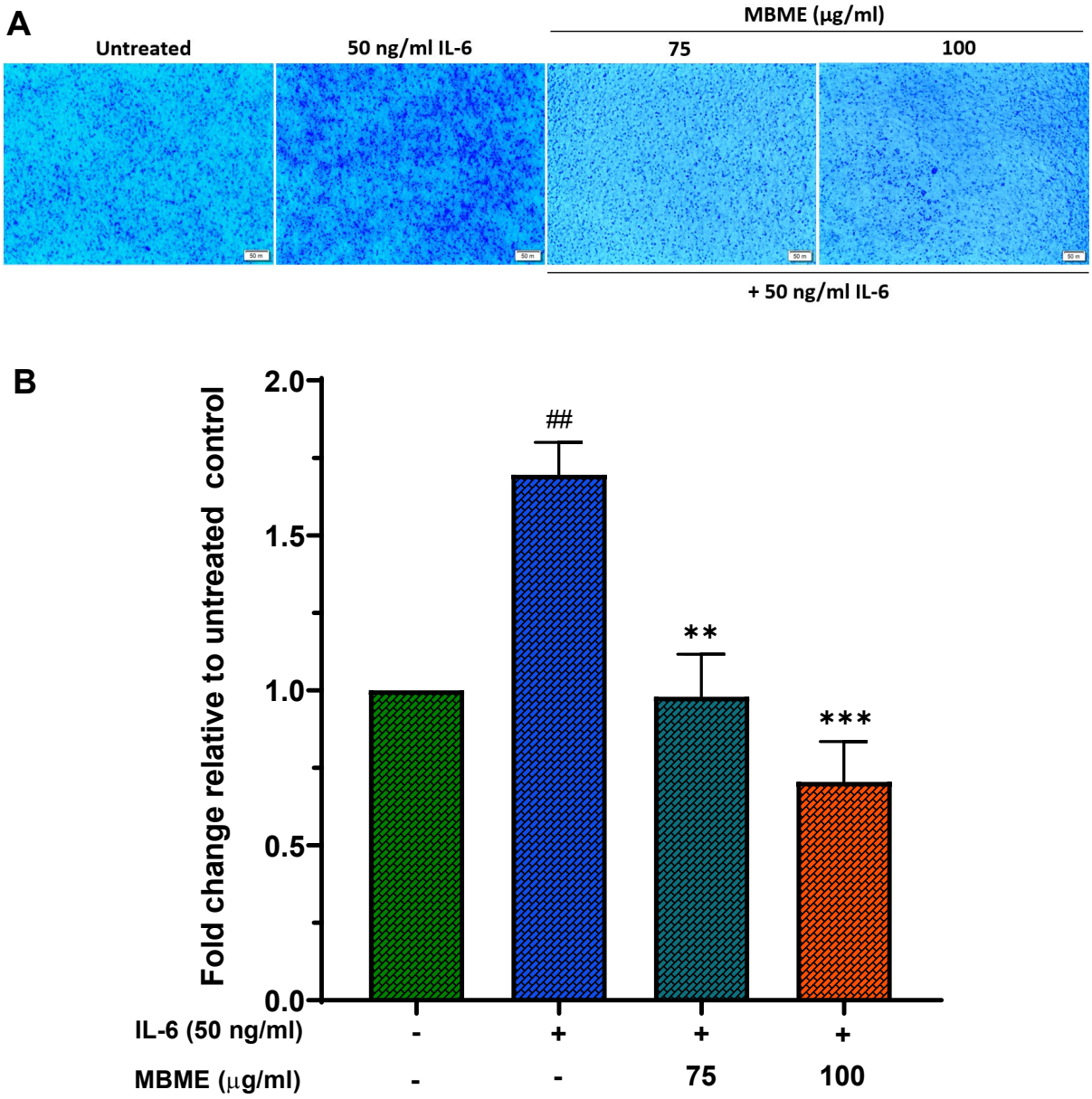
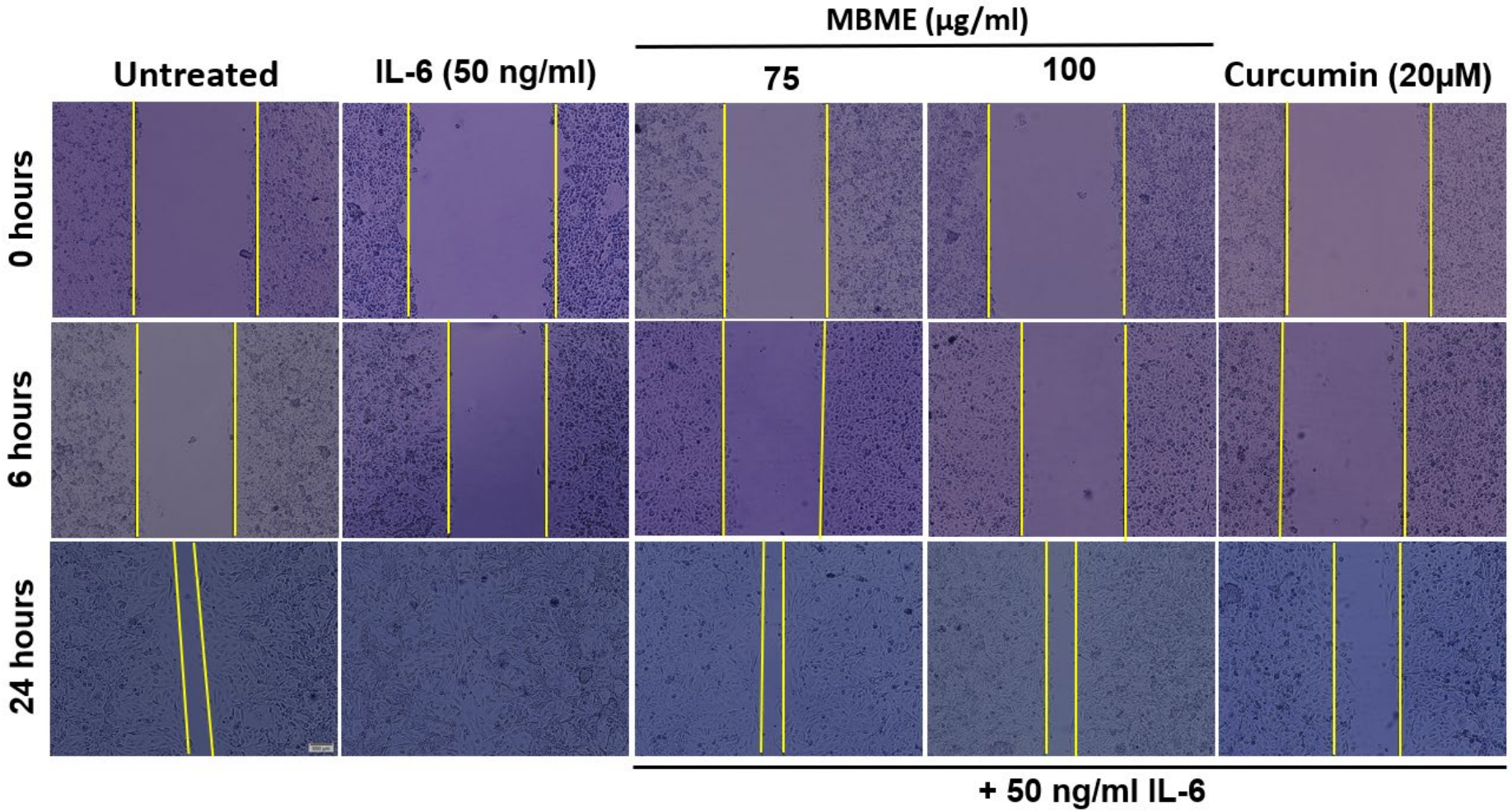


Figure 4.4: The *Momordica balsamina* leaf methanol extract inhibits IL-6-induced MDA-MB-231 cell invasiveness. Cells were activated with 50 ng/mL of IL-6 and treated with 75 or 100 µg/mL of MBME for 24 hours. A BME coated transwell plate was used to assess the invasiveness of IL-6-activated MDA-MB-231 cells after 24 hours of treatment. The invaded MDA-MB-231 cells were stained and photographed under a 4x objective using inverted microscope (**A**) and ImageJ was used to quantify the number of invaded cells. Data was expressed as Fold of cell invasion (**B**). Each data point is a representative of mean ± SEM of three independent experiments, performed in duplicates. $^{\#\#}p < 0.01$, indicates statistical difference to the untreated control and $^{**}p < 0.01$, $^{***}p < 0.001$, indicates statistical difference to the IL-6-activated cells.

4.5. The *Momordica balsamina* leaf methanol extract exhibits anti-migratory effects in IL-6-activated MDA-MB-231 cells.

Cancer cell migration is an essential process in the metastatic cascade that leads to the development of metastatic lesions (Wu *et al*., 2021). The wound healing assay was used to evaluate the effect of *M. balsamina* leaf methanol extract on the migratory abilities of IL-6-activated MDA-MB-231 cells. As depicted in **Figures 4.5A** treatment with 50 ng/mL IL-6 resulted in increased migration of MDA-MB-231 cells as shown by a complete closure of the wound area compared to the untreated control, which was still open at 24 hours. Treatment of IL-6-activated MDA-MB-231 cells with 75 or 100 µg/mL of MBME showed a concentration-dependent inhibition of cell migration (**Figures 4.5A and B**) at 6 and 24 hours. Furthermore, wound closure analysis (**Figure 4.5C**) revealed that the observed inhibition of IL-6-activated MDA-MB-231 cell migration was significant ($p < 0.0001$) at 6 and 24 hours of treatment. Moreover, 20 µM of curcumin inhibited the migration of IL-6-activated MDA-MB-231 cells in a time-dependent manner ($p < 0.001$).

A



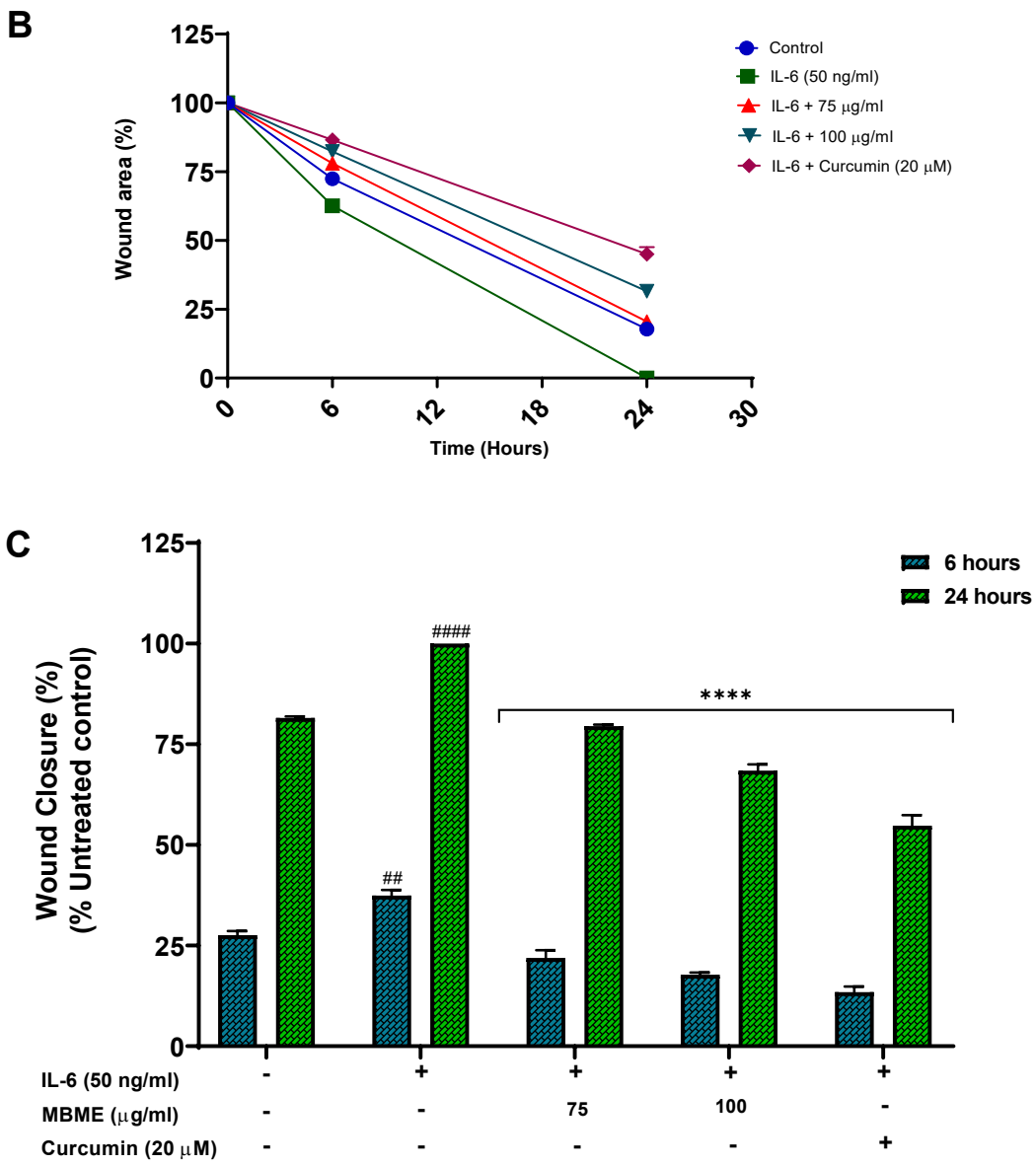


Figure 4.5: The *Momordica balsamina* leaf methanol extract exerts anti-migratory effects on IL-6-activated MDA-MB-231 cells. The confluent monolayer of MDA-MB-231 cells was wounded and then activated with 50 ng/mL of IL-6 and treated with 75 or 150 µg/mL of MBME or 20 µM curcumin for 24 hours. After 6 and 24 hours of incubation, cells were observed and photographed under a 10x objective using inverted microscope. Figure A represents cell migration pictures from one of three independent experiments photographed under a 10x objective using inverted microscope and LCmicro was used to measure the wound sizes. Data is expressed as percentage of wound area (B) and wound closure (C). Each data point is a representative of mean ± SEM of three independent experiments, performed in duplicates. $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$, indicates statistical difference to the untreated control and $^{****}p < 0.0001$, indicates statistical difference to the IL-6-activated cells.

4.6. The effect of *Momordica balsamina* leaf methanol extract on the mRNA and protein expression of *MMP-2* and *MMP-9* in IL-6-activated MDA-MB-231 cells.

The matrix metalloproteinase-2 and matrix metalloproteinase-9 promote the invasiveness of tumour cells by facilitating the degradation of the basement membrane and extracellular matrix (Dongre and Weinberg, 2019). The *Momordica balsamina* leaf methanol extract exhibited inhibitory effects on the invasiveness of IL-6-activated MDA-MB-231 cells. As such, to elucidate the mechanism behind the anti-invasiveness effects, the effect of MBME on the mRNA and protein expression levels of *MMP-2* and *MMP-9* was investigated using quantitative RT-PCR and western blotting, respectively. As observed in **Figure 4.6 and 4.7A**, the activation with 50 ng/mL of IL-6 significantly upregulated the mRNA and protein expression levels of both *MMP-2* and *MMP-9* in MDA-MB-231 cells. The quantification revealed that treatment with 75 or 100 µg/mL of MBME led to a significant downregulation of the IL-6-induced *MMP-2* and *MMP-9* expression at the mRNA and protein level in a concentration-dependent manner (**Figure 4.6 and 4.7B**).

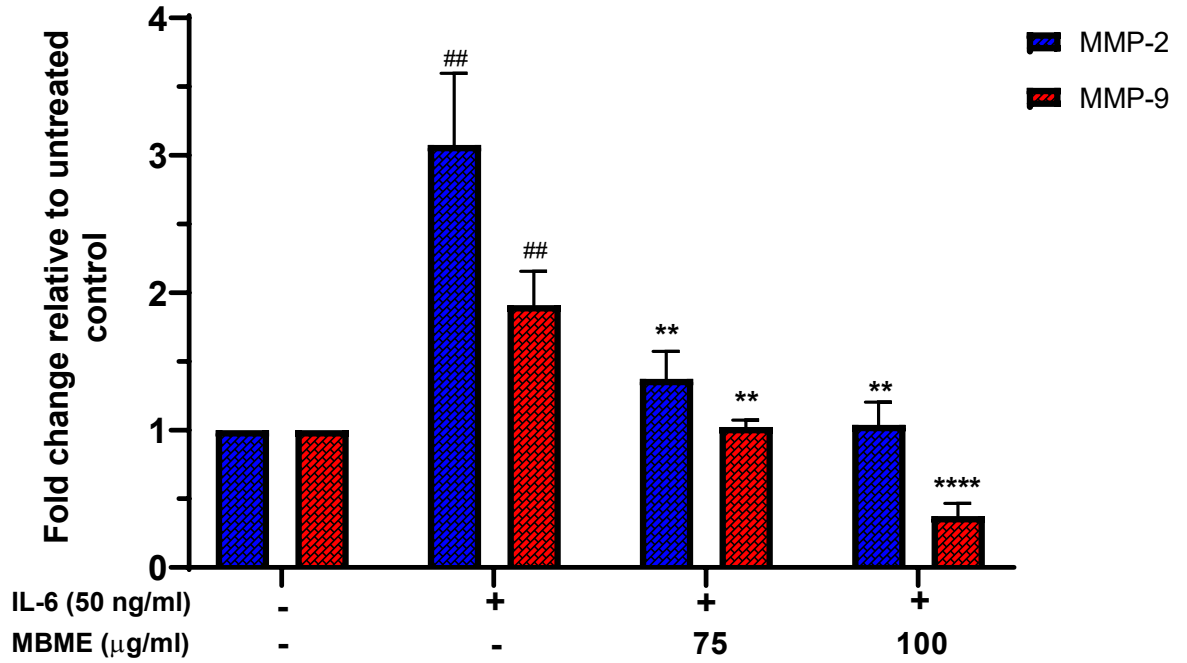


Figure 4.6: The *Momordica balsamina* leaf methanol extract downregulates the mRNA expression of *MMP-2* and *MMP-9* in IL-6-activated MDA-MB-231 cells. Cells were activated with 50 ng/mL of IL-6 and treated with 75 or 100 µg/mL of MBME for 24 hours. *MMP-2* and *MMP-9* expression was assessed using quantitative RT-PCR. Each data point is a representative of mean ± SEM of two independent experiments, performed in duplicates. $^{\#\#}p < 0.01$, indicates statistical difference to the untreated control and $^{**}p < 0.01$, $^{****}p < 0.0001$ indicates statistical difference to the IL-6-activated cells.

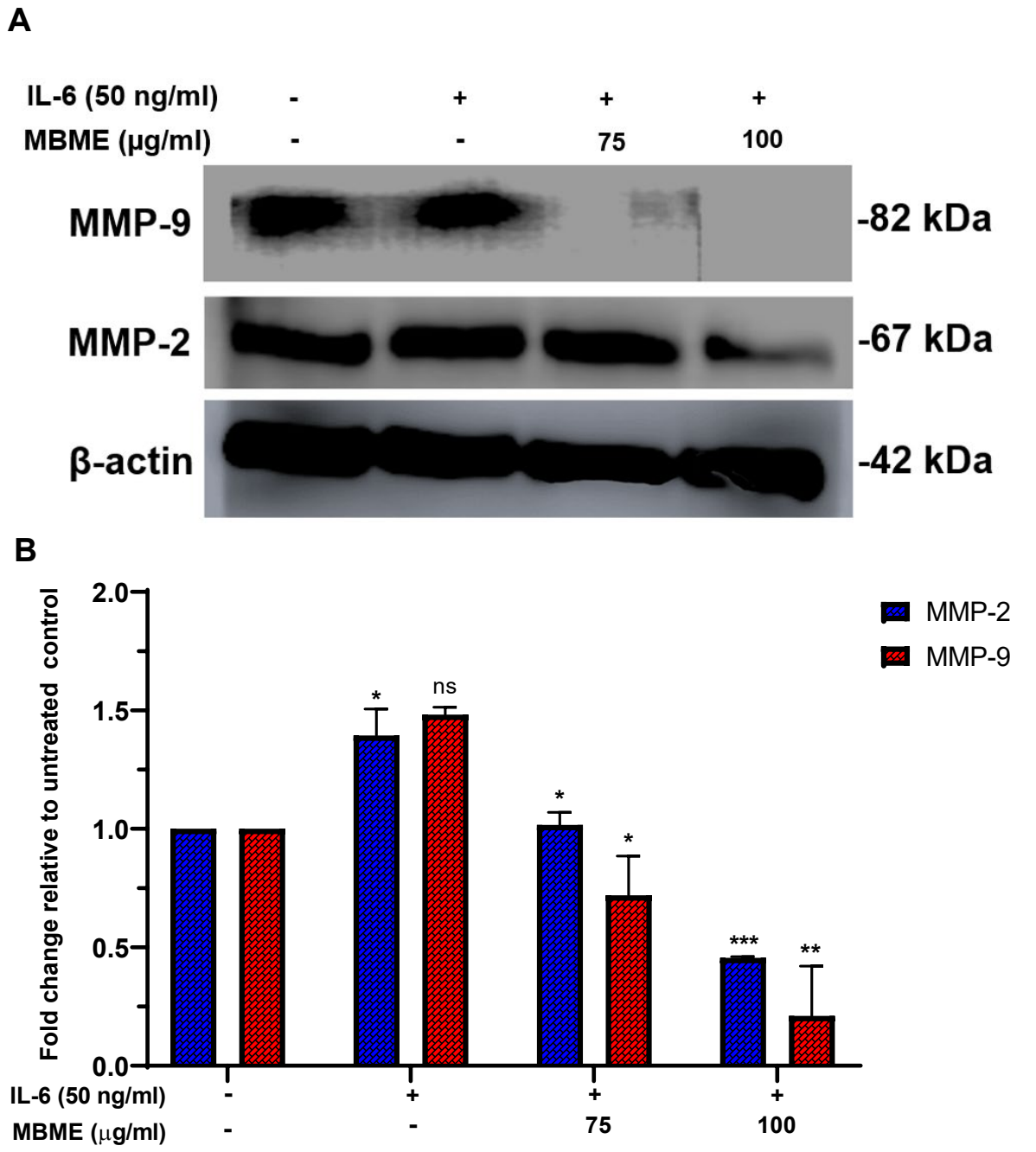


Figure 4.7: The *Momordica balsamina* leaf methanol extract downregulates MMP-2 and MMP-9 protein expression in IL-6-activated MDA-MB-231 cells. Cells were activated with 50 ng/mL of IL-6 and treated with 75 or 100 µg/mL of MBME for 24 hours. MMP-2 and MMP-9 expression was assessed using western blotting. ImageJ software was used to analyse the mean integrated density of bands, which were then quantified by normalizing to β-actin. Each data point represents the mean ± SEM of two independent experiments. $^{\#}p < 0.05$ indicates statistical difference to the untreated control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ indicates statistical difference to the IL-6-activated cells.

4.7. The effect of *Momordica balsamina* leaf methanol extract on the gelatinolytic activity of MMP-2 and MMP-9 in IL-6-activated MDA-MB-231 cells.

The proteolytic activity of matrix metalloproteinases against ECM components promotes cancer cell invasiveness and migration (Das *et al*., 2017). MMP-2 and MMP-9 are the important MMPs that facilitate the degradation of the ECM and BM (Deb *et al*., 2015). Therefore, the effect of MBME on the gelatinolytic activity of MMP-2 and MMP-9 in IL-6-activated MDA-MB-231 cells was determined using Gelatin-zymography assay. As depicted in **Figure 4.8A**, treatment of IL-6-activated MDA-MB-231 cells with 75 or 100 µg/mL of MBME reduced the gelatinolytic activity of MMP-2 and MMP-9 as compared to the untreated control. The densitometric analysis of the bands showed a significant concentration-dependant inhibition of the gelatinolytic activity of both MMP-2 ($p < 0.01$; $p < 0.001$) and MMP-9 ($p < 0.001$) (**Figure 4.8B**) after treatment with 75 or 100 µg/mL of MBME. Furthermore, as shown in **Figure 4.8**, there was no significant induction or reduction of the gelatinolytic activity of either MMP-2 and MMP-9 in cells activated with IL-6 as compared to the untreated control.

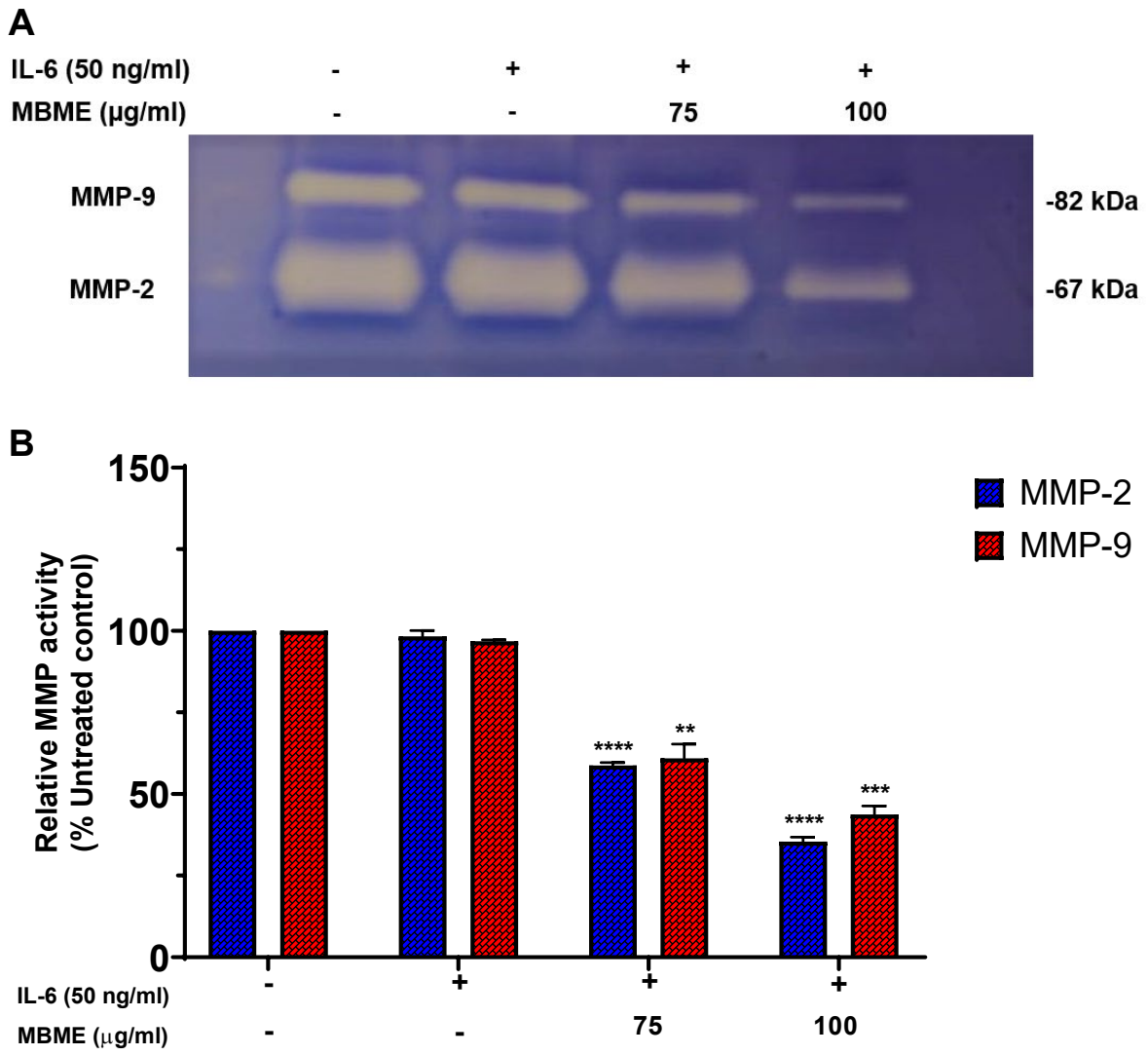


Figure 4.8: The *Momordica balsamina* leaf methanol extract inhibits the gelatinolytic activity of MMP-2 and MMP-9 in MDA-MB-231 cells. Cells were activated with IL-6 (50 ng/mL), then treated with 75 or 100 µg/mL of MBME for 24 hours, and the MMP-2 and MMP9 activity was assessed using gelatin-zymography. The mean integrated density of bands was analysed using ImageJ software. Each data point is a representative of mean ± SEM of two independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ indicates statistical difference to the IL-6-activated MDA-MB-231 cells.

4.8. The effect of *Momordica balsamina* leaf methanol extract on TIMP-3 expression in IL-6-activated MDA-MB-231 cells.

The progression cancer is determined by the balance between active MMPs and TIMPs, which regulates the total activity of MMPs (Deb *et al*., 2015). Tissue inhibitor of matrix metalloproteinases-3 negatively regulates the proteolytic activity of MMP-2 and MMP-9, to suppress cell invasion and metastasis (Groblewska *et al*., 2012; Loffek *et al*., 2011). Given the significance of TIMP-3 expression levels in regulating MMP activity and tumour invasiveness, western blotting was used to assess the effect of MBME on TIMP-3 protein expression levels in IL-6-activated MDA-MB-231 cells. As shown in **Figure 4.9A**, treatment with 75 or 100 µg/mL of MBME upregulated the protein expression of TIMP-3 in IL-6-activated MDA-MB-231 cells. The increased TIMP-3 protein expression levels were in a concentration-dependent manner **Figure 4.9B**.

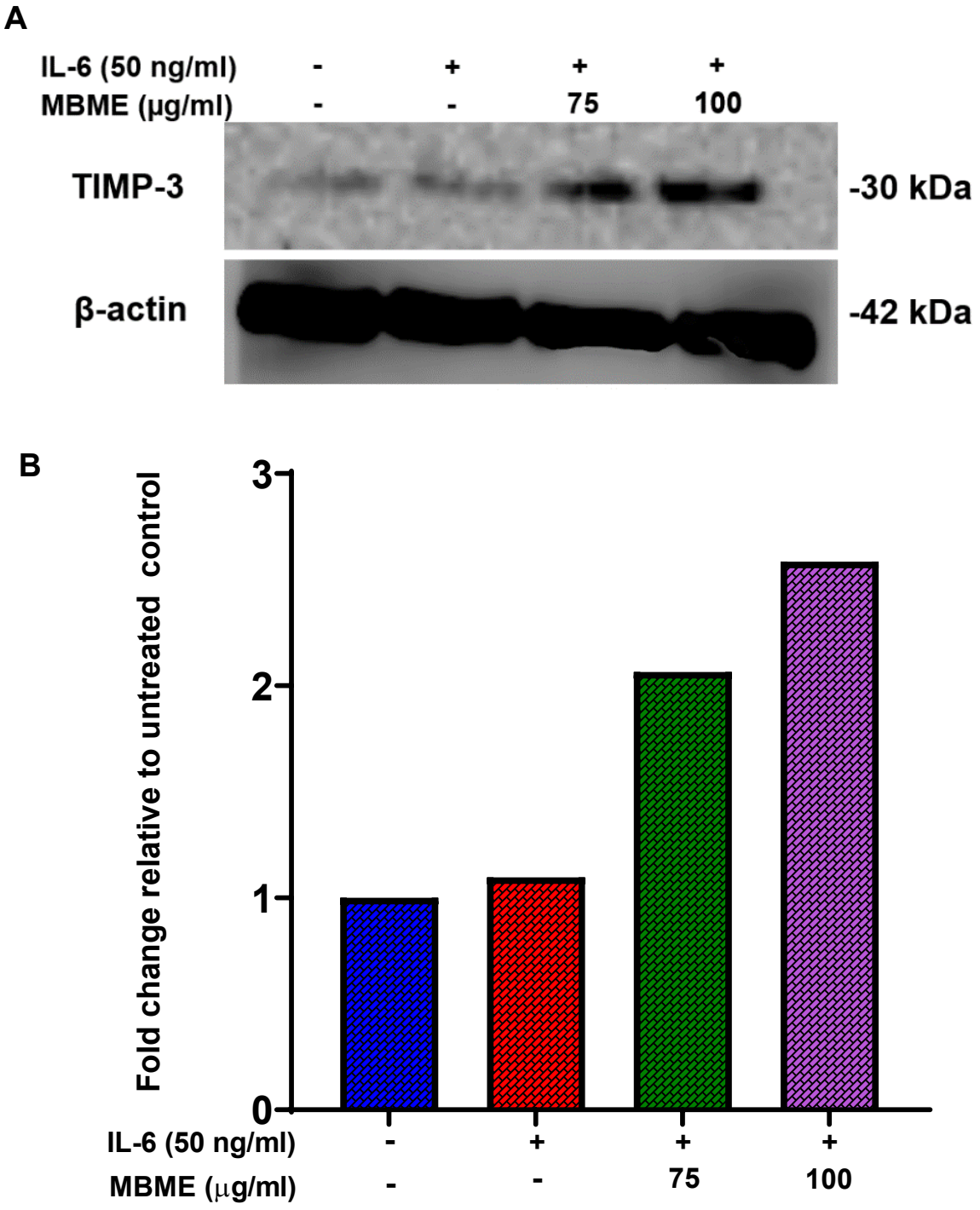


Figure 4.9: The *Momordica balsamina* leaf methanol extract upregulated TIMP-3 protein expression levels in IL-6-activated MDA-MB-231 cells. Cells were activated with 50 ng/mL of IL-6 and treated with 75 or 100 µg/mL of MBME for 24 hours. TIMP-3 expression was assessed using western blotting. ImageJ software was used to analyse the mean integrated density of bands, which were then quantified by normalizing to β-actin. This data shows results from single experiment.

4.9. The effect of *Momordica balsamina* leaf methanol extract on vimentin in IL-6-activated MDA-MB-231 cells.

Vimentin is a mesenchymal marker that promotes EMT, and its overexpression has been linked to enhanced metastatic abilities in solid cancers (Usman *et al*., 2021; Wu *et al*., 2018). The effect of the *M. balsamina* leaf methanol extract on vimentin protein expression levels in IL-6-activated MDA-MB-231 cells was investigated using western blotting. As depicted in **Figure 4.10A**, activation with 50 ng/mL of IL-6 upregulated vimentin protein expression levels in MDA-MB-231 cells. However, treatment with 75 or 100 µg/mL of MBME downregulated the IL-6-induced vimentin protein expression in MDA-MB-231 cells in a concentration-dependant manner **Figure 4.10A and B**.

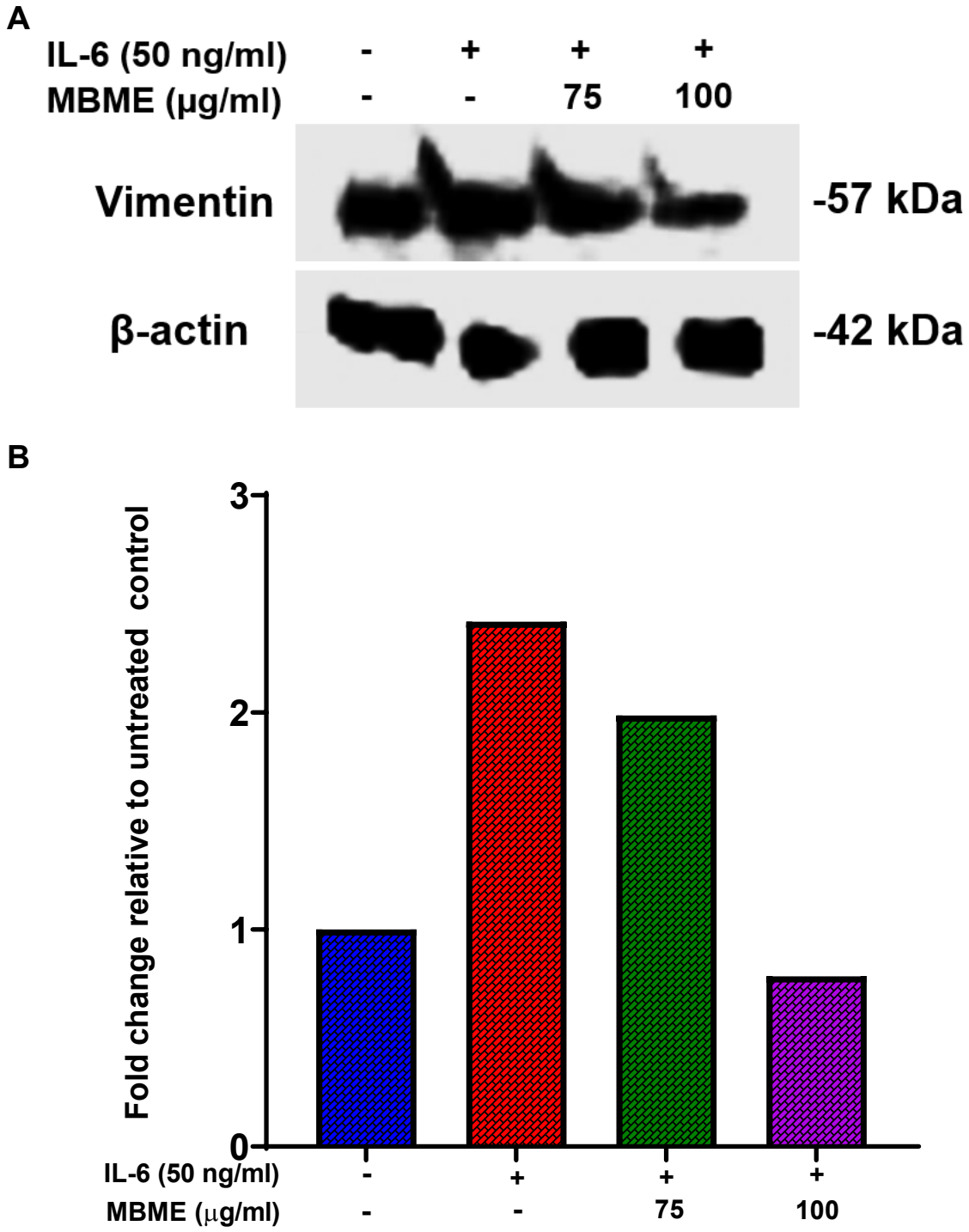


Figure 4.10: The *Momordica balsamina* leaf methanol extract downregulated the expression of vimentin in IL-6-activated MDA-MB-231 cells. Cells were activated with 50 ng/mL of IL-6 and treated with 75 or 100 µg/mL of MBME for 24 hours. Vimentin expression was assessed using western blotting. ImageJ software was used to analyse the mean integrated density of bands, which were then quantified by normalising to β-actin. This data shows results from single experiment.

4.10. The effect of *Momordica balsamina* leaf methanol extract on the mRNA expression of *Bax* and *Bcl-2* in IL-6-activated MDA-MB-231 cells.

The expression of *Bcl-2* and *Bax* not only mediates apoptosis but also cell invasiveness and metastatic progression (Denisenko *et al*., 2019). The effect of *Momordica balsamina* leaf methanol extract on *Bax* and *Bcl-2* mRNA expression levels in IL-6-activated MDA-MB-231 cells was assessed using quantitative RT-PCR. As shown in **Figure 4.11**, treatment with IL-6 significantly ($p < 0.01$) downregulated the mRNA expression of *Bax,* whereas treatment with MBME counteracted the IL-6 effect and significantly upregulated *Bax* mRNA expression in a concentration-dependent manner ($p < 0.01$). In contrast, MBME significantly ($p < 0.01$, $p < 0.001$) downregulated the IL-6-induced *Bcl-2* mRNA expression in IL-6-activated MDA-MB-231 cells in a concentration-dependent manner (**Figure 4.12**).

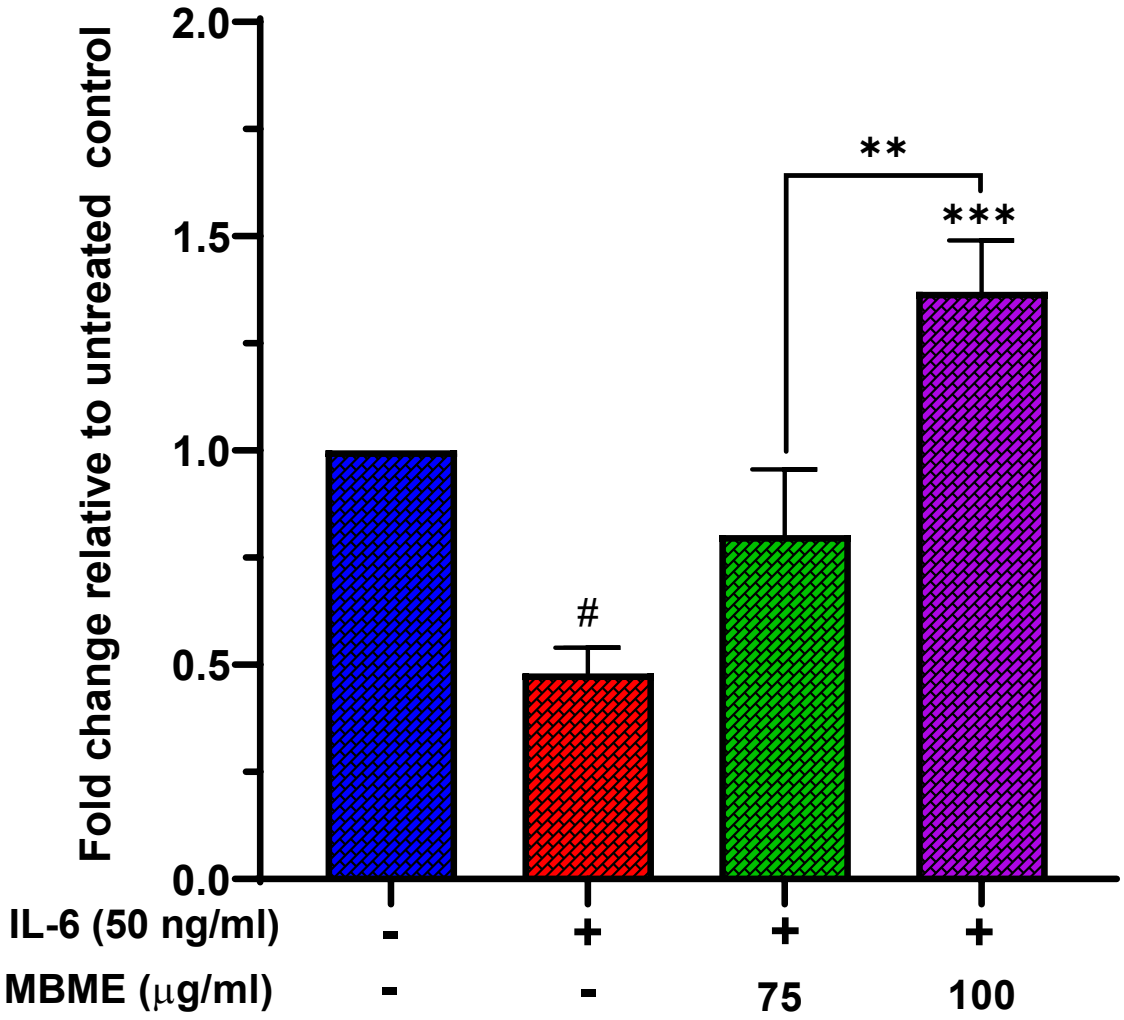


Figure 4.11. The *Momordica balsamina* leaf methanol extract upregulates *Bax* mRNA expression in IL-6-activated MDA-MB-231 cells. Cells were activated with IL-6 (50 ng/mL), then treated with 75 or 100 µg/mL of MBME for 24 hours. The effect of the MBME on the *Bax* mRNA expression was assessed using quantitative RT-PCR. Each data point is a representative of mean ± SEM of two independent experiments, performed in duplicates. $^{\#}p < 0.05$, indicates statistical difference to the untreated control and $^{***}p < 0.001$, indicates statistical difference to the IL-6-activated cells.

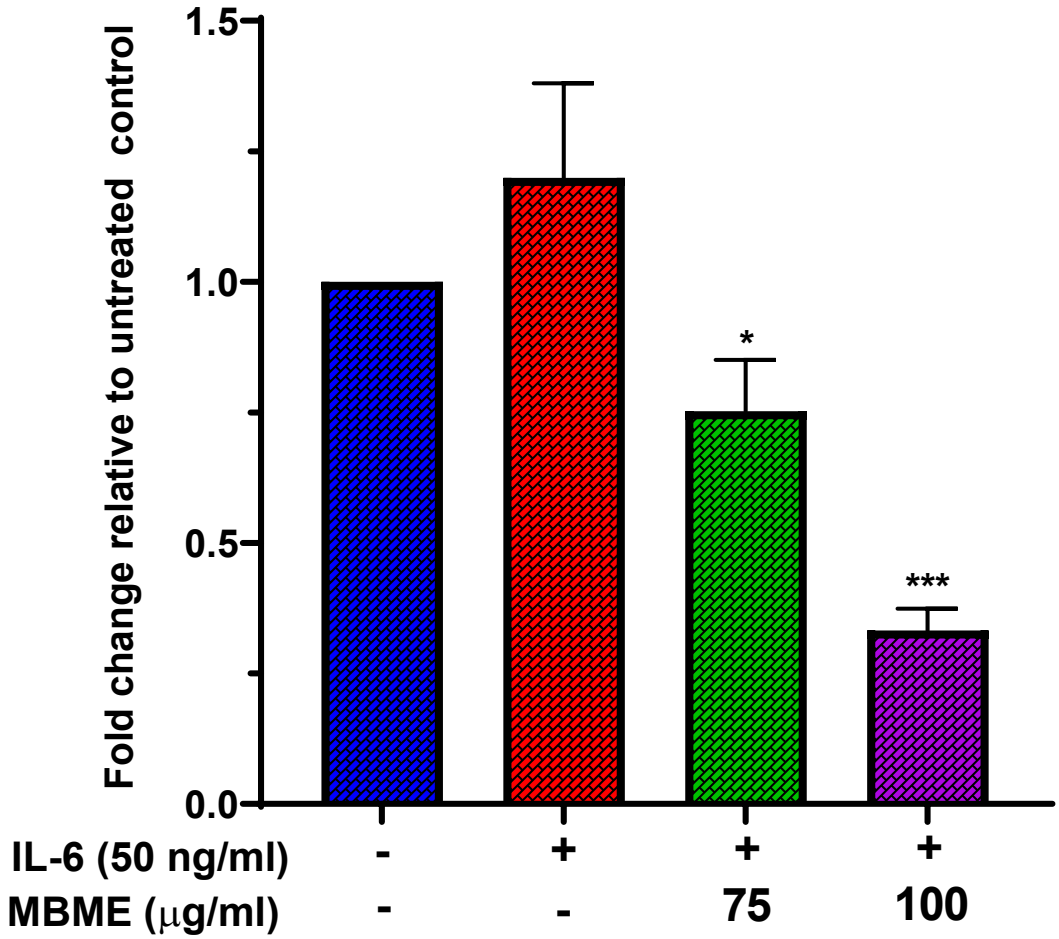


Figure 4.12. The *Momordica balsamina* leaf methanol extract downregulates *Bcl-2* mRNA expression in IL-6-activated MDA-MB-231 cells. Cells were activated with IL-6 (50 ng/mL), then treated with 75 or 100 µg/mL of MBME for 24 hours. The effect of the MBME on *Bcl-2* mRNA expression was assessed using quantitative RT-PCR. Each data point is a representative of mean ± SEM of two independent experiments, performed in duplicates. *p < 0.05, ***p < 0.001, indicates statistical difference to the IL-6-activated cells.

4.11. The effect of *Momordica balsamina* leaf methanol extract on the mRNA expression of *JAK2* and *STAT3* in IL-6-activated MDA-MB-231 cells.

The majority of IL-6-dependent metastatic effects are as a result of gene expression changes modulated by the transcriptional regulatory activity of STAT3 from the IL-6/JAK2/STAT3 pathway (Manore *et al*., 2022; Razidlo *et al*., 2018). The effect of *Momordica balsamina* leaf methanol extract on the transcription of *JAK2* and *STAT3* in IL-6-activated MDA-MB-231 cells was assessed using qRT-PCR. An increase in *JAK2* ($p < 0.01$) and *STAT3* mRNA expression levels was observed following activation of MDA-MB-231 with IL-6 (**Figures 4.13 and 4.14)**. However, treatment with 75 or 100 µg/mL of MBME resulted in a significant concentration-dependent decrease in *JAK2* ($p < 0.05$, $p < 0.01$) and *STAT3* ($p < 0.01$) mRNA expression in IL-6-activated MDA-MB-231 cells in a concentration-dependent manner.

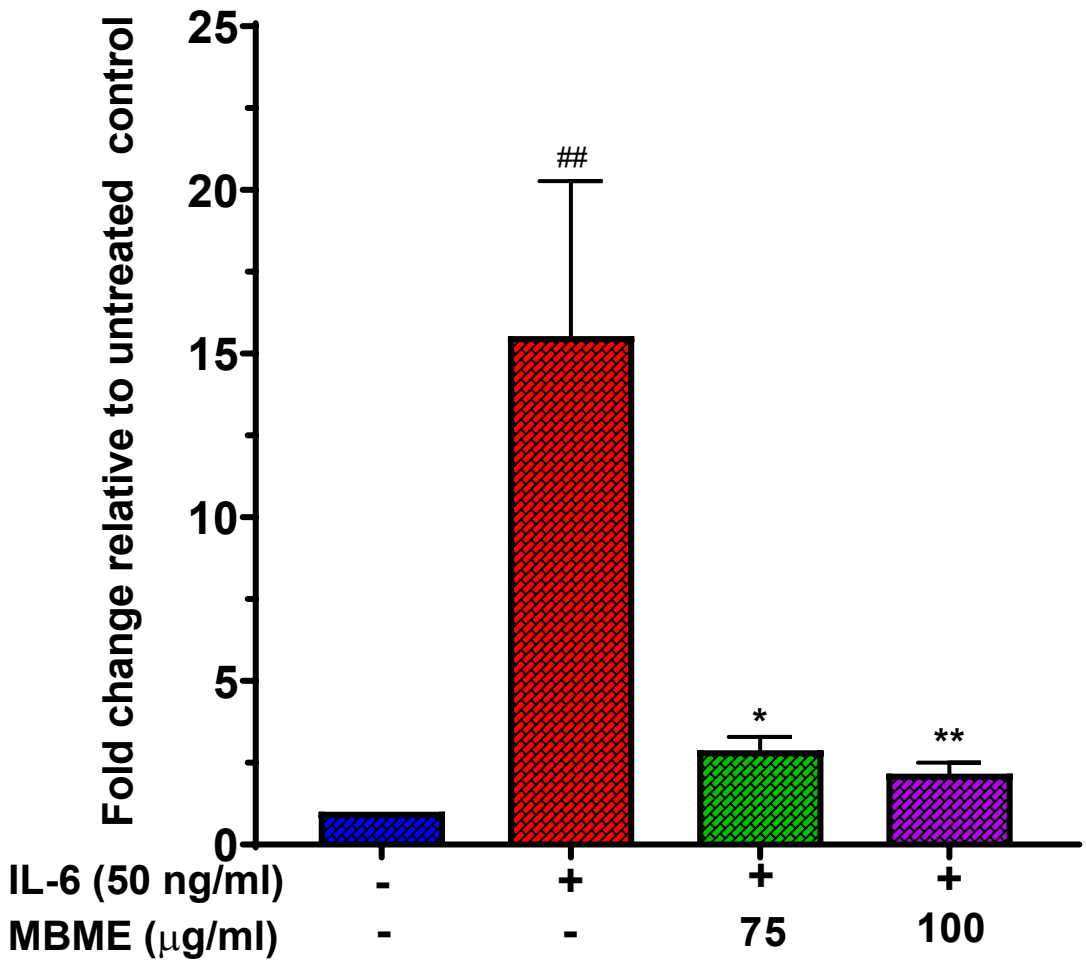


Figure 4.13: The *Momordica balsamina* leaf methanol extract downregulates the IL-6-induced *JAK2* mRNA expression in IL-6-activated MDA-MB-231 cells. Cells were activated with IL-6 (50 ng/mL), then treated with 75 or 100 µg/mL of MBME for 24 hours. The effect of the MBME on the mRNA expression of *JAK2* was assessed using real-time PCR. Each data point is a representative of mean ± SEM of two independent experiments, performed in duplicates. $^{\#\#}p < 0.01$, indicates statistical difference to the untreated control and $^{*}p < 0.01$, $^{***}p < 0.001$, indicates statistical difference to the IL-6-activated cells.

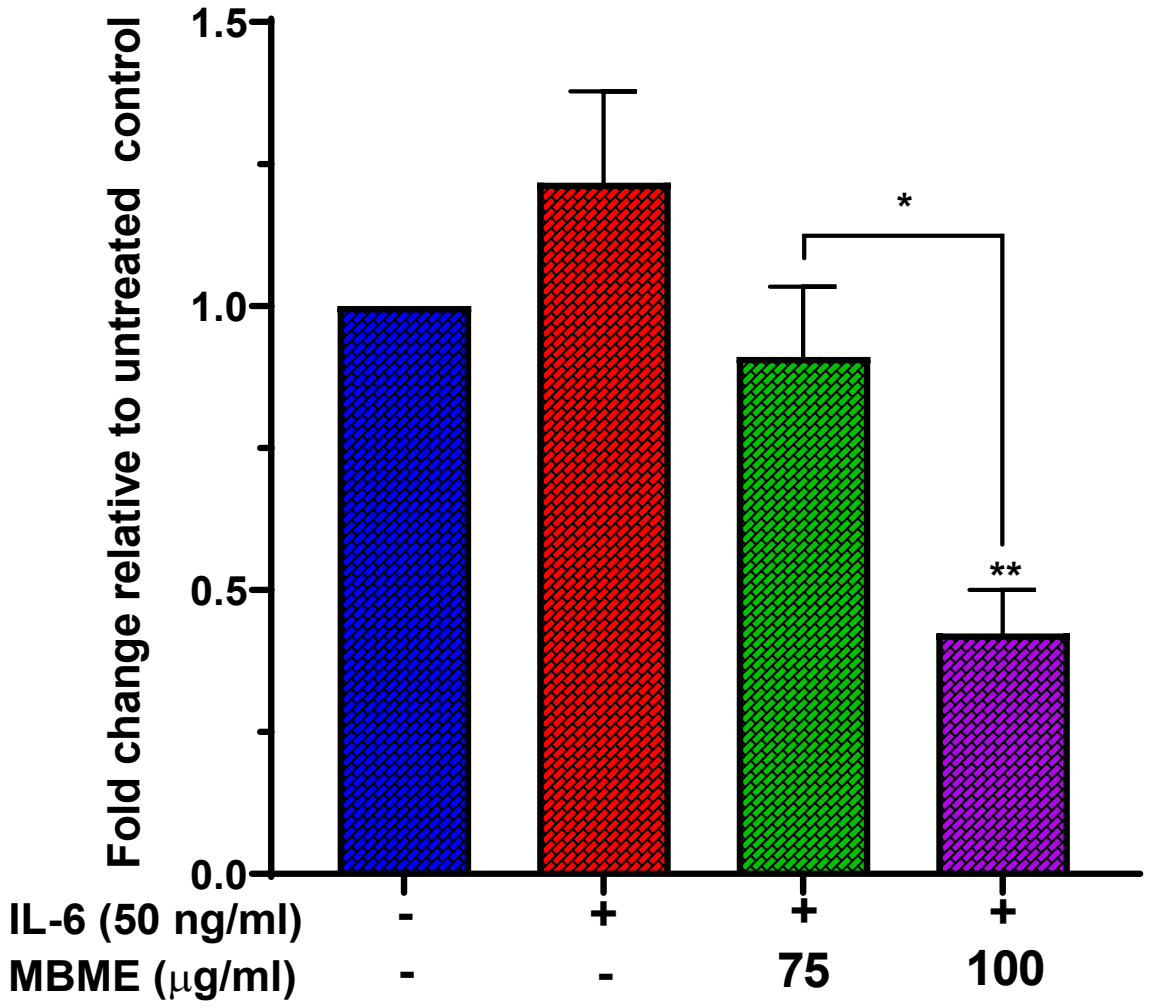


Figure 4.14. The *Momordica balsamina* leaf methanol extract downregulates the *STAT3* mRNA expression in IL-6-activated MDA-MB-231 cells. Cells were activated with IL-6 (50 ng/mL), then treated with 75 or 100 µg/mL of MBME for 24 hours. The effect of the MBME on the mRNA expression of *STAT3* was assessed using qRT-PCR. Each data point is a representative of mean ± SEM of two independent experiments, performed in duplicates. ***p* < 0.01, indicates statistical difference to the IL-6-activated cells.

4.12. The effect of *Momordica balsamina* leaf methanol extract on MDA-MB-231 cell adhesiveness.

The ability of cancer cells to adhere to the ECM is critical for the development of the secondary lesions (Holle *et al*., 2016). The anti-adhesiveness effects of *M. balsamina* leaf methanol extract on the IL-6-activated MDA-MB-231 cells was assessed using the cell adhesion assay. The enhanced MDA-MB-231 cell adhesiveness was observed following activation with IL-6, as demonstrated by the enhanced number of stained-adherent cells after activation with IL-6 in **Figure 4.15A** as compared to the untreated control. Treatment with 75 or 100 µg/mL of MBME resulted in a reduced cell adhesiveness of IL-6-activated MDA-MB-231 cells as indicated by the reduced number of stained-adherent cells in **Figure 3.15A** when compared to IL-6-activated MDA-MB-231 cells. The quantification indicated that IL-6 activation resulted in a significant increase ($p < 0.05$) in MDA-MB-231 cell adhesiveness (**Figure 3.15B**). However, treatment with 75 or 100 µg/mL of MBME resulted in a significant concentration-dependent reduction ($p < 0.05$, $p < 0.01$) in the IL-6-induced MDA-MB-231 cell adhesiveness (**Figure 3.15B**).

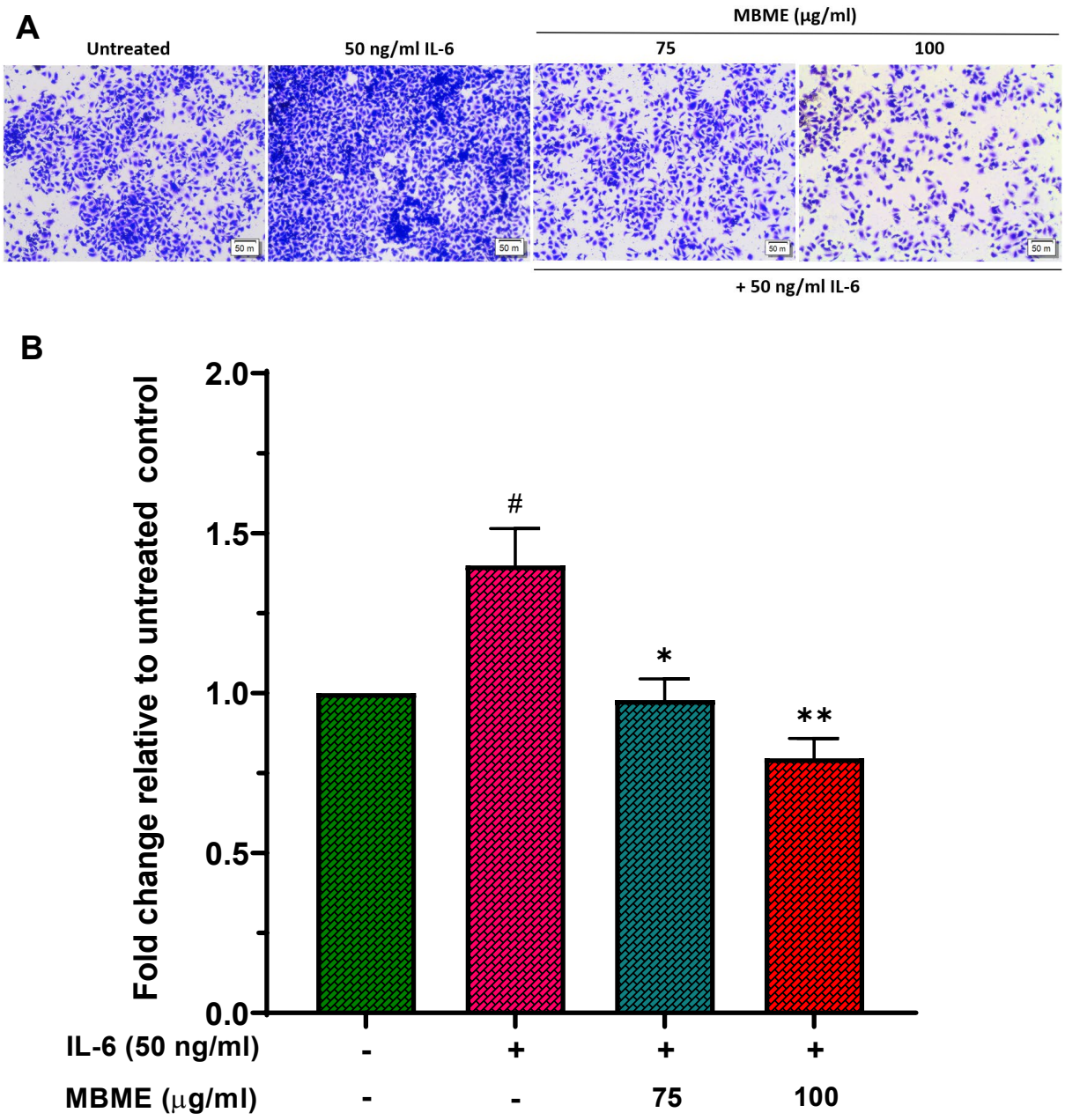


Figure 4.15: The *Momordica balsamina* leaf methanol extract inhibits IL-6-induced MDA-MB-231 cell adhesiveness. Cells were activated with 50 ng/mL of IL-6 and treated with 75 or 100 µg/mL of MBME for 24 hours. Cell attachment assay was used to assess the adhesiveness of MBME-treated IL-6-activated MDA-MB-231 cells. The attached cells were stained and photographed under a 4X objective using inverted light microscope (**A**) and quantified using equation (5) (**B**). Each data point is a representative of mean ± SEM of three independent experiments, performed in duplicates. $^{\#}p < 0.05$, indicates statistical difference to the untreated control and $^{*}p < 0.05$, $^{**}p < 0.01$, indicates statistical difference to the IL-6-activated cells.

4.13. The effect of *Momordica balsamina* leaf methanol extract on the attachment of IL-6-activated MDA-MB-231 to extracellular matrix proteins.

The attachment of cells to the extracellular matrix proteins promotes signalling pathways that supports tumour cell survival and development of successful secondary metastases (He *et al*., 2022). The effect of MBME on the attachment of IL-6-activated MDA-MB-231 to the ECM proteins was assessed using CHEMICON® ECM protein array kit. Activation of MDA-MB-231 cells with IL-6 significantly ($p < 0.05$) promoted the attachment of MDA-MB-231 to ECM proteins collagen I, II, and IV, fibronectin, tenascin, vitronectin ($p < 0.01$) and, laminin ($p < 0.001$) (**Figure 4.16**). Treatment with 75 or 100 µg/mL of MBME reduced the IL-6-induced MDA-MB-231 cell attachment to collagen I and IV, fibronectin, laminin, tenascin, and vitronectin in a concentration-dependent manner. Attachment to collagen I was significantly ($p < 0.05$) reduced following treatment with 100 µg/mL of MBME. Moreover, treatment with 75 or 100 µg/mL of MBME significantly reduced the attachment of IL-6-activated MDA-MB-231 cells to laminin ($p < 0.001$; $p < 0.0001$) and vitronectin ($p < 0.01$) in a concentration-dependent manner. On the contrary, treatment with 75 µg/mL of MBME promoted the attachment of IL-6-activated MDA-MB-231 cells to collagen II, however; the attachment of IL-6-activated MDA-MB-231 cells to collagen II were reduced following treatment with 100 µg/mL of MBME (**Figure 4.16**).

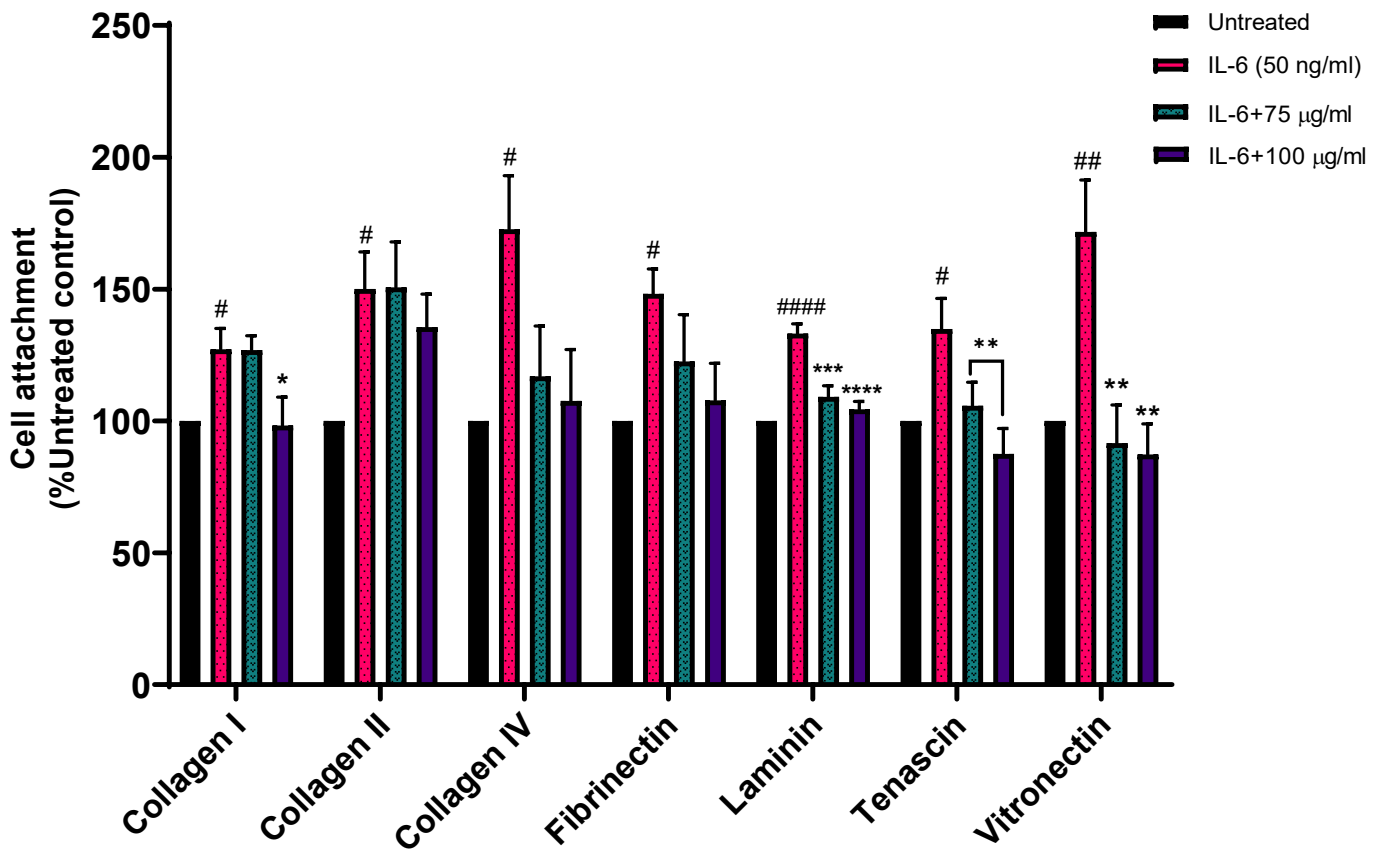


Figure 4.16: The *Momordica balsamina* leaf methanol extract reduced the attachment of IL-6-activated MDA-MB-231 cells to the ECM proteins. Cells were activated with IL-6 (50 ng/mL), then treated with 75 or 100 µg/mL of MBME for 24 hours, and then allowed to attach on the CHEMICON® ECM protein coated wells for 1hour. Each data point is a representative of mean ± SEM of two independent experiments, performed in triplicates. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$, indicates statistical difference to the untreated control and $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ indicates statistical difference to the IL-6-activated cells.

CHAPTER FIVE

5. Discussion

Metastasis is the key cause of cancer mortality and attributed to about 90% of cancer deaths from solid tumours (Entschladen *et al*., 2004; Kramer *et al*., 2013). Although solid tumours are associated with invasiveness and metastasis, the evaluation of potential drugs for solid tumours is predominantly based on the induction of a rarely sustainable tumour shrinkage via apoptosis which is not indicative of anti-metastatic effects rather than the potential to inhibit local invasion and colonisation at the secondary site (Gandalovičová *et al*., 2017). As such, the emphasis in the development of cancer therapeutics should be on drugs that target the most lethal attribute of malignant cancer (migrastatics); the spreading of cancer cells from their primary site and their ability to colonise at distant sites (Gandalovičová *et al*., 2017; Kramer *et al*., 2013; Solomon *et al*., 2021). Migrastatics are expected to be administered continuously to inhibit cell invasiveness, as such they must exhibit minimal toxicity as compared to cytotoxic drugs (Gandalovičová *et al*., 2017). The regular consumption of medicinal plants has been demonstrated to mitigate a variety of ailments, making them a safer alternative for development of novel migrastatics and anti-cancer agents as natural plants are more tolerable than synthetic drugs (Olalere and Gan, 2020). Although 60% of currently used anticancer drugs are plant-derived, only a few exhibits anti-metastatic effects, thus the urge to continuously investigate plant extracts and/or plant-derived compounds for the development of novel migrastatics for cancer therapy persists. In this regard, this study investigated the potential anti-invasiveness effects of *Momordica balsamina* leaf methanol extract on IL-6-activated triple-negative MDA-MB-231 breast cancer cell line.

An enhanced cellular proliferation is a notable feature of cancerous cells; hence the anticancer agents are engineered to exert their cytotoxic and cytostatic effects by decreasing the viability and/or proliferation of highly proliferative cells (Gandalovičová *et al*., 2017; Singh *et al*., 2016). However, the effects of these anticancer agents are also toxic to highly proliferative non-cancerous cells (Singh *et al*., 2016). Thus, the search for an ideal anticancer agent that selectively targets cancerous cells while remaining non-toxic to highly proliferating cells persists. As such, the *Momordica*

balsamina leaf methanol extract was evaluated for its potential selective toxicity using the *in vitro* toxicology model Human embryonic kidney (HEK-293) and metastatic breast MDA-MB-231 cancer cells. Our findings revealed that the extract did not selectively affect MDA-MB-231 cells. The HEK-293 cells were found to be more sensitive to the *M. balsamina* leaf methanol extract (MBME) as compared to the MDA-MB-231 cells, as a significant decrease in the viability of HEK-293 cells was observed in concentrations above 100 µg/mL, whereas a significant decrease in the viability of MDA-MB-231 cells was observed at 150 µg/mL (**Figure 4.1**). However, concentrations ≤100 µg/mL of MBME exhibited minimal toxicity to both MDA-MB-231 and HEK-293 cells. A decrease in the viability of cells is associated with the inhibition of metabolism or definite cell death (Sazonova *et al*., 2022). In this regard, the mode of cell death induced by the extract was investigated. The externalisation of the inward facing phosphatidylserine to the outer surface of the cell membrane, while the cell membrane remains intact is an early indicator of cells undergoing apoptosis (Chen *et al*., 2018; Wong, 2011). The staining of treated cells by annexin V suggest that there was externalisation of PS, indicative of apoptotic cell death (**Figure 4.2**). Thus, these findings suggest that the observed decrease in viability (**Figure 4.1**) was associated with non-significant induction of the apoptotic cell death. Based on these findings, the sub-lethal concentrations of MBME (75 and 100 µg/mL) were chosen to assess the anti-metastatic effects of *M. balsamina* leaf methanol extract.

The epithelial-to-mesenchymal transition is an essential first step in the development of metastatic cancer (Pearson, 2019), that leads to the loss of apical-basal cell polarity and acquisition of mesenchymal features which enhances cancer cell invasiveness and metastatic abilities (Scheau *et al*., 2019). Vimentin is a well-known key biomarker and mediator of EMT whose overexpression is associated with enhanced TNBC invasiveness, migration, and metastasis (Winter *et al*., 2021). Overexpression of vimentin further promotes a successful metastasis by shielding cancer cells from mechanical stresses during invasion and migration (Usman *et al*., 2021). In the present study, vimentin expression was enhanced following activation with IL-6 in MDA-MB-231 cells (**Figure 4.10**). However, treatment with 75 or 100 µg/mL of MBME reduced the IL-6-induced vimentin expression in MDA-MB-231 cells, suggesting the potential of MBME to inhibit EMT.

Cell invasion is another fundamental process in the development of metastases that provides a passageway for the dissemination of detached cells through the restructuring of the ECM and BM (Thakur and Venkateswara, 2016). Matrix metalloproteinases-2 and -9, whose expression and activity are enhanced in breast cancer, facilitate the proteolysis of ECM and BM and thereby enhancing the invasiveness of tumour cells and their migration to distant organs. (Jiang and Li, 2021; Laronha and Caldeira, 2020). Thus, inhibiting MMP-2, MMP-9 and subsequently cell invasiveness serves as a potentially effective approach of inhibiting cancer metastasis (Hong *et al*., 2022). In the present study, the treatment of IL-6-activated MDA-MB-231 cells with MBME reduced the IL-6-induced MMP-2 and MMP-9 expression at the mRNA and protein levels **(Figure 4.6 and 4.7**), as well as inhibited MMP-2 and -9 protein activity **(Figure 4.8)**. These findings suggests that the observed significant inhibition in cell invasiveness **(Figure 4.4)** may be attributed to the downregulation of *MMP-2* and *-9* expression as well as the inhibition of MMP-2 and -9 activity.

The proteolytic activity of MMPs is tightly regulated by their specific endogenous inhibitors called TIMPs (Groblewska *et al*., 2012). Tissue inhibitors of matrix metalloproteinases prevent the activation of latent MMPs by binding to the active site of the protein with high affinity and specificity (Wang and Khalil, 2018). Research shows the prevalence of *TIMP-3* gene silencing in breast cancer. The repression of the ECM-bound TIMP-3 is associated with enhanced MMP-2 and MMP-9 activity, tumour invasiveness, and metastasis (Cabral-Pacheco *et al*., 2020; Deb *et al*., 2015). On the contrary, the increased expression of *TIMP-3* is linked to suppressed tumour growth, angiogenesis, invasiveness, and consequently reduced metastasis (Deb *et al*., 2015; Han *et al*., 2018) by negatively regulating the proteolytic activity of MMP-2 and MMP-9 (Groblewska *et al*., 2012; Loffek *et al*., 2011). Interestingly, Han *et al*., 2018 reported that the activation of STAT3 by IL-6 leads to the inhibition of TIMP-3 expressions. Consistent with the reported findings, there was relatively low protein expression of TIMP-3 in both non-activated and IL-6-activated MDA-MB-231 cells as shown in **Figure 4.9A**. However, treatment with MBME upregulated TIMP-3 protein expression levels in IL-6-activated MDA-MB-231 cells (**Figure 4.9A and B**). Therefore, the suppressed proteolytic activity of MMP-2 and MMP-9 (**Figure 4.8**) may be attributed to the upregulation of TIMP-3 protein expression levels induced by treatment with the extract (**Figure 4.9**).

Despite the complexity of the metastatic cascade, a common feature in all stages of metastasis is cancer cell migration, which strongly orchestrates cancer progression in patients (Liew *et al*., 2017; Stoletov *et al*., 2020). It is therefore of utmost relevance to target cell migration in search for anti-metastatic agents. In this study, the results demonstrated that IL-6 enhanced the MDA-MB-231 cell migratory abilities, but MBME treatment significantly inhibited the IL-6-induced MDA-MB-231 cell migration in a concentration and time-dependent manner (**Figure 4.5**). The reduced migratory abilities can be attributed to the suppression of vimentin (EMT marker), *MMP-2*, *MMP-9* expression, and activity as well as cell invasiveness by MBME.

The pro-survival and pro-apoptotic members of the Bcl-2 family have also been reported to regulate cell invasiveness and migration (Um, 2016). The expression of *Bcl-2* and *Bax* therefore not only regulates apoptosis but also metastatic progression. Upregulation of Bcl-2 and downregulation of Bax protein expression levels enhances the expression and activity of uPA, MMP-2 and MMP-9 thereby resulting in increased cell invasiveness (Denisenko *et al*., 2019; Lee *et al*., 2010; Um, 2016). However, co-expression of Bcl-2 proteins with Twist, c-Myc, and exposure to hypoxic conditions is required to sufficiently induce cell invasiveness (Denisenko *et al*., 2019). Enhanced tumour proliferation causes hypoxia in the TME, resulting in persistently low levels of nitric oxide, which further accelerates tumour progression (Huang *et al*., 2017). However, elevated levels of nitric oxide have been reported to attenuate the expression and activity of MMP-2 (Chen and Wang, 2004; Jacob-Ferreira *et al*., 2013) and MMP-9 expression (Cheng *et al*., 2014). Elevated NO levels reacts with ROS and produce Peroxynitrite, and high levels of Peroxynitrite are reported by Jacob-Ferreira *et al*. (2013) to inhibit MMP-2 activity. Nonetheless, the effects of Bcl-2 and NO on cell invasiveness differ based on cell lines. As a result, the effects of MBME on the mRNA expression of *Bcl-2* and *Bax* as well as the production of NO in IL-6-activated MDA-MB-231 cells were investigated. In the current study, MBME treatment led to a concentration-dependent induction in intratumoral nitric oxide production in IL-6-activated MDA-MB-231 cells (**Figure 4.3**). Furthermore, IL-6 activation downregulated the mRNA expression of *Bax* while upregulating mRNA expression of *Bcl-2* in MDA-MB-231 cells. On the contrary, the MBME then upregulated *Bax* mRNA expression while downregulating *Bcl-2* mRNA expression in IL-6-activated MDA-MB-231 cells (**Figure 4.11 and 4.12**). These findings imply that the observed anti-migratory and

anti-invasive effects of MBME in IL-6-activated MDA-MB-231 cells could therefore be associated with increased nitric oxide and *Bax* levels, as well as downregulated *Bcl-2* levels. Additionally, our findings suggest that MBME contain phytochemicals that are capable of reversing the apoptosis evasion promoted by IL-6 as a survival mode by upregulating *Bax* (**Figure 4.11**) and downregulating *Bcl-2* (**Figure 4.12**) in MDA-MB-231 cells.

The constitutive overexpression and activation of the IL-6/JAK2/STAT3 signalling axis is associated with enhanced cell migration, invasion, metastasis, and evasion of apoptosis in approximately 80% of all TNBC cells (Qin *et al*., 2019; Xie *et al*., 2019). This is because it culminates in STAT3 protein translocating to the nucleus and facilitating the transcriptional expression of target genes, such as *Bcl-2*, *MMP-2*, *MMP-9,* and *VIM* (vimentin), promoting cancer cell survival and metastasis (Johnson *et al*., 2018; Manore *et al*., 2022; Xu *et al*., 2021). Consistent with the reported findings, following IL-6 activation the mRNA expression levels of *JAK2* and *STAT3* were upregulated in MDA-MB-231 cells. Our results further revealed a significant downregulation in *JAK-2* and *STAT3* mRNA expression in IL-6-activated MDA-MB-231 cells following treatment with MBME (**Figure 4.13 and 4.14**). These findings suggest that the observed inhibition of MDA-MB-231 cell invasiveness, migration and decrease in viability could be associated with the downregulation of the IL-6/JAK2/STAT3 pathway which in turn downregulate downstream target genes such as *Bcl-2*, *MMP-2*, *MMP-9*, and *VIM*.

The ECM is the most significant component of the TME that greatly influences cancer progression, and it is rich in proteins such as type I, II, IV collagen, laminin, fibronectin, tenascin, and vitronectin (Xiong and Xu, 2016). The binding of tumour cells to the ECM proteins initiates an intracellular signalling which promotes tumour survival, progression, invasion, and development of metastatic outgrowth at the secondary site (Läubli and Borsig, 2019). The ECM proteins play a role in ECM remodelling, which is critical for determining the pre-metastatic niche and survival of disseminated cells. Tenascin has been linked to the homing of disseminated tumour cells as well as the development of lung metastatic lesions (Läubli and Borsig, 2019; Winkler *et al*., 2020). Collagen has been shown to activate signalling in breast cancer, promoting colonisation and proliferation of disseminated tumour cells and awakening dormant tumour cells in the lungs (Parker and Cox, 2020). The binding of tumour cells to laminin

and collagen IV which make up the BM, establishes and maintains their epithelial cell polarity (Kai *et al*., 2019; Xiong and Xu, 2016). In our study, IL-6 activation enhanced the binding of MDA-MB-231 cells to type I, II, IV collagen, fibronectin, laminin, vitronectin, and tenascin, while treatment with MBME reduced the binding of the IL-6-activated MDA-MB-231 cells to type I, II and IV collagen, fibronectin, laminin, tenascin, and vitronectin (**Figure 4.16**). Thus, the observed significant inhibition in IL-6-induced MDA-MB-231 cell adhesiveness (**Figure 4.15**) may be associated with the reduced ability of extract-treated IL-6-activated MDA-MB-231 cells to attach to the ECM proteins. These results suggest the potential of MBME to hinder MET process through inhibition of binding to the BM, colonisation, and development of metastatic outgrowth of the disseminated cells at the secondary site.

In summary, the findings have demonstrated that IL-6 promotes metastatic behaviour in MDA-MB-231 breast cancer cell line. *Momordica balsamina leaf methanol extract* inhibited the IL-6 mediated metastatic abilities in MDA-MB-231 by downregulating the transcription of *JAK2* and *STAT3* of the IL-6/JAK2/STAT3 signalling pathway and down-stream processes associated with the activation of the IL-6/JAK2/STAT3 signalling pathway (**Figure 5.1**).

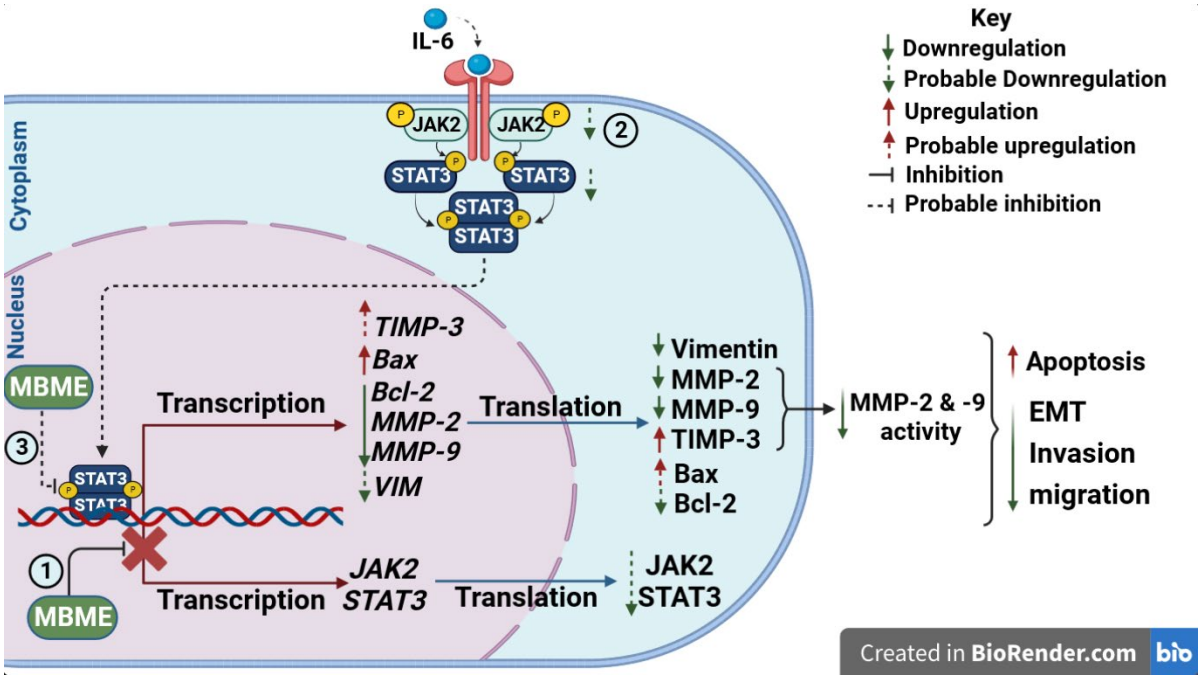


Figure 5.1: A schematic diagram of the proposed mechanism *Momordica balsamina leaf methanol extract* (MBME) in IL-6-activated MDA-MB-231 cells. The findings of the

current study suggested that **(1)** MBME inhibited the transcription of *JAK2* and *STAT3*, potentially reducing JAK2 and STAT3 protein expression levels. **(2)** The reduced expression of *JAK2* and *STAT3* could potentially result in the reduced activation of JAK2 and STAT3 following IL-6 signalling, **(3)** which would then result in suppressed transcription of STAT3 target genes (*Bcl-2, MMP-2, MMP-9, VIM*). The expression of *TIMP-3* and *Bax* is upregulated when the transcriptional activity of STAT3 is suppressed. MBME further reduced MMP-2, MMP-9, and vimentin protein expression levels with TIMP-3 upregulation, and reduced MMP-2 and MMP-9 proteolytic activity. When combined, these events could contribute to reduced EMT, invasiveness, migration, and enhanced sensitivity to apoptotic cell death. The diagram was created using BioRender.com.

In conclusion, the findings suggest that *in vitro* methanol extract may contain candidate compounds with potential for development into candidates for treatment of metastasis exacerbated by an inflammatory tumour microenvironment.

Recommendations

Further studies may be conducted with the following objectives: Firstly, investigating the effect of the *Momordica balsamina leaf methanol extract* on the protein expression of phosphorylated-JAK2 and -STAT3, to further validate the effect of the extract on the IL-6/JAK2/STAT3 pathway. Secondly, the antimetastatic effects of *Momordica balsamina leaf methanol extract* may further be confirmed using the *in vivo* animal models. Lastly, the *in vitro* analysis of the effect of the water extract of *Momordica balsamina* in IL-6-activated MDA-MB-231, to confirm the potential antimetastatic effects of the herbal concoction and/or water extract in treating triple-negative breast cancer.

6. References

Abdel-Hamid, N.M., Abass, S.A., 2021. Matrix metalloproteinase contribution in management of cancer proliferation, metastasis and drug targeting. Mol Biol Rep 48, 6525–6538.

Adekoya, T.O., Richardson, R.M., 2020. Cytokines and Chemokines as Mediators of Prostate Cancer Metastasis. Int J Mol Sci 21, 4449.

Ajji, P.K., Binder, M.J., Walder, K., Puri, M., 2017. Balsamin induces apoptosis in breast cancer cells via DNA fragmentation and cell cycle arrest. Mol Cell Biochem 432, 189–198.

Akula, S., Odhav, B., 2008. In vitro 5-Lipoxygenase inhibition of polyphenolic antioxidants from undomesticated plants of South Africa.

Banerjee, K., Resat, H., 2016. Constitutive activation of STAT3 in breast cancer cells: A review. Int J Cancer.

Bardia, A., Mayer, I.A., Vahdat, L.T., Tolaney, S.M., Isakoff, S.J., Diamond, J.R., O'Shaughnessy, J., Moroose, R.L., Santin, A.D., Abramson, V.G., Shah, N.C., Rugo, H.S., Goldenberg, D.M., Sweidan, A.M., Iannone, R., Washkowitz, S., Sharkey, R.M., Wegener, W.A., Kalinsky, K., 2019. Sacituzumab Govitecan-hziy in Refractory Metastatic Triple-Negative Breast Cancer. N Engl J Med 380, 741–751.

Bartholomeusz, C., Gonzalez-Angulo, A.M., Liu, P., Hayashi, N., Lluch, A., Ferrer-Lozano, J., Hortobágyi, G.N., 2012. High ERK protein expression levels correlate with shorter survival in triple-negative breast cancer patients. Oncologist 17, 766–74.

Berrington, D., Lall, N., 2012. Anticancer Activity of Certain Herbs and Spices on the Cervical Epithelial Carcinoma (HeLa) Cell Line. Evidence-Based Complementary and Alternative Medicine 2012, 1–11.

Bonati, L., Tang, L., 2021. Cytokine engineering for targeted cancer immunotherapy. Curr Opin Chem Biol 62, 43–52.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., Jemal, A., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68, 394–424.

Cabral-Pacheco, G.A., Garza-Veloz, I., Castruita-De la Rosa, C., Ramirez-Acuña, J.M., Perez-Romero, B.A., Guerrero-Rodriguez, J.F., Martinez-Avila, N., Martinez-Fierro, M.L., 2020. The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. Int J Mol Sci 21, 9739.

Cao, H., Xu, E., Liu, H., Wan, L., Lai, M., 2015. Epithelial–mesenchymal transition in colorectal cancer metastasis: A system review. Pathol Res Pract 211, 557–569.

Cao, Z., Livas, T., Kyprianou, N., 2016. Anoikis and EMT: Lethal "Liaisons" during Cancer Progression. Crit Rev Oncog 21, 155–168.

Charlier, C., Michaux, C., 2003. Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. Eur J Med Chem 38, 645–659.

Chen, H.-H., Wang, D.L., 2004. Nitric Oxide Inhibits Matrix Metalloproteinase-2 Expression via the Induction of Activating Transcription Factor 3 in Endothelial Cells. Mol Pharmacol 65, 1130–1140.

Chen, M.B., Whisler, J.A., Jeon, J.S., Kamm, R.D., 2013. Mechanisms of tumor cell extravasation in an in vitro microvascular network platform. Integrative Biology 5, 1262.

Chen, Q., Kang, J., Fu, C., 2018. The independence of and associations among apoptosis, autophagy, and necrosis. Signal Transduct Target Ther 3, 18.

Chen, S.-L., Yu, H., Luo, H.-M., Wu, Q., Li, C.-F., Steinmetz, A., 2016. Conservation and sustainable use of medicinal plants: problems, progress, and prospects. Chin Med 11, 37.

Cheng, H., Wang, L., Mollica, M., Re, A.T., Wu, S., Zuo, L., 2014. Nitric oxide in cancer metastasis. Cancer Lett 353, 1–7.

Chinthan, K.N., Manjunathagowda, D.C., Rathod, V., Devan, S.R., Anjanappa, M., 2022. Southern balsam pear (Momordica balsamina L.): Characterization of underutilized cucurbitaceous vegetable. South African Journal of Botany 145, 95–98.

Cragg, G.M., Newman, D.J., 2005. Plants as a source of anti-cancer agents. J Ethnopharmacol 100, 72–79.

Crusz, S.M., Balkwill, F.R., 2015. Inflammation and cancer: Advances and new agents. Nat Rev Clin Oncol 12, 584–596.

Cui, N., Hu, M., Khalil, R.A., 2017. Biochemical and Biological Attributes of Matrix Metalloproteinases. In: Progress in Molecular Biology and Translational Science. Elsevier B.V., pp. 1–73.

Das, A., Monteiro, M., Barai, A., Kumar, S., Sen, S., 2017. MMP proteolytic activity regulates cancer invasiveness by modulating integrins. Sci Rep 7, 14219.

Das, S., Amin, S.A., Jha, T., 2021. Inhibitors of gelatinases (MMP-2 and MMP-9) for the management of hematological malignancies. Eur J Med Chem 223, 113623.

Datta, A., Deng, S., Gopal, V., Yap, K.C.-H., Halim, C.E., Lye, M.L., Ong, M.S., Tan, T.Z., Sethi, G., Hooi, S.C., Kumar, A.P., Yap, C.T., 2021. Cytoskeletal Dynamics in Epithelial-Mesenchymal Transition: Insights into Therapeutic Targets for Cancer Metastasis. Cancers (Basel) 13, 1882.

Deb, G., Thakur, V.S., Limaye, A.M., Gupta, S., 2015. Epigenetic induction of tissue inhibitor of matrix metalloproteinase-3 by green tea polyphenols in breast cancer cells. Mol Carcinog 54, 485–99.

Denisenko, T. V., Gorbunova, A.S., Zhivotovsky, B., 2019. Mitochondrial Involvement in Migration, Invasion and Metastasis. Front Cell Dev Biol 7, 1–17.

Deryugina, E.I., Quigley, J.P., 2015. Tumor angiogenesis: MMP-mediated induction of intravasation- and metastasis-sustaining neovasculature. Matrix Biol 44–46, 94–112.

Desai, A., Qazi, G., Ganju, R., El-Tamer, M., Singh, J., Saxena, A., Bedi, Y., Taneja, S., Bhat, H., 2008. Medicinal Plants and Cancer Chemoprevention. Curr Drug Metab 9, 581–591.

Dongre, A., Weinberg, R.A., 2019. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol 20, 69–84.

El-Sahli, S., Hua, K., Sulaiman, A., Chambers, J., Li, L., Farah, E., McGarry, S., Liu, D., Zheng, P., Lee, S.-H., Cui, J., Ekker, M., Côté, M., Alain, T., Li, X., D'Costa, V.M., Wang, L., Gadde, S., 2021. A triple-drug nanotherapy to target breast cancer cells, cancer stem cells, and tumor vasculature. Cell Death Dis 12, 8–18.

Entschladen, F., Drell, T.L., Lang, K., Joseph, J., Zaenker, K.S., 2004. Tumour-cell migration, invasion, and metastasis: navigation by neurotransmitters. Lancet Oncol 5, 254–258.

Esquivel-Velázquez, M., Ostoa-Saloma, P., Palacios-Arreola, M.I., Nava-Castro, K.E., Castro, J.I., Morales-Montor, J., 2015. The Role of Cytokines in Breast Cancer Development and Progression. Journal of Interferon & Cytokine Research 35, 1–16.

Fares, J., Fares, M.Y., Khachfe, H.H., Salhab, H.A., Fares, Y., 2020. Molecular principles of metastasis: a hallmark of cancer revisited. Signal Transduct Target Ther 5, 28–44.

Fujihara, K., Bennett, J.L., de Seze, J., Haramura, M., Kleiter, I., Weinshenker, B.G., Kang, D., Mughal, T., Yamamura, T., 2020. Interleukin-6 in neuromyelitis optica spectrum disorder pathophysiology. Neurology(R) neuroimmunology & neuroinflammation 7, 1–11.

Gandalovičová, A., Rosel, D., Fernandes, M., Veselý, P., Heneberg, P., Čermák, V., Petruželka, L., Kumar, S., Sanz-Moreno, V., Brábek, J., 2017. Migrastatics-Anti-metastatic and Anti-invasion Drugs: Promises and Challenges. Trends Cancer 3, 391–406.

Groblewska, M., Siewko, M., Mroczko, B., Szmitkowski, M., 2012. The role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. Folia Histochem Cytobiol 50, 12–9.

Gu, G., Dustin, D., Fuqua, S.A., 2016. Targeted therapy for breast cancer and molecular mechanisms of resistance to treatment. Curr Opin Pharmacol 31, 97–103.

Guo, Y., Fan, Y., Pei, X., 2020. Fangjihuangqi Decoction inhibits MDA-MB-231 cell invasion in vitro and decreases tumor growth and metastasis in triple-negative breast cancer xenografts tumor zebrafish model. Cancer Med 9, 2564–2578.

Han, X., Mo, H., Liu, X., Li, Y., Du, L., Qiao, H., Fan, Q., Zhao, J., Zhang, S., Tang, T., 2018. TIMP3 Overexpression Improves the Sensitivity of Osteosarcoma to Cisplatin by Reducing IL-6 Production. Front Genet 9, 1–13.

He, X., Lee, B., Jiang, Y., 2022. Extracellular matrix in cancer progression and therapy. Medical Review 2, 125–139.

Holle, A.W., Young, J.L., Spatz, J.P., 2016. In vitro cancer cell-ECM interactions inform in vivo cancer treatment. Adv Drug Deliv Rev 97, 270–9.

Hong, O.-Y., Jang, H.-Y., Lee, Y.-R., Jung, S.H., Youn, H.J., Kim, J.-S., 2022. Inhibition of cell invasion and migration by targeting matrix metalloproteinase-9 expression via sirtuin 6 silencing in human breast cancer cells. Sci Rep 12, 12125.

Hrabia, A., Wolak, D., Kwaśniewska, M., Kieronska, A., Socha, J.K., Sechman, A., 2019. Expression of gelatinases (MMP-2 and MMP-9) and tissue inhibitors of metalloproteinases (TIMP-2 and TIMP-3) in the chicken ovary in relation to follicle development and atresia. Theriogenology 125, 268–276.

Huang, Z., Fu, J., Zhang, Y., 2017. Nitric Oxide Donor-Based Cancer Therapy: Advances and Prospects. J Med Chem 60, 7617–7635.

Jackson, H.W., Defamie, V., Waterhouse, P., Khokha, R., 2017. TIMPs: versatile extracellular regulators in cancer. Nat Rev Cancer 17, 38–53.

Jacob-Ferreira, A.L., Kondo, M.Y., Baral, P.K., James, M.N.G., Holt, A., Fan, X., Schulz, R., 2013. Phosphorylation status of 72 kDa MMP-2 determines its structure and activity in response to peroxynitrite. PLoS One 8, e71794.

Jiang, H., Li, H., 2021. Prognostic values of tumoral MMP2 and MMP9 overexpression in breast cancer: a systematic review and meta-analysis. BMC Cancer 21, 149–161.

Jin, W., 2020. Role of JAK/STAT3 Signaling in the Regulation of Metastasis, the Transition of Cancer Stem Cells, and Chemoresistance of Cancer by Epithelial–Mesenchymal Transition. Cells 9, 217.

Johnson, D.E., O'Keefe, R.A., Grandis, J.R., 2018. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. Nat Rev Clin Oncol 15, 234–248.

Jonkman, J.E.N., Cathcart, J.A., Xu, F., Bartolini, M.E., Amon, J.E., Stevens, K.M., Colarusso, P., 2014. An introduction to the wound healing assay using live-cell microscopy. Cell Adh Migr 8, 440–51.

Kadiri Okai Jonathan, Okafor Sylvester Ignatius, Ogaji Joshua Ikoni, 2020. Safety and health benefits profile studies of leaf extracts of Momordica balsamina Linn (Cucurbitaceae) found in North Central Nigeria. GSC Biological and Pharmaceutical Sciences 11, 278–286.

Kai, F., Drain, A.P., Weaver, V.M., 2019. The Extracellular Matrix Modulates the Metastatic Journey. Dev Cell 49, 332–346.

Karatoprak, G.Ş., Küpeli Akkol, E., Genç, Y., Bardakci, H., Yücel, Ç., Sobarzo-Sánchez, E., 2020. Combretastatins: An Overview of Structure, Probable Mechanisms of Action and Potential Applications. Molecules 25, 1–33.

Khan, F.H., Dervan, E., Bhattacharyya, D.D., McAuliffe, J.D., Miranda, K.M., Glynn, S.A., 2020. The Role of Nitric Oxide in Cancer: Master Regulator or NOt? Int J Mol Sci 21, 1–30.

Kooti, W., Servatyari, K., Behzadifar, M., Asadi-Samani, M., Sadeghi, F., Nouri, B., Zare Marzouni, H., 2017. Effective Medicinal Plant in Cancer Treatment, Part 2: Review Study. J Evid Based Complementary Altern Med 22, 982–995.

Kramer, N., Walzl, A., Unger, C., Rosner, M., Krupitza, G., Hengstschläger, M., Dolznig, H., 2013. In vitro cell migration and invasion assays. Mutat Res 752, 10–24.

Krock, B.L., Skuli, N., Simon, M.C., 2011. Hypoxia-Induced Angiogenesis: Good and Evil. Genes Cancer 2, 1117–1133.

Kumari, N., Dwarakanath, B.S., Das, A., Bhatt, A.N., 2016. Role of interleukin-6 in cancer progression and therapeutic resistance. Tumor Biology 37, 11553–11572.

Lambert, A.W., Pattabiraman, D.R., Weinberg, R.A., 2017. Emerging Biological Principles of Metastasis. Cell 168, 670–691.

Lan, T., Chen, L., Wei, X., 2021. Inflammatory Cytokines in Cancer: Comprehensive Understanding and Clinical Progress in Gene Therapy. Cells 10, a028662.

Landskron, G., de la Fuente, M., Thuwajit, P., Thuwajit, C., Hermoso, M.A., 2014. Chronic Inflammation and Cytokines in the Tumor Microenvironment. J Immunol Res 2014, 1–19.

Laronha, H., Caldeira, J., 2020. Structure and Function of Human Matrix Metalloproteinases. Cells 9, 1076–1093.

Läubli, H., Borsig, L., 2019. Altered Cell Adhesion and Glycosylation Promote Cancer Immune Suppression and Metastasis. Front Immunol 10, 1–9.

Lee, G.-H., Yan, C., Shin, S.-J., Hong, S.-C., Ahn, T., Moon, A., Park, S.J., Lee, Y.C., Yoo, W.H., Kim, H.-T., Kim, D.-S., Chae, S.-W., Kim, H.-R., Chae, H.-J., 2010. BAX inhibitor-1 enhances cancer metastasis by altering glucose metabolism and activating the sodium-hydrogen exchanger: the alteration of mitochondrial function. Oncogene 29, 2130–41.

Lee, H.H., Jung, J., Moon, A., Kang, H., Cho, H., 2019. Antitumor and Anti-Invasive Effect of Apigenin on Human Breast Carcinoma through Suppression of IL-6 Expression. Int J Mol Sci 20, 3143–3158.

Li, H., Qiu, Z., Li, F., Wang, C., 2017. The relationship between MMP-2 and MMP-9 expression levels with breast cancer incidence and prognosis. Oncol Lett 14, 5865–5870.

Li, R., Wen, A., Lin, J., 2020. Pro-Inflammatory Cytokines in the Formation of the Pre-Metastatic Niche. Cancers (Basel) 12, 3752.

Liew, S.K., Azmi, M.N., In, L. la, Awang, K., Nagoor, N.H., 2017. Anti-proliferative, apoptotic induction, and anti-migration effects of hemi-synthetic 1'S-1'-acetoxychavicol acetate analogs on MDA-MB-231 breast cancer cells. Drug Des Devel Ther 11, 2763–2776.

Liubomirski, Y., Lerrer, S., Meshel, T., Rubinstein-Achiasaf, L., Morein, D., Wiemann, S., Körner, C., Ben-Baruch, A., 2019. Tumor-Stroma-Inflammation Networks Promote Pro-metastatic Chemokines and Aggressiveness Characteristics in Triple-Negative Breast Cancer. Front Immunol 10, 1–24.

Loffek, S., Schilling, O., Franzke, C.-W., 2011. Biological role of matrix metalloproteinases: a critical balance. European Respiratory Journal 38, 191–208.

Lugano, R., Ramachandran, M., Dimberg, A., 2020. Tumor angiogenesis: causes, consequences, challenges and opportunities. Cellular and Molecular Life Sciences 77, 1745–1770.

Mabasa, X.E., Mathomu, L.M., Madala, N.E., Musie, E.M., Sigidi, M.T., 2021. Molecular Spectroscopic (FTIR and UV-Vis) and Hyphenated Chromatographic (UHPLC-qTOF-MS) Analysis and In Vitro Bioactivities of the Momordica balsamina Leaf Extract. Biochem Res Int 2021, 1–12.

Maeshiro, M., Shinriki, S., Liu, R., Nakachi, Y., Komohara, Y., Fujiwara, Y., Ohtsubo, K., Yoshida, R., Iwamoto, K., Nakayama, H., Matsui, H., 2021. Colonization of distant organs by tumor cells generating circulating homotypic clusters adaptive to fluid shear stress. Sci Rep 11, 6150.

Mahomoodally, M.F., 2013. Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants. Evidence-Based Complementary and Alternative Medicine 2013, 1–14.

Majidpoor, J., Mortezaee, K., 2021. Steps in metastasis: an updated review. Medical Oncology 38, 3.

Manore, S.G., Doheny, D.L., Wong, G.L., Lo, H.-W., 2022. IL-6/JAK/STAT3 Signaling in Breast Cancer Metastasis: Biology and Treatment. Front Oncol 12, 1–17.

Marciel, M.P., Hoffmann, P.R., 2017. Selenoproteins and Metastasis. In: Advances in Cancer Research. Academic Press Inc., pp. 85–108.

Masjedi, A., Hashemi, V., Hojjat-Farsangi, M., Ghalamfarsa, G., Azizi, G., Yousefi, M., Jadidi-Niaragh, F., 2018. The significant role of interleukin-6 and its signaling pathway in the immunopathogenesis and treatment of breast cancer. Biomedicine & Pharmacotherapy 108, 1415–1424.

Massagué, J., Obenauf, A.C., 2016. Metastatic colonization by circulating tumour cells. Nature 529, 298–306.

Mbele, M., Hull, R., Dlamini, Z., 2017. African medicinal plants and their derivatives: Current efforts towards potential anti-cancer drugs. Exp Mol Pathol 103, 121–134.

Medeiros, B., Allan, A.L., 2019. Molecular Mechanisms of Breast Cancer Metastasis to the Lung: Clinical and Experimental Perspectives. Int J Mol Sci 20, 2272–2288.

Meirson, T., Gil-Henn, H., 2018. Targeting invadopodia for blocking breast cancer metastasis. Drug Resist Updat 39, 1–17.

Mshelia, H.S., Karumi, Y., Dibal, N.I., 2017. Therapeutic effect of Momordica balsamina leaf extract on ethanol-induced gastric ulcer in Wistar rats. Ann Res Hosp 1, 1–1.

Multhoff, G., Molls, M., Radons, J., 2012. Chronic Inflammation in Cancer Development. Front Immunol 2, 1–17.

Munn, L.L., 2017. Cancer and inflammation. Wiley Interdiscip Rev Syst Biol Med 9, e1370.

Muz, B., de la Puente, P., Azab, F., Azab, A.K., 2015. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia (Auckl) 3, 83–92.

Ndhlala, A.R., Finnie, J.F., van Staden, J., 2011. Plant composition, pharmacological properties and mutagenic evaluation of a commercial Zulu herbal mixture: Imbiza ephuzwato. J Ethnopharmacol 133, 663–674.

Nordin, F.J., Pearanpan, L., Chan, K.M., Kumolosasi, E., Yong, Y.K., Shaari, K., Rajab, N.F., 2021. Immunomodulatory potential of Clinacanthus nutans extracts in the co-culture of triple-negative breast cancer cells, MDA-MB-231, and THP-1 macrophages. PLoS One 16, e0256012.

Odenthal, J., Takes, R., Friedl, P., 2016. Plasticity of tumor cell invasion: governance by growth factors and cytokines. Carcinogenesis 37, 1117–1128.

Olalere, O.A., Gan, C.Y., 2020. Multi-step reflux extraction of bio-pharmaceutical phenolic bioactives from balsam apple (Momordica balsamina L.). Journal of Taibah University for Science 14, 227–234.

Oronsky, B., Fanger, G.R., Oronsky, N., Knox, S., Scicinski, J., 2014. The Implications of Hyponitroxia in Cancer. Transl Oncol 7, 167–173.

O'Sullivan, S., Medina, C., Ledwidge, M., Radomski, M.W., Gilmer, J.F., 2014. Nitric oxide-matrix metaloproteinase-9 interactions: biological and pharmacological significance--NO and MMP-9 interactions. Biochim Biophys Acta 1843, 603–17.

Palhares, R.M., Gonçalves Drummond, M., Dos Santos Alves Figueiredo Brasil, B., Pereira Cosenza, G., das Graças Lins Brandão, M., Oliveira, G., 2015. Medicinal plants recommended by the

world health organization: DNA barcode identification associated with chemical analyses guarantees their quality. PLoS One 10, e0127866.

Park, J.Y., Shin, M.-S., 2020. Inhibitory Effects of Pectic Polysaccharide Isolated from Diospyros kaki Leaves on Tumor Cell Angiogenesis via VEGF and MMP-9 Regulation. Polymers (Basel) 13, 64.

Parker, A.L., Cox, T.R., 2020. The Role of the ECM in Lung Cancer Dormancy and Outgrowth. Front Oncol 10, 1–22.

Pearson, G.W., 2019. Control of Invasion by Epithelial-to-Mesenchymal Transition Programs during Metastasis. J Clin Med 8, 646.

Piotrowski, I., Kulcenty, K., Suchorska, W., 2020. Interplay between inflammation and cancer. Reports of Practical Oncology & Radiotherapy 25, 422–427.

Qian, B.-Z., 2017. Inflammation fires up cancer metastasis. Semin Cancer Biol 47, 170–176.

Qin, J.-J., Yan, L., Zhang, J., Zhang, W.-D., 2019. STAT3 as a potential therapeutic target in triple negative breast cancer: a systematic review. Journal of Experimental & Clinical Cancer Research 38, 195.

Raina, H., Soni, G., Jauhari, N., Sharma, N., Bharadvaja, N., 2014. Phytochemical importance of medicinal plants as potential sources of anticancer agents. Turk J Botany 38, 1027–1035.

Ramalhete, C., Gonçalves, B.M.F., Barbosa, F., Duarte, N., Ferreira, M.-J.U., 2022. Momordica balsamina: phytochemistry and pharmacological potential of a gifted species. Phytochemistry Reviews 21, 617–646.

Raykin, J., Snider, E., Bheri, S., Mulvihill, J., Ethier, C.R., 2017. A modified gelatin zymography technique incorporating total protein normalization. Anal Biochem 521, 8–10.

Razidlo, G.L., Burton, K.M., McNiven, M.A., 2018. Interleukin-6 promotes pancreatic cancer cell migration by rapidly activating the small GTPase CDC42. Journal of Biological Chemistry 293, 11143–11153.

Reeh, H., Rudolph, N., Billing, U., Christen, H., Streif, S., Bullinger, E., Schliemann-Bullinger, M., Findeisen, R., Schaper, F., Huber, H.J., Dittrich, A., 2019. Response to IL-6 trans- and IL-6 classic signalling is determined by the ratio of the IL-6 receptor α to gp130 expression: fusing experimental insights and dynamic modelling. Cell Communication and Signaling 17, 46.

Rose-John, S., 2020. Interleukin-6 signalling in health and disease. F1000Res 9, 1013.

Sağlam, Ö., Ünal, Z.S., Subaşı, C., Ulukaya, E., Karaöz, E., 2015. IL-6 originated from breast cancer tissue-derived mesenchymal stromal cells may contribute to carcinogenesis. Tumor Biology 36, 5667–5677.

Sathiya Kamatchi, T., Mohamed Subarkhan, M.K., Ramesh, R., Wang, H., Małecki, J.G., 2020. Investigation into antiproliferative activity and apoptosis mechanism of new arene Ru(II) carbazole-based hydrazone complexes. Dalton Transactions 49, 11385–11395.

Sazonova, E. v., Chesnokov, M.S., Zhivotovsky, B., Kopeina, G.S., 2022. Drug toxicity assessment: cell proliferation versus cell death. Cell Death Discov 8, 417.

Scheau, C., Badarau, I.A., Costache, R., Caruntu, C., Mihai, G.L., Didilescu, A.C., Constantin, C., Neagu, M., 2019. The Role of Matrix Metalloproteinases in the Epithelial-Mesenchymal Transition of Hepatocellular Carcinoma. Analytical Cellular Pathology 2019, 1–10.

Schumacher, N., Rose-John, S., 2022. ADAM17 orchestrates Interleukin-6, TNFα and EGF-R signaling in inflammation and cancer. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1869, 119141.

Semwal, D.K., Chauhan, A., Kumar, Ankit, Aswal, S., Semwal, R.B., Kumar, Abhimanyu, 2019. Status of Indian medicinal plants in the International Union for Conservation of Nature and the future of Ayurvedic drugs: Shouldn't think about Ayurvedic fundamentals? J Integr Med 17, 238–243.

Senggunprai, L., Thammaniwit, W., Kukongviriyapan, V., Prawan, A., Kaewseejan, N., Siriamornpun, S., 2016. Cratoxylum formosum Extracts Inhibit Growth and Metastasis of Cholangiocarcinoma Cells by Modulating the NF-κB and STAT3 Pathways. Nutr Cancer 68, 328–41.

Serala, K., Steenkamp, P., Mampuru, L., Prince, S., Poopedi, K., Mbazima, V., 2021. In vitro antimetastatic activity of Momordica balsamina crude acetone extract in HT-29 human colon cancer cells. Environ Toxicol 36, 2196–2205.

Seyfried, T.N., Huysentruyt, L.C., 2013. On the Origin of Cancer Metastasis. Crit Rev Oncog 18, 43–73.

Shan, J., Chouchane, A., Mokrab, Y., Saad, M., Boujassoum, S., Sayaman, R.W., Ziv, E., Bouaouina, N., Remadi, Y., Gabbouj, S., Roelands, J., Ma, X., Bedognetti, D., Chouchane, L., 2019. Genetic Variation in CCL5 Signaling Genes and Triple Negative Breast Cancer: Susceptibility and Prognosis Implications. Front Oncol 9, 1–13.

Simo Richard, T., Hervé Nwabo Kamdje, A., Mukhtar, F., Herve Nwabo Kamdje, A., 2015. Medicinal Plants in Breast Cancer Therapy. Journal of Diseases and Medicinal Plants 1, 19–23.

Singh, S., Sharma, B., Kanwar, S.S., Kumar, A., 2016. Lead Phytochemicals for Anticancer Drug Development. Front Plant Sci 7, 1–13.

Sinka, I., Kiss, A., Mernyák, E., Wölfling, J., Schneider, G., Ocsovszki, I., Kuo, C.-Y., Wang, H.-C., Zupkó, I., 2018. Antiproliferative and antimetastatic properties of 3-benzyloxy-16-hydroxymethylene-estradiol analogs against breast cancer cell lines. Eur J Pharm Sci 123, 362–370.

Sirotković-Skerlev, M., Kulić, A., Bradić, L., Čačev, T., 2012. Protumor effects of proinflammatory mediators in breast cancer. Period Biol 114, 489–496.

Solomon, J., Raškova, M., Rösel, D., Brábek, J., Gil-Henn, H., 2021. Are We Ready for Migrastatics? Cells 10, 1–8.

Sonnessa, M., Cioffi, A., Brunetti, O., Silvestris, N., Zito, F.A., Saponaro, C., Mangia, A., 2020. NLRP3 Inflammasome From Bench to Bedside: New Perspectives for Triple Negative Breast Cancer. Front Oncol 10, 1–11.

Souda, S., George, S., Mannathoko, N., Goercke, I., Chabaesele, K., 2018. Antioxidant and Antibacterial Activity of Methanol Extract of Momordica Balsamina. IRA-International Journal of Applied Sciences (ISSN 2455-4499) 10, 7–17.

Stoletov, K., Beatty, P.H., Lewis, J.D., 2020. Novel therapeutic targets for cancer metastasis. Expert Rev Anticancer Ther 20, 97–109.

Strilic, B., Offermanns, S., 2017. Intravascular Survival and Extravasation of Tumor Cells. Cancer Cell.

Su, Z., Yang, Z., Xu, Y., Chen, Y., Yu, Q., 2015. Apoptosis, autophagy, necroptosis, and cancer metastasis. Mol Cancer 14, 48.

Sullivan., N.J., 2011. Interleukin-6 in the Breast Tumor Microenvironment. In: Breast Cancer - Focusing Tumor Microenvironment, Stem Cells and Metastasis. InTech, pp. 165–184.

SUN, W., LIU, D.-B., LI, W.-W., ZHANG, L.-L., LONG, G.-X., WANG, J.-F., MEI, Q., HU, G.-Q., 2014. Interleukin-6 promotes the migration and invasion of nasopharyngeal carcinoma cell lines and upregulates the expression of MMP-2 and MMP-9. Int J Oncol 44, 1551–1560.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F., 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 71, 209–249.

Szczerba, B.M., Castro-Giner, F., Vetter, M., Krol, I., Gkountela, S., Landin, J., Scheidmann, M.C., Donato, C., Scherrer, R., Singer, J., Beisel, C., Kurzeder, C., Heinzelmann-Schwarz, V., Rochlitz, C., Weber, W.P., Beerenwinkel, N., Aceto, N., 2019. Neutrophils escort circulating tumour cells to enable cell cycle progression. Nature 566, 553–557.

Sznurkowska, M.K., Aceto, N., 2021. The gate to metastasis: key players in cancer cell intravasation. FEBS J 1–19.

Tang, K., Xin, Y., Li, K., Chen, X., Tan, Y., 2021. Cell Cytoskeleton and Stiffness Are Mechanical Indicators of Organotropism in Breast Cancer. Biology (Basel) 10, 259.

Tesfahuneygn, G., Gebreegziabher, G., 2019. Medicinal Plants Used in Traditional Medicine by Ethiopians A Review Article Journal of Respiratory Medicine and Lung Disease 2, 18–21.

Thakur, G.S., Pandey, M., Sharma, R., Sanodiya, B.S., Prasad, G.B.K.S., Bisen, P.S., 2011. Factors affecting in vitro propagation of Momordica balsamina: a medicinal and nutritional climber. Physiology and Molecular Biology of Plants 17, 193–197.

Thakur, M., Venkateswara, R.G., 2016. Tumour Invasion and Metastasis: A review. Review Article Saudi Journal of Pathology and Microbiology 1, 65–72.

Tsuji, W., Plock, J.A., 2017. Breast Cancer Metastasis. In: Introduction to Cancer Metastasis. Elsevier, pp. 13–31.

Um, H.-D., 2016. Bcl-2 family proteins as regulators of cancer cell invasion and metastasis: a review focusing on mitochondrial respiration and reactive oxygen species. Oncotarget 7, 5193–5203.

Usman, S., Waseem, N.H., Nguyen, T.K.N., Mohsin, S., Jamal, A., Teh, M.-T., Waseem, A., 2021. Vimentin Is at the Heart of Epithelial Mesenchymal Transition (EMT) Mediated Metastasis. Cancers (Basel) 13, 4985.

Valastyan, S., Weinberg, R.A., 2011. Tumor Metastasis: Molecular Insights and Evolving Paradigms. Cell 147, 275–292.

Venter, P., Malemela, K., Mbazima, V., Mampuru, L.J., Muller, C.J.F., Riedel, S., 2021. An RP-LC-UV-TWIMS-HRMS and Chemometric Approach to Differentiate between Momordicabalsamina Chemotypes from Three Different Geographical Locations in Limpopo Province of South Africa. Molecules 26, 1896.

Viallard, C., Larrivée, B., 2017. Tumor angiogenesis and vascular normalization: alternative therapeutic targets. Angiogenesis 20, 409–426.

Wang, X., Bove, A.M., Simone, G., Ma, B., 2020. Molecular Bases of VEGFR-2-Mediated Physiological Function and Pathological Role. Front Cell Dev Biol 8, 1–12.

Wang, X., Khalil, R.A., 2018. Matrix Metalloproteinases, Vascular Remodeling, and Vascular Disease. In: Advances in Pharmacology. Academic Press Inc., pp. 241–330.

Winkler, J., Abisoye-Ogunniyan, A., Metcalf, K.J., Werb, Z., 2020. Concepts of extracellular matrix remodelling in tumour progression and metastasis. Nat Commun 11, 5120.

Winter, M., Meignan, S., Völkel, P., Angrand, P.-O., Chopin, V., Bidan, N., Toillon, R.-A., Adriaenssens, E., Lagadec, C., Le Bourhis, X., 2021. Vimentin Promotes the Aggressiveness of Triple Negative Breast Cancer Cells Surviving Chemotherapeutic Treatment. Cells 10, 1–16.

Wong, R.S., 2011. Apoptosis in cancer: from pathogenesis to treatment. Journal of Experimental & Clinical Cancer Research 30, 87.

Wu, J., Jiang, J., Chen, B., Wang, K., Tang, Y., Liang, X., 2021. Plasticity of cancer cell invasion: Patterns and mechanisms. Transl Oncol 14, 100899.

Wu, S., Du, Y., Beckford, J., Alachkar, H., 2018. Upregulation of the EMT marker vimentin is associated with poor clinical outcome in acute myeloid leukemia. J Transl Med 16, 170.

Xie, Q., Yang, Z., Huang, X., Zhang, Z., Li, J., Ju, J., Zhang, H., Ma, J., 2019. Ilamycin C induces apoptosis and inhibits migration and invasion in triple-negative breast cancer by suppressing IL-6/STAT3 pathway. J Hematol Oncol 12, 60.

Xiong, G.-F., Xu, R., 2016. Function of cancer cell-derived extracellular matrix in tumor progression. J Cancer Metastasis Treat 2, 357.

Xu, J., Lin, H., Wu, G., Zhu, M., Li, M., 2021. IL-6/STAT3 Is a Promising Therapeutic Target for Hepatocellular Carcinoma. Front Oncol 11, 1–14.

Yadav, L., Puri, N., Rastogi, V., Satpute, P., Ahmad, R., Kaur, G., 2014. Matrix Metalloproteinases and Cancer - Roles in Threat and Therapy. Asian Pacific Journal of Cancer Prevention 15, 1085–1091.

Yahayo, W., Supabphol, A., Supabphol, R., 2013. Suppression of human fibrosarcoma cell metastasis by Phyllanthus emblica extract in vitro. Asian Pac J Cancer Prev 14, 6863–7.

Yari, K., Rahimi, Ziba, Moradi, M.T., Rahimi, Zohreh, 2014. The MMP-2 -735 C Allele is a Risk Factor for Susceptibility to Breast Cancer. Asian Pacific Journal of Cancer Prevention 15, 6199–6203.

Yeung, K.T., Yang, J., 2017. Epithelial-mesenchymal transition in tumor metastasis. Mol Oncol 11, 20–39.

Yin, L., Duan, J.-J., Bian, X.-W., Yu, S., 2020. Triple-negative breast cancer molecular subtyping and treatment progress. Breast Cancer Research 22, 61.

Zavyalova, M. v., Denisov, E. v., Tashireva, L.A., Savelieva, O.E., Kaigorodova, E. v., Krakhmal, N. v., Perelmuter, V.M., 2019. Intravasation as a Key Step in Cancer Metastasis. Biochemistry (Moscow) 84, 762–772.

Zhang, H., Liu, L., Jiang, C., Pan, K., Deng, J., Wan, C., 2020. MMP9 protects against LPS-induced inflammation in osteoblasts. Innate Immun 26, 259–269.

APPENDIX: SUPPLEMENTARY DATA

7.1. Main reagents and consumables

Table 7.1: The list of main reagents and consumables used in the study.

Reagents/consumables and Kits	Company and Country of Origin
Accuris™ qMax First strand cDNA Synthesis Kit	Accuris, USA
Annexin-V-FLUOS staining kit	Roche, Germany
Antibodies (MMP-2, MMP-9, Vimentin, β-actin)	Santa Cruz Biotechnology, RSA
Anti-h-TIMP-3 antibody	R&D systems, USA
Bicinchoninic Acid Kit	Sigma-Aldrich, USA
Bovine Serum Albumin (BSA)	Sigma-Aldrich, USA
Cell culture flasks and plates	Nest Biotechnology, USA
Chemicon® Cell adhesion array kit	Merck, USA
Cultrex® BME	R&D systems, USA
Curcumin	Sigma-Aldrich, Germany
Dimethyl sulfoxide (DMSO)	EMD Millipore, Japan
Dulbecco's Modified Eagle Medium	Lonza, USA
Fixation solution	Abcam, UK
Foetal bovine serum (FBS)	Biowest, USA
Interleukin-6	Sigma-Aldrich, USA
Mitomycin C	Tocris, UK
Multiskan Sky microplate spectrophotometer	Thermo Scientific, USA
Muse® Count and Viability Kit	Luminex Corporations, USA
Muse® Nitric oxide Kit	Luminex Corporations, USA
Novex™ 10% Zymogram Plus (Gelatin) gel	Thermo Fisher Scientific, USA
Penicillin and Streptomycin solution	Biowest, USA
Phosphate Buffered Saline (PBS)	Lonza, USA
Primers	Inqaba biotec, RSA

Protease inhibitor cocktail tablets	Roche, Germany
PVDF Transfer membrane	Thermo Scientific, USA
RNeasy® Mini Kit	Qiagen, Germany
SensiMix™ SYBR No-ROX Kit	Bioline, UK
Transwell® permeable supports	Corning Incorporated, USA
Triton X-100	E. Merck, Germany
Trypan Blue stain	Thermo Fisher Scientific, USA
Trypsin	Lonza, USA
Tween 20	Roche, Germany

7.2. Main equipment

- Benchtop centrifuge HERMLE Z 326 K (HERMLE Laboetechnik GmbH, Germany).
- Biological safety cabinet, class II type A2, (Heal Force Bio-meditech, Shanghai, China).
- C-Digit® Blot Scanner for chemilumiscent western blot (LI-COR Biosciences, USA).
- Corbett Real-Time PCR Rotor-gene 6000 (Qiagen, Germany).
- Countess II FL automated cell counter (Life Technologies, USA).
- Heracell 150i Carbon dioxide incubator (Thermo Fisher Scientific, USA).
- MUSE™ Cell Analyser (Merck-Millipore, Germany).
- Nanodrop One spectrophotometer (Thermo Fisher Scientific, USA).
- Olympus CKX53 inverted microscope (Olympus, Japan).
- Zeiss Primo Vert inverted microscope (Carl Zeiss Microscopy GmbH, Germany).

7.3. PCR Primer sequences

Table 7.2: Primer sequences used in real-time quantitative PCR.

Gene	Primer sequence (5'-3')	Annealing temperature (°C)
Bax	**F**: GGGTGGTTGGGTGAGACTC **R**: AGACACGTAAGGAAAACGCATTA	54
Bcl-2	**F**: GCACCGGGCATCTTCTCCTC **R**: CCGAGATGTCCAGCCAGCTG	57
JAK2	**F**: AGGATGCCCAGATGAGATTTATG **R**: CAGCTATACTGTCCCGGATTTG	56
MMP-2	**F**: GTTCAACGGTCGGGAATACA **R**: GCCATACTTGCCATCCTTCT	51.8
MMP-9	**F**: CTGGAACTCACACGACATCTT **R**: TCCACCTTGTTCACCTCATTT	51.8
STAT3	**F**: TGCCTGCGGCATCCTTCTGC **R**: ACAGGCGTGAGCCACCATGC	58
GAPDH	**F**: AGCTGAACGGGAAGCTCACT **R**: TGCTGTAGCCAAATTCGTTG	60

7.4. Buffer preparations

1. **RIPA lysis buffer:**

   10 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, and 1 tablet of protease inhibitor cocktail (1 tablet in 50 mL).

2. **Tris-glycine running buffer (10X)-500 mL:**

   15 g Tris-base, 72 g Glycine, and 5 g SDS in sterile distilled-water, pH 8.4.

   1X running buffer (1 L)

   100 mL of 10X Tris-glycine transfer buffer, 200 mL methanol, and 700 mL dH_2O.

3. **Tris-glycine transfer buffer (10X)-500 mL:**

   15 g Tris-base and 72 g Glycine in sterile distilled-H_2O, pH 8.4.

   1X transfer buffer (1L)

   100 mL of 10X Tris-glycine transfer buffer, 200 mL methanol, and 700 mL dH_2O.

4. **Tris buffered saline (10X TBS)**

   24 g Tris and 88 g NaCl

0.1% TBS-Tween 20

100 mL 10X TBS, 900 mL dH_2O, and 1 mL Tween-20

5. **2X SDS-sample buffer (Western blot)**

   126 mM Tris-HCl pH 6.8, 10% glycerol, 1% SDS, 5% 2-mecarptoethanol, and 0.01% bromophenol blue.

6. **2X SDS sample buffer (Gelatin zymography)**

   62.5 mM Tris-HCl, 1% SDS, 10% glycerol, and 0.01% bromophenol blue.

7. **Wash buffer (Gelatin zymography)**

   2.5% Triton X-100

8. **Developing buffer**

   50 mM Tris-HCl pH 8, 150 mM NaCl, and 10 mM $CaCl_2$.

9. **Destaining solution**

   50% methanol, 10% glacial acetic acid, and 40% dH_2O.