MOMORDICA BALSAMINA CRUDE ACETONE LEAF EXTRACT IMPEDES THE HUMAN HT-29 COLON CANCER CELL INVASIVENESS, MIGRATION AND ADHESION BY INHIBITING ROS-MEDIATED TNF-α/NF-κB/MMP-2/-9 SIGNALLING PATHWAY

MASTER OF SCIENCE

K. SERALA

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by

KARABO SERALA

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Prof. S. Prince (UCT)

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Biochemistry has not previously been submitted by me for a degree at this or any University; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Serala K	21/12/2020
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Surname and Initial	Date

DEDICATION

This work is dedicated to my parents, **Serala Letjatji Donald** and **Machipi Makoma Johanna**, for believing me and their endless love, motivation and unreserved support throughout my studies.

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*"**With man this is impossible, but with God, all things are possible**"* ~ Matthew 19:26.

LIST OF CONFERENCES AND PRESENTATIONS

Serala K., Mampuru L.J., Prince S. and Mbazima V.G. The anti-metastatic effects of *Momordica balsamina* L. crude acetone leaf extract in human HT-29 colon cancer cells. University of Limpopo Faculty of Science and Agriculture research day, Fusion Boutique, Polokwane, September 20–21, 2018.

Serala K., Mampuru L.J., Prince S. and Mbazima V.G. The anti-metastatic effects of *Momordica balsamina* L. crude acetone leaf extract in human HT-29 colon cancer cells. SAMRC 12th Annual Early Career Scientific Convention, Parow, Cape Town, October 17–19, 2018.

Serala K., Mampuru L.J., Prince S. and Mbazima V.G. *Momordica balsamina* L. inhibits HT-29 colon cancer cell invasiveness by suppressing ROS-mediated TNF-α/NF-κB/MMP-2/-9 signalling pathway. 4th Annual joint RCDI-SAMRC Research Conference, Salt Rock Hotel, Durban, July 24–27, 2019.

Serala K., Mampuru L.J., Prince S. and Mbazima V.G. The *in vitro* anti-migratory and anti-adhesive effects of *Momordica balsamina* L. crude acetone leaf extract in human HT-29 colon cancer cells. 1st Biennial Health Science Research Conference, The Ranch Resort, Limpopo, September 11–13, 2019.

Serala K., Mampuru L.J., Prince S. and Mbazima V.G. *Momordica balsamina* L. inhibits HT-29 colon cancer cell invasiveness by suppressing ROS-mediated TNF-α/NF-κB/MMP-2/-9 signalling pathway. 10th Faculty of Science and Agriculture research day, The Ranch Resort, Limpopo, September 19–20, 2019.

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LIST OF ABBREVIATION

BCA	Bicinchoninic acid
BM	Basement membrane
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
CANSA	Cancer Association of South Africa
CO_2	Carbon dioxide
CTCs	Circulating tumour cells
$DFCH_2$-DA	Dichloro-dihydro-fluorescein diacetate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Medium
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor-kappa B
PA	Plasmin activator
PBS	Phosphate buffered saline
PSN	Penicillin, streptomycin and neomycin
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TIMPs	Tissue inhibitor of matrix metalloproteinases
TNF-α	Tumour necrosis factor-alpha
WHO	World Health Organisation

ABSTRACT

Metastatic cancer remains incurable and accounts for 90% of cancer-related deaths (He *et al.*, 2019). Therefore, there's an urgent need to find anti-metastatic drugs with novel therapeutic targets (Zhang *et al.*, 2018). Medicinal plants are promising sources of novel compounds with anti-metastatic activity (Tungsukruthai *et al.*, 2018). This study investigated the anti-metastatic effects of *Momordica balsamina* L. crude acetone leaf extract in human HT-29 colon cancer cells. Powdered leaves of *M. balsamina* were macerated in acetone and reconstituted in dimethyl sulfoxide (>99.9%). The cytotoxic effect of the *M. balsamina* extract was investigated using the MTT assay. The acridine orange/ethidium bromide dual staining assay was used to show that the chosen concentrations of the *M. balsamina* extract do not induce apoptosis. The effect of the *M. balsamina* extract on reactive oxygen species formation and epithelial to mesenchymal transition-related morphological changes were assessed by the $DCFH_2$-DA assay and light microscopy, respectively. The anti-invasive, anti-migratory and anti-adhesive effects of the *M. balsamina* extract were investigated using the cell invasion, wound-healing and cell adhesion assays, respectively. The adhesion of HT-29 cells to collagen I, II and IV, fibronectin, laminin, tenascin C and vitronectin was assessed using the ECM-cell adhesion array kit. Furthermore, western blotting was used to assess the effect of the *M. balsamina* extract on the expression of TNF-α, NF-κB, MMP-2, MMP-9 and TIMP-3. The findings revealed that the *M. balsamina* extract significantly inhibited the viability of HT-29 cells at concentrations above 50 µg/ml but had no effect on the viability of C3A liver cells at 40 and 80 µg/ml. Apoptotic features such as cell shrinkage, nuclei condensation, loss of membrane function and formation of apoptotic bodies were observed at 48 hours exposure to the *M. balsamina* extract. Reactive oxygen species formation, epithelial to mesenchymal transition, invasiveness, migration and adhesion were suppressed in HT-29 cells treated with the *M. balsamina* extract for 24 hours. The adhesion of HT-29 cells were varied amongst different extracellular matrix proteins. Furthermore, HT-29 cells treated with the *M. balsamina* extract showed a reduction in the expression of TNF-α, NF-κB, MMP-2 and MMP-9 proteins and an upregulation of TIMP-3 protein. In conclusion, the *M. balsamina* extract tested in this study impedes the metastatic cascade in HT-29 colon cancer cells by inhibiting the reactive oxygen species-mediated TNF-α/NF-κB/MMP-2/MMP-9 pathway. The findings suggest that

M. balsamina L. may be a source of compounds with potential therapeutic use for the treatment of metastatic colon cancer.

GRAPHICAL ABSTRACT

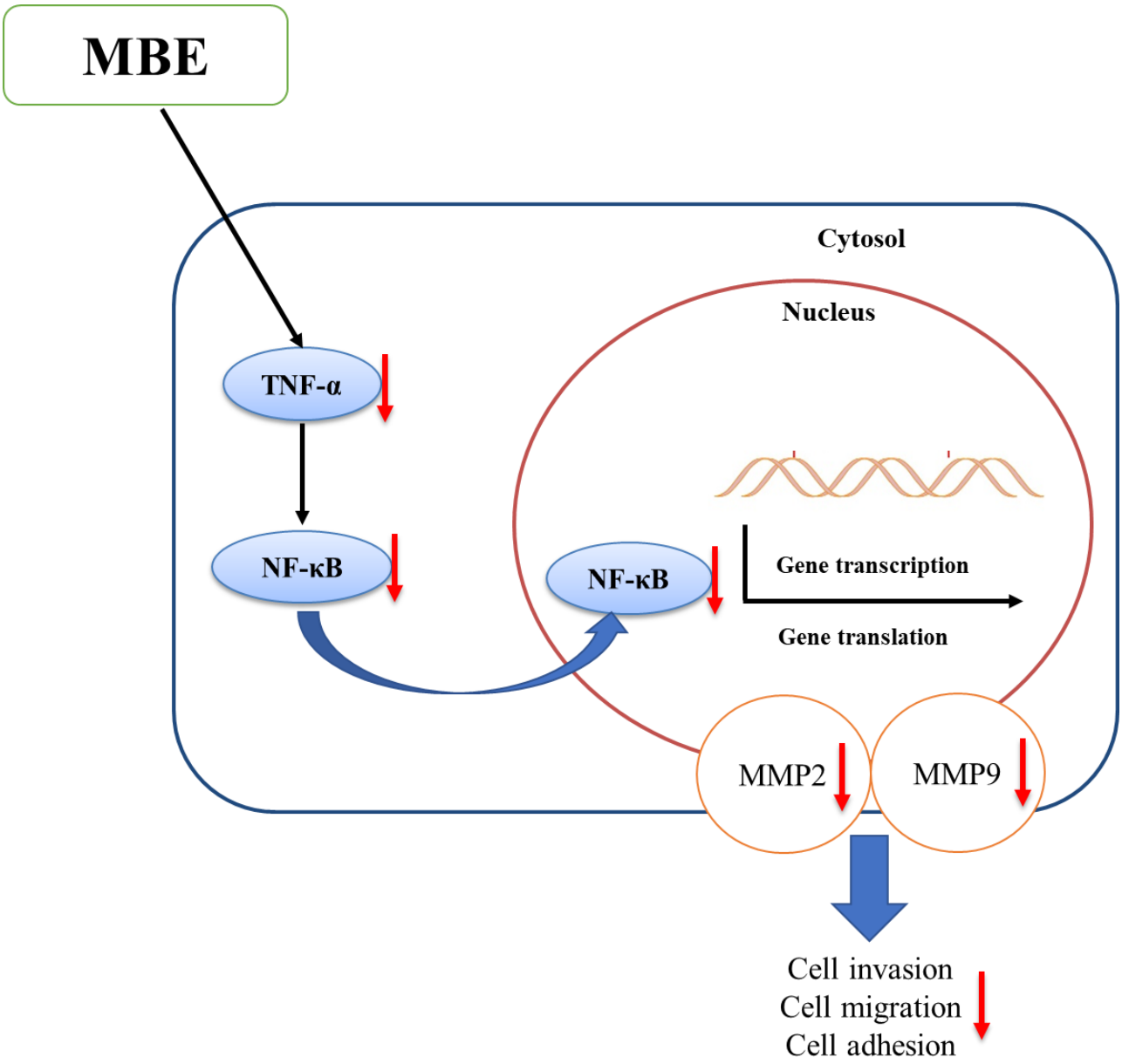

Figure 1: Mechanism of action of *Momordica balsamina* extract in HT-29 colon cancer cells.

CHAPTER ONE

1 Introduction

Cancer has become a significant healthcare burden and contributes to high mortality rates across the globe (Brand *et al.*, 2018). Colon cancer ranks sixth in cancer-related mortality, worldwide (GLOBOCON, 2012). In 2018, 140 250 new colon cancer cases and 50 630 colon cancer deaths were estimated, worldwide (Mehta and Patel, 2019). In South Africa (SA), the cumulative lifetime risk of developing colon cancer is 1.24 and 0.74 for males and females, respectively and the crude incident risk is 7.17/100 000/year for men and 5.80/100 000/year for women. This makes colon cancer the fourth most common cancer in SA (CANSA, 2012).

Treatment and survival of colon cancer patients depend on its advancement (Stein *et al.*, 2011). In South Africa, the currently used treatment methods include surgical resection, chemotherapy and radiotherapy (Nasrallah and El-Sibai, 2014). However, these are only applicable for stages I, II and III colon cancer which have five-year survival rates of 93.2%, 72.2–84.7% and 52.3–83.4%, respectively (Brand *et al.*, 2018). Stage IV colon cancer can only be managed through palliative care and has a five-year survival rate of 8.1% (Brand *et al.*, 2018; Nasrallah and El-Sibai, 2014). This is because cancer has already spread to distant organs through the process of metastasis, which accounts for 90% of all cancer-related deaths (Khan and Mukhtar, 2010; Van Cutsem *et al.*, 2014)

Clinically used anti-metastatic drugs are reported to lose their efficacy with prolonged use and, therefore, fail to render a long-lasting curative response (Peitzsch *et al.*, 2017). This is due to mutations occurring in the target sites of the drugs, thus rendering cancer cells resistant or insensitive to the drugs (Weber, 2012). Furthermore, the drugs have poor selectivity for cancer cells and therefore, cause toxicity to normal cells, especially those in highly proliferative tissues such as bone marrow, gut, gonads and to organs such as liver, kidneys and heart (Fraczkowska *et al.*, 2018). Because of these, the therapeutic value of most drugs is diminished and rejected by the United States Food and Drug Administration (FDA) (Fraczkowska *et al.*, 2018; Nakisige *et al.*, 2017). Therefore, it is critical to find novel therapeutic strategies for the treatment of metastatic cancer (Zhang *et al.*, 2018).

Plant-derived compounds, known as phytochemicals are the most promising candidates for cancer treatment (Manoharan *et al.*, 2014). They possess low toxicity compared to most chemically synthesised drugs (Agbabiaka *et al.*, 2016; Nasri, 2013). However, there are challenges such as cutting down more than 12 000 *Taxus brevifolia* trees to produce 2.5 kg Taxol from 27 000 tons of bark (Rates, 2001). Also, compounds such as Camptothecin have poor solubility and therefore, lead to severe toxicities (Gurib-Fakim, 2006). Therefore, there is an urgent need to screen plants for novel compounds or analogues which would have high solubility, be environmentally friendly and that can be produced at a lower cost (Khazir *et al.*, 2014).

Momordica balsamina L., commonly known as Basalm apple is a perennial climber common in most parts of SA except the Western Cape (Thakur *et al.*, 2009). It has tremendous nutritional and medicinal properties (Souda *et al.*, 2018). Extracts of various parts of the plant have been shown to have anti-viral, anti-inflammatory, shigellocidal, anti-diarrheal, antiseptic, antibacterial and antimicrobial properties (Thakur *et al.*, 2009). In our lab, Boshielo (2017) and Mudalahothe (2018) have shown that the crude acetone extract of *M. balsamina* has pro-apoptotic and anti-metastatic activities against MCF-7 breast and pro-apoptotic activity against A549 lung cancer cells, respectively. Given the differences in the sensitivity of different cancer cell lines towards treatment, this study investigated the potential anti-metastatic effects of the *M. balsamina* crude acetone leaf extract in human HT-29 colon cancer cells.

1.1 Cancer

Cancer is a group of diseases characterised by unrestrained cell proliferation caused by mutations in the genetic control of the cell division cycle, particularly proto-oncogenes and tumour suppressor genes (Hustedt and Durocher, 2017). The unrestricted cell proliferation results in tumours, which can either be benign or malignant. Benign tumours are immotile and therefore, do not spread and invade nearby or distant tissues. In contrast, malignant tumours are motile, hence they spread and invade nearby and distant tissues through a process known as metastasis (Talmadge *et al.*, 2014).

1.2 Metastasis

Cancer metastasis is a dissemination process by which cancer cells detach and migrate from their natural margins to distal organs (Massague *et al.*, 2017). It is a process comprised of multiple distinctive but functionally interrelated steps termed the metastasis cascade. The cascade consists of five stages: cell detachment, invasion, migration, cell adhesion and angiogenesis **(Figure 1)** (Alizadeh *et al.*, 2014).

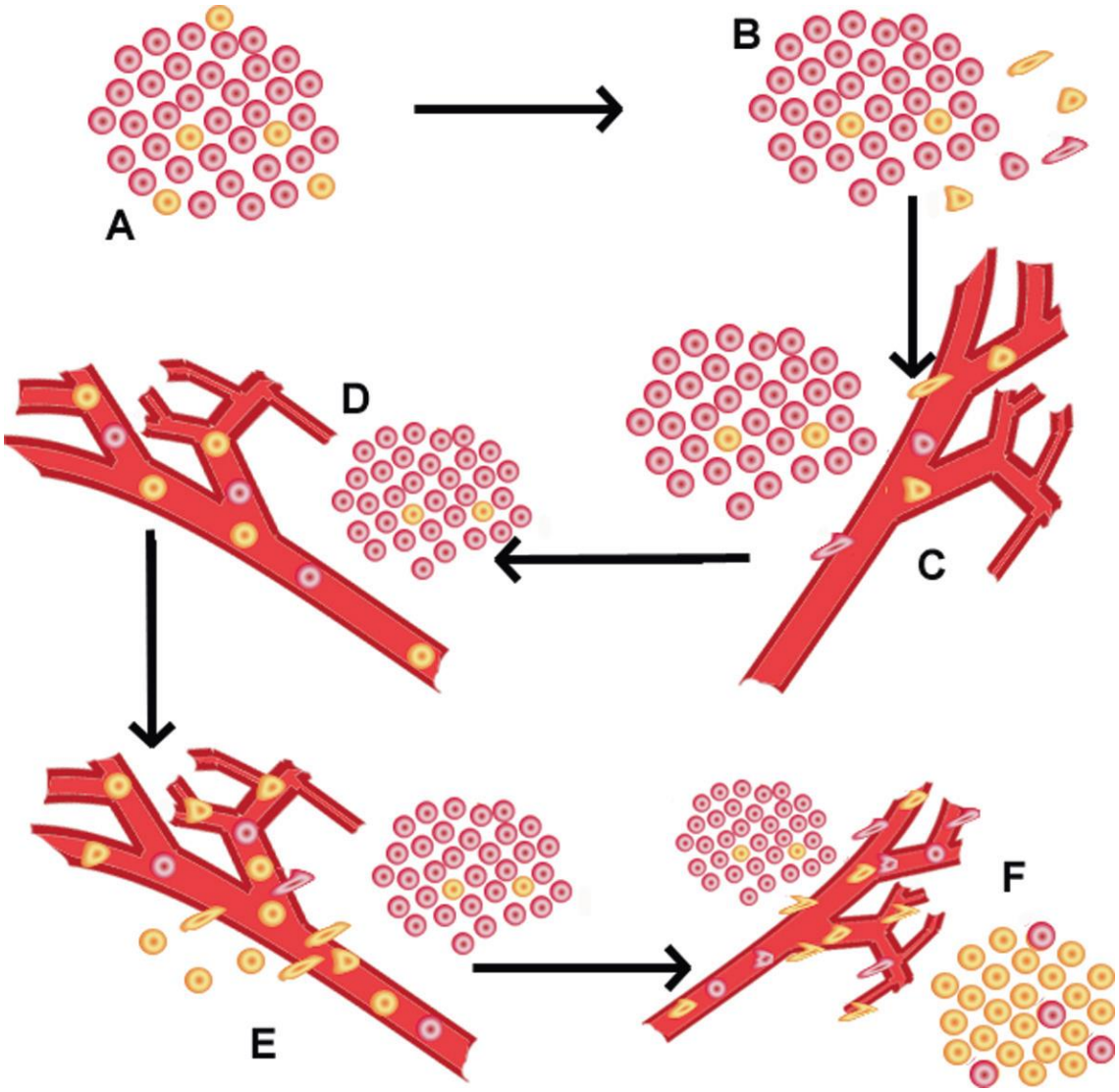

Figure 2: The metastatic cascade. **(A)** Primary (benign) tumour resulting from unrestrained cell proliferation, **(B)** detachment of cells from the primary tumour and epithelial to mesenchymal transition (EMT), **(C)** intravasation of tumour cells into the bloodstream, **(D)** migration of tumour cells to the distal site through the circulatory system, **(E)** extravasation of tumour cells at the distal site and **(F)** tumour growth at the secondary site (Ray and Jablons, 2009).

1.2.1 Cell detachment

Cell detachment is the first step of the metastatic cascade which occurs as a result of pressure exerted by tumour growth onto the extracellular matrix (ECM) (Bhattacharya *et al.*, 2018). It involves mechanical forces and protease-mediated cleavage of the ECM and basement membrane (BM) (Guan, 2015). Mechanical forces are actomyosin-driven and facilitate the dissociation of cells at cytosolic and extracellular sites. Cytosolic dissociation of cell-substrate adhesion is achieved by posttranslational modification of integrins and cytosolic adapter proteins (Kirfel *et al.*, 2004). In contrast, dissociation of extracellular cell-substrate adhesion is performed by matrix proteases which facilitate proteolytic cleavage of matrix constituents such as collagens, fibronectin, laminin, fibrinogen and vitronectin (Guan, 2015; Kirfel *et al.*, 2004). When detached from the substrate, eukaryotic cells undergo a programmed mode of cell death termed anoikis. Hence, they must resist anoikis for survival following the detachment process (Buchheit *et al.*, 2014). Anoikis resistance and other cancer cell property changes are collectively termed epithelial to mesenchymal transition (Gupta and Massague, 2006).

Epithelial to mesenchymal transition (EMT) is a highly dynamic process by which endothelial cells undergo biochemical changes that transform their morphology to a mesenchymal state (Nistico *et al.*, 2012; Roche, 2018). It occurs during normal embryonic development, tissue regeneration, organ fibrosis and wound healing, in response to factors such as hypoxia, cytokines, stromal crosstalk, metabolic changes, the immune system and growth factors secreted by the microenvironment (De Craene and Berx, 2013; Kalluri and Weinberg, 2009). These factors trigger the Wnt, transforming growth factor-β (TGF-β), nuclear factor-kappa B (NF-κB), Hedgehog and Notch signalling pathways, which orchestrate concerted gene and protein programmes necessary for the disassembly and establishment of the epithelial and mesenchymal phenotype, respectively (Gavert and Ben-Ze'ev, 2008; Wu and Zhou, 2008). The disassembly of the epithelial phenotype is due to low expression of epithelial markers such as E-cadherin, occludin, claudin, desmoplakin, cytokeratin-8, −9, −18 and mucin-1 (Kumar *et al.*, 2018). The latter is characterised by reduced cell-cell and cell-ECM adhesion and cell motility (Wu and Zhou, 2008). In contrast, the mesenchymal phenotype is characterised by the overexpression of N-cadherin, vimentin, vitronectin and fibronectin (Kumar *et al.*, 2018). High levels of these proteins

result in elevated resistance to apoptosis, greatly increased production of ECM components, enhanced cell motility and invasiveness (Salehi *et al.*, 2019).

1.2.2 Cell invasiveness

Cell invasiveness is a rate-limiting process during which metastatic cells perforate the ECM and BM through mesenchymal or amoeboid cell invasion (Gupta and Massague, 2006; Nguyen *et al.*, 2009). Amoeboid cell invasion is protease-independent and uses mechanical forces to create a path for cells instead of degrading the ECM (Pepper *et al.*, 2003). In contrast, mesenchymal cell invasion employs proteases to degrade the ECM and is, therefore, protease-dependent. Enzymes involved in mesenchymal cell migration include matrix metalloproteinases and serine proteases (Pepper *et al.*, 2003; Sahai, 2005).

Matrix metalloproteinases (MMPs) represent a large family of zinc-dependent, proteolytic endopeptidases which are synthesised and secreted as zymogens (Kirchhain *et al.*, 2019). They are involved in physiological and pathological processes including tumorigenic processes such as proliferation, invasion and angiogenesis (Alaseem *et al.*, 2017). The MMPs structure is characterised by a pro-peptide domain, a catalytic domain and a hemopexin-like domain (Bourboulia and Stetler-Stevenson, 2010). The pro-peptide domain consists of 80 amino acids and a cysteine-switch motif which maintains the MMPs latency as zymogens. The catalytic domain has 170 amino acids consisting of a zinc-binding site and a methionine residue for zinc-binding support. The hemopexin-like domain is nearly 200 amino acids long and is bound to the catalytic domain by a flexible proline-rich hinge region (Alaseem *et al.*, 2017). Additionally, the hemopexin-like domain facilitates non-catalytic MMP activities as well as other activities such as substrate specificity and activation, inhibition, anchoring and dimerization (Brinckerhoff and Matrisian, 2002; Kessenbrock *et al.*, 2010).

Matrix metalloproteinases are specific for their substrates and are, therefore, categorised based on substrate preference (Naim *et al.*, 2017). For instance, MMP-2 and MMP-9 target gelatin and are, therefore, categorised into gelatinases. Other MMPs are categorised into collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), stromelysins (MMP-3, MMP-10, MMP-11 and MMP-17), matrilysins (MMP-7 and MMP-26) and membrane-type-MMPs (MT-MMP-14, MMP-15, MMP-16, MMP-24 and

MMP-25). Uncharacterised MMPs include MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-28 and MMP-29 (Chaudhary *et al.*, 2013; Sekhon, 2010).

Of all the MMPs, gelatinases (also known as type IV collagenases) are primarily involved in tumour cell invasiveness, where they influence ECM remodelling and degrade all ECM and BM components (Alizadeh *et al.*, 2014). Extracellular matrix components include elastin, fibronectin and vitronectin, and BM components include the triple-helical regions of type IV collagen and laminin (Alaseem *et al.*, 2017). Uniquely, the catalytic domain of gelatinases has a fibronectin type II which is needed for gelatin digestion (Vandooren *et al.*, 2013). Matrix metalloproteinase-2, also known as gelatinase A, is a 72 kDa type IV collagenase which is strongly expressed in tumour and stromal cells (Alizadeh *et al.*, 2014; Chaudhary *et al.*, 2013). It is synthesised as a 631 amino acid proenzyme and its activation involves the removal of the first 80 amino acids (Kupai *et al.*, 2010). When active, MMP-2 is involved in the constant remodelling of the ECM (Alizadeh *et al.*, 2014). On the other hand, MMP-9 (gelatinase B) is an 84 kDa gelatinase belonging to the metzincin enzyme family (Hallett *et al.*, 2013; Kupai *et al.*, 2010). Pro-MMP-9 is 707 amino acids long and has a molecular weight of 92 kDa. Its activation involves the removal of an 8 kDa segment (Kupai *et al.*, 2010). Active MMP-9 preferentially degrades type IV collagen and is expressed in tumour cells and some normal cells (Alizadeh *et al.*, 2014).

The level and activity of MMPs are accurately regulated at various levels including transcriptional, proteolytic activation of the latent form and inhibition of the active enzyme (Chaudhary *et al.*, 2013). Matrix metalloproteinases are expressed in response to multiple stimuli, including inflammatory cytokines, growth factors, glucocorticoids and retinoids, which activate various transcriptional factors, including NF-κB and signal transducers and activators of transcription (Fanjul-Fernández *et al.*, 2010; Vincenti and Brinckerhoff, 2007; Yan and Boyd, 2007). Nuclear factor-kappa B is a pleiotropic transcription factor composed of five distinctive but structurally related subunits viz., Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100), which interact with DNA elements to form homo- and heterodimers (Ma *et al.*, 2015; Shi *et al.*, 2014). The p50 (NF-κB1)/p65 (RelA) is the most common and abundant dimer, which interacts with cytoplasmic IkB proteins and keep NF-κB in an inactive state (Hoesel and Schmid, 2013). Upon stimulation, mostly by tumour necrosis factor-alpha (TNF-α), IkB is phosphorylated,

polyubiquitinated and degraded by IkB kinases, ubiquitin ligase complex and 26S proteasome, respectively (Hoesel and Schmid, 2013; Karin and Ben-Neriah, 2000). This releases an active NF-κB which translocates to the nucleus and bind to the AP-1 site leading to the facilitation of MMP gene expression (Shi *et al.*, 2014).

The activity of MMPs can also be regulated by both physiological and synthetic inhibitors (Bjorklund and Koivunen, 2005). Tissue inhibitors of MMPs (TIMPs) are relatively small, tissue-specific and cysteine-rich proteins commonly expressed at tumour sites as physiological inhibitors of MMPs (Arpino *et al.*, 2015; Bjorklund and Koivunen, 2005). Tissue inhibitors of MMPs consist of four homologs: TIMP-1, TIMP-2, TIMP-3 and TIMP-4, which vary in tissue-specific expression and their ability to inhibit various MMPs (Benjamin and Khalil, 2012; Bjorklund and Koivunen, 2005). Tissue inhibitors of MMP-1 strongly inhibit the activity of MMP-1, MMP-3, MMP-7 and MMP-9. Tissue inhibitors of MMP-2 inhibit the activity of MMP-2 stronger than TIMP-1, while TIMP-3 preferentially inhibit the activity of MMP-9. Furthermore, TIMP-4 inhibits the activity of MMP-2 and MT1-MMP (Alizadeh *et al.*, 2014; Hrabia *et al.*, 2019). Inhibiting the activity of MMPs, blocks intravasation, a rate-limiting step by which cells enter the circulatory systems for migration to distal sites (Alizadeh *et al.*, 2014; Malemud, 2019).

1.2.3 Cell migration

Cell migration is a pivotal step by which metastatic cells move within the circulatory system by directional and non-directional flow to a susceptible metastatic organ (Aceto *et al.*, 2014; Cho *et al.*, 2012). Non-directional movement occurs in response to chemoattractant gradients by a process known as chemotaxis (Alizadeh *et al.*, 2014). During this process, cells enter a motility cycle where they polymerise new actin-rich filaments and extend protrusions which direct their movement towards a chemoattract. The actin-rich protrusions are stabilised by adhesion of cells to the endothelium. This transmits a mechanical force which pulls the cells towards the direction of motion (Hanna and El-Sibai, 2013).

In circulation, cells migrate as single cells or clusters and must evade the immune system, survive shear flow and resist apoptosis for survival (Katt *et al.*, 2018). Several pathways have been proposed for immune protection of circulating tumour cells (CTCs). These include the binding of platelets to CTCs and formation of microthrombi,

which provide a physical barrier and protection from immune components, particularly natural killer cells (Kitamura *et al.*, 2015). Once the shear flow gets low, CTCs get arrested from circulation by rolling adhesion or occlusion (Falanga *et al.*, 2015). Rolling adhesion occurs in large vessels and it is mediated by the upregulation of adhesion molecules on the endothelium (Strell and Entschladen, 2008). Following an endothelial arrest, cells migrate against the direction of flow along the lumen and squeeze through the capillaries (Stoletov *et al.*, 2010). In contrast, occlusion occurs at branch points, on the first capillary bed encountered by CTCs and through the formation of tumour-associated thrombi (Chambers *et al.*, 2002; Kitamura *et al.*, 2015; Yamauchi *et al.*, 2006). The formation of thrombi at neutrophil extracellular traps promote adhesion of tumour cells to the endothelium, thus, providing a mechanism for CTC extravasation (Yamauchi *et al.*, 2006). Extravasation is a complex process by which tumour cells emigrate the circulatory system into the susceptible organ (Alizadeh *et al.*, 2014). It involves modulation of the endothelial barrier, transendothelial migration as well as active adhesion processes (Strilic and Offermanns, 2017).

1.2.4 Cell adhesion

Cell adhesion is a process by which metastatic cells interacts with the ECM at the secondary site (Vlahakis and Debnath, 2017). These interactions are facilitated by cell adhesion molecules (CAMs), which are made of an intracellular, transmembrane and extracellular domain. Cell adhesion molecules can either be calcium-dependent (cadherins, selectins and integrins) or calcium-independent (immunoglobulin superfamily and lymphocyte homing receptors (Guan, 2015).

Integrins are heterodimeric receptors responsible for the adhesion of cells to ECM molecules such as fibronectin, laminin, collagen, fibrinogen and vitronectin (Alizadeh *et al.*, 2014). They are made of a large extracellular domain which binds to ECM components and an intracellular domain which binds to the cytoskeleton by intracellular focal adhesion (FAs) (Bravo-Cordero *et al.*, 2014). The binding of integrins to ECM components transmits signals from the intracellular network to intracellular and extracellular environments. This transmission is mediated by integrin-activated molecules such as focal adhesion kinase (FAK) and phosphatidylinositol-3-kinase (PI3K) (Sakamoto and Kyprianou, 2010). On the other hand, cadherins mediate homophilic cell-cell adhesion wherein they tightly hold adjacent cells together in a non-covalent manner (Alizadeh *et al.*, 2014; Li and Feng, 2011). There are more than

20 cadherin molecules classified based on their cell type-specific expression. These include E-, N-, VE-, P- and R-cadherins in endothelial, mesenchymal, vascular endothelial, placental and renal tissues, respectively (Guan, 2015). The extracellular domain of E-cadherin consists of five repeats and calcium-binding sites. Its intracellular domain is linked to the cytoskeleton through linker proteins thus, forming a complex which stabilises cell-cell adhesion (Rivard, 2009). At the secondary site, tumour cells can grow into a micrometastasis mode, die or become dormant, depending on nutrient and oxygen availability (Kienast *et al.*, 2010).

1.2.5 Angiogenesis

Angiogenesis is a complex process in which a new vascular network is generated from pre-existing blood vessels to supply nutrients, oxygen and facilitates the removal of waste products from the tumour **(Figure 2)** (Liekens *et al.*, 2001). In most cases, angiogenesis occurs in response to hypoxia, which induces the expression of proangiogenic proteins, resulting in an imbalance between the positive and negative angiogenic factors (Warmke *et al.*, 2018). The proangiogenic factors activate the endothelial cells of existing blood vessels to enzymatically digest the underlying BM by the cooperative activity of the plasmin activator (PA) system and MMPs (Liekens *et al.*, 2001). The PA system is comprised of serine proteases: tPAs and uPAs, which regulate the fibrinolytic activity in blood as well as the activation of tissue plasminogen to plasmin, respectively (Guan, 2015; Mignatti and Rifkin, 1996). Plasmin degrades ECM components and activates several MMPs which also facilitate the degradation of the BM (Prager and Poettler, 2012). This paves a way for endothelial cells to migrate through the degraded matrix and proliferate in response to growth factors, some of which have been released from the degraded ECM (Liekens *et al.*, 2001). These include the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiogenin, transforming growth factor-alpha and -beta (TGF-α and -β), TNF-α, interleukin-8, platelet-derived endothelial factor, placental growth factor, hepatocyte growth factor and epidermal growth factor (Nishida *et al.*, 2006). Under homeostatic conditions, the above-mentioned proangiogenic factors are balanced by antiangiogenic factors (Liekens *et al.*, 2001). The physiological antiangiogenic factors include angiostatin, endostatin, interferons, interleukin-1 and -12, TIMPs and retinoic acid (Nishida *et al.*, 2006).

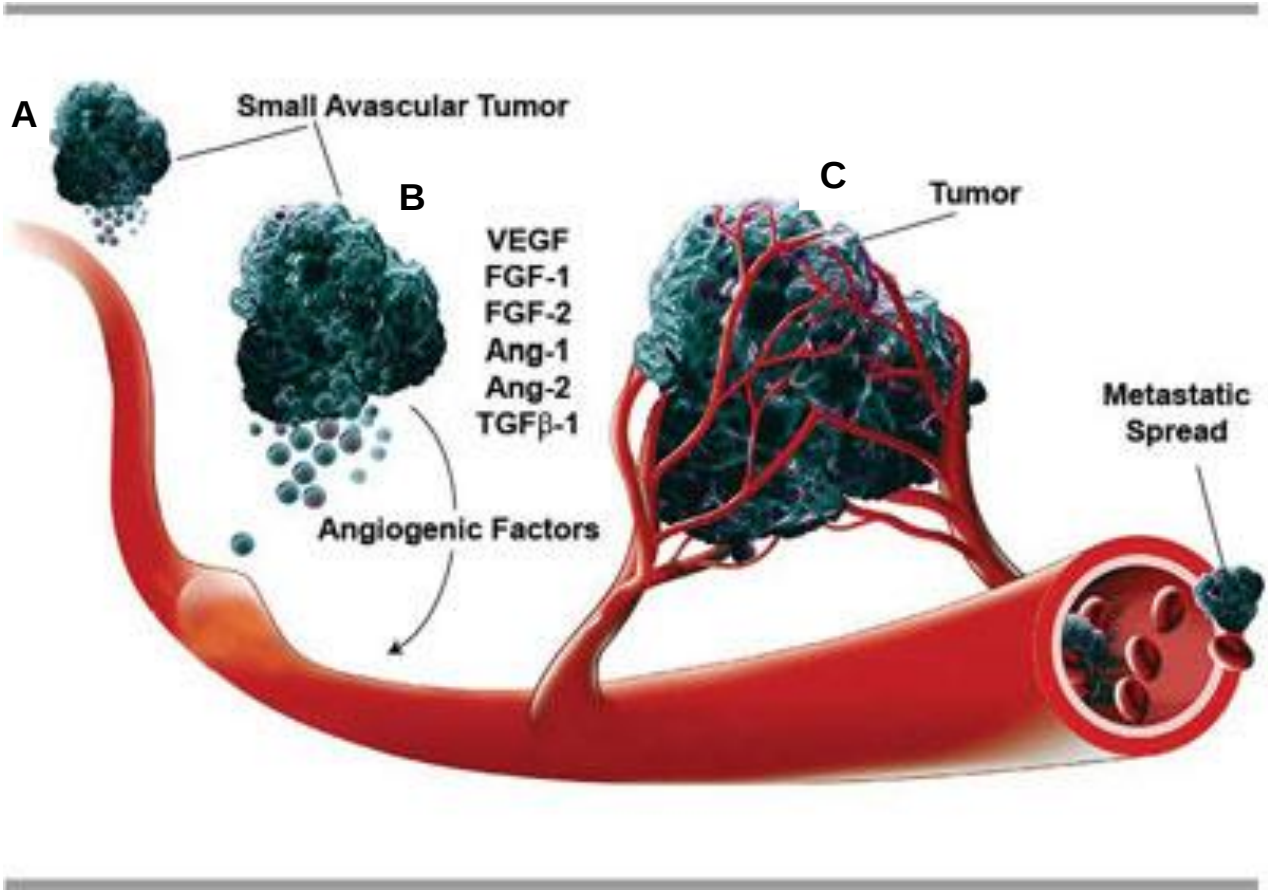

Figure 3: Angiogenesis. **(A)** Hypoxia stimulates tumour cells to release the proangiogenic factors which **(B)** cause vascular sprouting by inducing the proliferation of endothelial cells on the vessel walls. This forms a new vascular network **(C)** which supplies oxygen, nutrients and removes waste from the tumour (AdipoGen®, 2015).

1.3 Reactive oxygen species

Reactive oxygen species (ROS) are short-lived molecules resulting from oxygen-consuming metabolic reactions that occur in peroxisomes, endoplasmic reticulum and the mitochondria (Moloney and Cotter, 2018; Peiris-Pages *et al.*, 2015). Reactive oxygen species comprise of unstable and highly reactive superoxide ($O_2^{\bullet-}$) and hydroxyl ($HO^{\bullet}$) radicals and more stable, freely diffusible non-radicals such as hydrogen peroxide (H_2O_2) and hypochlorous acid (Dickinson and Chang, 2011; Jayavelu *et al.*, 2016). Due to their higher reactivity, ROS can react with all types of biological molecules, therefore, elevated ROS levels can cause oxidative damage to proteins, nucleic acids, lipids and carbohydrates (Chio and Tuveson, 2017). The oxidation of nucleic acids, particularly DNA, causes mutations which alter the normal gene expression patterns (Brieger *et al.*, 2012). Furthermore, oxidation of proteins forms insoluble protein aggregates which have been implicated in carcinogenesis, neurodegeneration, atherosclerosis, diabetes and ageing (Brieger *et al.*, 2012; Chio and Tuveson, 2017; Prasad *et al.*, 2017).

In carcinogenesis, ROS result from increased metabolic activity, mitochondrial dysfunction, oncogenic activity, increased oxidase activity and through crosstalk with infiltrating tumour cells (Liou and Storz, 2010). Reactive oxygen species play a vital role in cell proliferation, survival and inflammation (Storz, 2005). Elevated ROS levels stimulate several signalling molecules which contribute to a greater metastatic potential (Kumari *et al.*, 2018). The signalling molecules include Src kinase, FAK and integrin-associated kinases involved in adhesion, the mitogen-activated protein kinase (MAPK) family of proteins, extracellular signal-regulated kinase, c-jun NH-2 terminal kinase and the p-38 MAPK in migration as well as transcription factors (AP-1, Ets-1 and NF-κB) in invasion (Tochhawng *et al.*, 2013). The impact of high ROS levels can be minimised or reduced through detoxification by antioxidants, which can either be enzymatic or non-enzymatic (Kumari *et al.*, 2018; Liou and Storz, 2010). Enzymatic antioxidants, such as superoxide dismutase, superoxide reductase, catalase, glutathione peroxidase, glutathione reductase, peroxiredoxin and thioredoxin, are highly specific to different kinds of ROS (Brieger *et al.*, 2012; Liou and Storz, 2010). On the other hand, non-enzymatic antioxidants, such as glutathione, flavonoids, vitamins A, C and E are less specific and are of plant origin (Chio and Tuveson, 2017).

1.4 Medicinal plants

The use of herbal medicines has been increasing at an alarming rate (Netshiluvhi and Eloff, 2019). According to the World Health Organisation (WHO), 70–95% of the world's population still use plants as a source of medicine, particularly in developing and underdeveloped countries (Ait-Sidi-Brahim *et al.*, 2019). People from these regions prefer herbal medicine because it is affordable, easily accessible and believed to be more efficient than western medicine (Cragg and Newman, 2013; Hilonga *et al.*, 2019). South Africa is well known for its unique flora and botanical diversity (Viljoen *et al.*, 2019). The South African plant kingdom has 30 000 species of higher plants, and 3000 of these species have the potential to be used medicinally (van Wyk and Prinsloo, 2018).

Medicinal plants continue to have a great impact in drug discovery (Sameiyan *et al.*, 2019). About 75% of the drugs in the global market are derived from plants or knowledge of traditional medicine (Semwal *et al.*, 2019). Drugs derived from medicinal plants are categorised as pure compounds or complex mixtures such as infusions, essential oils, tinctures and extracts (Sengupta *et al.*, 2018). The complex mixture consists of secondary metabolites such as alkaloids, terpenes, phenolics, flavonoids, organosulfur and nitrogen-containing compounds (Adorjan and Buchbauer, 2010; Asif *et al.*, 2016). These metabolites have diverse biological activities and are the greatest sources of drugs (Prakash *et al.*, 2018; Sengupta *et al.*, 2018). Morphine is the first pharmacologically active drug to be isolated from plants in 1805 (Yehya *et al.*, 2017). Since then, medicinal plants have played a very important role in the amelioration of various ailments (Balunas and Kinghorn, 2005). A review by Prakash *et al*. (2018) outlined some of the recently explored plant-derived compounds and their biological activities. This review listed Taxol, Maytansine, Ergoflavin, Cytoskyrins, Aplidine, Trabectedin and Palmerolide A isolated from *Taxus brevifolia*, *Maytenus serrata*, *Solanum spp.*, *Cytospora sp.*, *Aplidium albicans*, *Ecteinascidia turbinate* and *Synocium adareanum*, respectively as some of the plant-compounds with anticancer activity. In addition, species in the *Momordica* genus have shown a great potential as anticancer agents (Nagarani *et al.*, 2014a).

1.5 *Momordica* genus

The *Momordica* genus, belonging to the Cucurbitaceae family has approximately 60 species of annual and perennial climbers well known for their bitter taste due to a high quantity of phytochemicals such as alkaloids and cucurbitacins (Ghosh *et al.*, 2018; Madala *et al.*, 2016). Species in this genus grow indigenously in warmer parts of the world including East and Southern Africa, Asia, India and South America, where they are consumed as vegetables (Khazir *et al.*, 2014). Apart from their nutritional value, *Momordica* species are well-known for their medicinal properties (Nagarani *et al.*, 2014b). For instance, *Momordica charantia* is used for the treatment of wounds, diabetes, colic, jaundice, leprosy, kidney stones, gout, eczema, pneumonia and rheumatism and psoriasis (Lubinska-Szczygeł *et al.*, 2019). Animal models have shown *Momordica cymbalaria* to have anti-diabetic activity, cardioprotective activity, hepatoprotective activity, nephroprotective effects (Elangovan *et al.*, 2019). Also, *Momordica balsamina*, commonly known as Basalm apple has been used in traditional medicine to treat various ailments, such as mental illness, skin diseases, intercostal pains, wounds, fever and yaws (Puri, 2010). The plant grows natively in Namibia, Botswana, Swaziland and all provinces in South Africa except the Western Cape (Ludidi *et al.*, 2019). The *M. balsamina* plant is characterised by pale yellow, deeply veined flowers and it's round, bright orange fruits and it can grow to an altitude of 1.293 metres and has globous to slightly hairy stems which are about 4–5 mm in length **(Figure 3)** (Thakur *et al.*, 2009).

Figure 4: The *Momordica balsamina* plant.

The mature green leaves and fruits are the commonly consumed parts of the *M. balsamina* plant and are an important source of nutrients such as carbohydrates, proteins, vitamins and minerals (Nagarani *et al.*, 2014b). In our lab, phytochemical analysis by Mohale (2019) revealed that the crude acetone extract of *M. balsamina* leaves contains several bioactive compounds such as flavonoids, tannins, terpenoids, saponins, steroids and coumarins. These compounds attest to the plants anti-plasmodial, shigellocidal, anti-diarrheal, anti-bacterial, anti-viral, anti-inflammatory, anti-microbial, anti-oxidant, analgesic and hepato-protective activities (Kushwaha *et al.*, 2012; Ramalhete *et al.*, 2016). Studies in our lab further revealed that the *M. balsamina* extract has pro-apoptotic and anti-metastatic activities against MCF-7 breast cancer cells (Boshielo, 2017) and pro-apoptotic activity against A549 lung cancer cells (Mudalahothe, 2018). However, its anti-metastatic activities and mechanism of action against HT-29 colon cancer cells are unknown.

2 Purpose of the study

2.1 Aim

The study aimed to investigate the potential anti-metastatic effects of the *Momordica balsamina* extract on human HT-29 colon cancer cells.

2.2 Objectives

The objectives of the study were to assess the effect of the *M. balsamina* extract on:

i. the viability of HT-29 and C3A liver cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.
ii. cell and nuclei morphology of HT-29 cells using the acridine orange/ethidium bromide dual staining assay.
iii. reactive oxygen species formation in HT-29 cells using the dichloro-dihydro-fluorescein diacetate ($DFCH_2$-DA) assay.
iv. epithelial to mesenchymal transition-related morphological changes in HT-29 cells using light microscopy.
v. the invasiveness of HT-29 cells using the cell invasion assay.
vi. the migration of HT-29 cells using the wound healing assay.
vii. the attachment of HT-29 cells using the cell adhesion assay.
viii. the attachment of HT-29 cells to ECM proteins using the ECM cell adhesion array kit.
ix. the expression of TNF-α, NF-κB, MMP-2, MMP-9 and TIMP-3 using western blotting.

CHAPTER TWO

2 Materials and methods

2.1 Main reagents

Acetone was purchased from Rochelle Chemicals (Johannesburg, SA). Filter papers from Munktell (Ahlstrom, Germany). Dimethyl sulfoxide (DMSO), paraformaldehyde and ethidium bromide from Sigma-Aldrich (St. Louis, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM) were purchased from HyClone Laboratories (South Logan, USA). Penicillin, streptomycin and neomycin (PSN) cocktail, Fetal Bovine Serum (FBS) and Trypsin from Gibco (Life Technologies, USA). Phosphate-buffered saline (PBS) from Lonza (Verviers, Belgium). Acridine orange from Carl Roth GmbH + Co. KG, (Karlsruhe, Germany). QCM™ cell invasion assay kit and CHEMICON® ECM cell adhesion array kit from Millipore (Massachusetts, USA). Pierce® Bicinchoninic Acid (BCA) protein assay kit from Thermo Fisher Scientific (Waltham, USA). Nitrocellulose membranes from Amersham Biosciences (Buckinghamshire, UK). Antibodies: NF-κB, TNF-α and β-Actin from Santa Cruz Biotechnology (Texas, USA) and MMP-2, MMP-9 and TIMP-3 from Novus Biotechnologicals (Colorado, USA). All tissue culture plates and other plastic ware were purchased from (Nest Biotechnology Co., Ltd, USA).

2.2 Main equipment

Waring commercial blender (Model 32BL79, Dynamics Corporation, New Hartford, Connecticut, USA). Carbon dioxide (CO_2) incubator (Steri-Cycle i160) and Benchtop centrifuge (Micro CL 17R, Thermo Fisher Scientific, Rockford, USA). GloMax®-Multi+Detection system microtiter plate reader (Promega, Madison, USA). Phase contrast inverted light and fluorescence microscope (Nikon ECLIPSE T*i*, Tokyo, Japan). Inverted light microscope (U-CMAD3 T7, Olympus, Tokyo, Japan). Rotary Shaker 3081U (Labcon, SA).

2.3 Plant collection and extraction

Momordica balsamina L. plant leaves were collected from Mankweng, 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 washed and dried at room temperature, ground into a fine powder using a waring commercial blender and exhaustively extracted as described by Azwanida (2015). Briefly, 200 ml

of acetone was added to 20 g of the powdered leaves, continuously shaken using a rotary shaker at 150 rpm for overnight at room temperature then filtered using a Whatman no. 1 filter paper. The filtrate was air-dried and dissolved in DMSO (>99.9%) to a stock concentration of 100 mg/ml, which was then aliquoted and stored at -20°C until use.

2.4 Cell culture maintenance and treatment

The HT-29 colon cancer cells and C3A liver cells were cultured in DMEM and MEM, respectively. The media were supplemented with 10% FBS and 1% PSN. Cell lines were grown and maintained at 37°C in a humidified tissue culture incubator containing 5% CO_2 and sub-cultured at 80–90% confluency after 3 days to avoid over confluence. For cell-based assays, the *M. balsamina* extract and curcumin (positive control) were diluted to experimental concentrations using cell culture media and filtered using a 0.45 µm syringe filter before treatment.

2.5 Cell viability assay

The cytotoxic effects of the *M. balsamina* extract were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The MTT assay measures cell viability based on the reduction of MTT by mitochondrial NAD(P)H-dependent cellular oxidoreductase enzymes active only in live cells (Mosmann, 1983). Briefly, HT-29 and C3A liver cells were seeded in 96-well culture plates at a density of 1×10^4 cells/well and incubated overnight to attach. To avoid exposing the cells to the extract for more than 24 and 48 hours, HT-29 cells were treated for 20 and 44 hours with 0–200 µg/ml of the *M. balsamina* extract, vehicle control [0.2% (v/v) DMSO] or 20 µM curcumin as a positive control. Liver C3A cells were treated for 20 and 44 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin. After treatment, 20 µl of 2 mg/ml MTT was added to each well and incubated for a further 4 hours. The media was discarded and 100 µl of DMSO (>99.9%) was added to each well to solubilise the formazan crystals. Absorbance was measured at 560 nm using a GloMax®-Multi+Detection system microtiter plate reader. Data were expressed as percentage cell viability using the following formula:

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

2.6 Cell and nuclei morphology assay

The effect of the *M. balsamina* extract on HT-29 cell and nuclei morphology was assessed using the acridine orange/ethidium bromide (AO/EB) dual staining assay. The assay uses acridine orange and ethidium bromide to differentiate between live, apoptotic and necrotic cells. Acridine orange is membrane permeable and stains cells as well as the nuclei green whereas ethidium bromide is membrane impermeable and stains the nuclei of cells with a compromised membrane red (Kasibhatla *et al.*, 2006). HT-29 cells were seeded in 48-well culture plates at a density of 1.5×10^5 cells/well, incubated overnight and treated for 24 and 48 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin. Following incubation, cells were washed twice with 300 µl of 1× PBS and stained with 100 µl of 1 µg/ml acridine orange and ethidium bromide for 10 minutes, in the dark. The cells were then washed as described above, viewed and images acquired under a 10× objective of a fluorescence microscope.

2.7 Reactive oxygen species formation assay

Reactive oxygen species formation in HT-29 cells was assessed using the dichloro-dihydro-fluorescein diacetate ($DFCH_2$-DA) assay. The assay measures the intensity of green DFC fluorescence which is relative to the amount of ROS produced within the cells (Mao *et al.*, 2016). HT-29 cells were seeded in 96-well culture plates at a density of 1.5×10^4 cells/well, incubated overnight and treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin in the presence of 50 µM H_2O_2. After treatment, cells were washed twice with 100 µl of 1× PBS and stained with 20 µl of 10 µM $DCFH_2$-DA at 37°C for 30 minutes, in the dark. After washing as described above, cells were viewed and images acquired under 10× objective of a fluorescence microscope. Total cellular fluorescence was measured at an excitation/emission spectra of 495/529 nm using a GloMax®-Multi+Detection system microtiter plate reader. Data were expressed as percentage ROS formation using the following formula:

$$\text{ROS formation (\%)} = \frac{\text{excitation/emission (495/529) of treated cells}}{\text{excitation/emission (495/529) of untreated cells}} \times 100$$

2.8 Epithelial to mesenchymal transition assay

Epithelial to mesenchymal transition-related morphological changes of HT-29 cells were analysed using light microscopy. The assay exploits the loss of cell-cell junctions as well as a change in morphology from an epithelial to a fibroblast state during EMT. HT-29 cells were seeded in 48-well culture plates at a density of 2×10^5 cells/well using serum-free media, incubated overnight and treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin. Following treatment, cells were washed twice with 300 µl of 1× PBS, viewed and images acquired under a 10× objective of a phase-contrast inverted light microscope.

2.9 Cell invasiveness assay

The effect of the *M. balsamina* extract on the invasiveness of HT-29 cells was assessed using the QCM™ cell invasion assay kit. The assay measures cell invasiveness based on the ability of cells to perforate an ECM coated transwell chamber in response to a chemoattractant. HT-29 cells were seeded in 25 cm^3 culture flasks overnight and treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin. After treatment, cells (2×10^4 /well) were seeded in the upper chamber of the transwell plate using serum-free media and placed in a 96-well culture plate containing DMEM supplemented with 10% FBS for a further 24 hours. Following incubation, cells in the upper chamber were gently removed using a cotton swab. Cells in the lower chamber were fixed with 3.7% (w/v) paraformaldehyde, stained with 0.1% (w/v) crystal violet, viewed and images acquired under a 4× objective of an inverted light microscope. The invasive cells were quantified using ImageJ software and data expressed as percentage cell invasion using the following formula:

$$\text{Cell invasion } (\%) = \frac{\text{Crystal violet intensity of treated cells}}{\text{Crystal violet intensity of untreated cells}} \times 100$$

2.10 SDS-PAGE and western blotting

The effect of the *M. balsamina* extract on TNF-α, NF-κB, MMP-2, MMP-9 and TIMP-3 expression levels was assessed using western blotting. HT-29 cells were cultured in a 25 cm^3 culture flask and treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin. Attached cells were harvested with 1× trypsin-EDTA and centrifuged at 3000 ×g for 5 minutes. The cell pellet was resuspended in a RIPA lysis

buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% deoxycholate powder] supplemented with complete protease inhibitor cocktail (aprotinin, pepstatin and 100 nM phenylmethylsulphonyl fluoride), incubated for 30 minutes on ice and centrifuged at 12 000 rpm for 20 minutes at 4°C. The total cellular protein content was quantified using a BCA kit. Proteins were separated on 8–12% sodium dodecyl sulphate-polyacrylamide gel at 100 volts for 2 hours 30 minutes. Proteins were transferred to Hybond® enhanced chemiluminescence™ (ECL) nitrocellulose membranes for 90 minutes. Membranes were blocked with 5% fat-free milk for 1 hour and incubated in the presence of primary antibodies in Tris-buffered saline-tween-20 (TBST)-milk at 4°C, overnight. The antibodies used were mouse anti-NF-κB (1:1000), goat anti-TNF-α (1:500), mouse anti-MMP-2 (1:500), mouse anti-MMP-9 (1:1000) and rabbit anti-TIMP-3 (1:1000). The membranes were washed three times at 10-minute intervals using 1× TBST and incubated in the presence of HRP-conjugated mouse-(anti-goat, anti-mouse and anti-rabbit) secondary antibodies (1:5000) in 1× TBST for 1 hour at room temperature, with constant shaking in the dark. After incubation, the proteins were visualised using the ECL detection system and scanned. Mean band densities were measured using the ImageJ software and quantitative analysis done by normalising to β-Actin. Data were expressed as relative protein expression.

2.11 Cell migration assay

The effect of the *M. balsamina* extract on HT-29 cell migration was assessed using the wound healing assay. The assay measures the cell's ability to migrate and close a wound created on a confluent cell monolayer (Justus *et al.*, 2014). HT-29 cells were seeded in 24-well culture plates and incubated overnight to form a monolayer. The cell monolayer was wounded using a sterile pipette tip, washed three times with 200 µl of 1× PBS and treated for 6 and 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin. After each incubation period, the wounded monolayers were viewed and images acquired under a 4× objective of an inverted light microscope. The wound sizes were measured using the LCmicro software and data expressed as percentage wound area or wound closure using the following formula:

$$\text{Wound closure/area (\%)} = \frac{\text{Wound size (To)} - \text{Wound size (Tn)}}{\text{Wound size (To)}} \times 100$$

2.12 Cell adhesion assay

The effect of the *M. balsamina* extract on HT-29 cell adhesion was assessed using the cell adhesion assay which measures the ability of cells to bind to Matrigel-coated culture plates (Humphries, 2009). Briefly, 48-well culture plates were coated with 5 µg/ml matrigel for overnight at 37°C and blocked with 2% (w/v) bovine serum albumin (BSA) for 2 hours at room temperature. HT-29 cells (2×10^5 /well) treated for 6 and 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin in serum-free media were seeded in the Matrigel-coated plates for an hour and washed twice with 300 µl of 1× PBS. Attached cells were viewed and images acquired under a 10× objective of a phase-contrast inverted light microscope and quantified by the MTT assay (Wang *et al.*, 2019). Data were expressed as percentage cell adhesion using the following formula:

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

2.13 Extracellular matrix protein adhesion assay

The effect of the *M. balsamina* extract on the adhesion of HT-29 cells to ECM proteins was assessed using the CHEMICON® ECM cell adhesion array kit. This assay uses different ECM proteins to capture cells expressing their specific integrins. Briefly, HT-29 cells (2×10^5 /well) treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM curcumin in serum-free media were seeded in the ECM array plate and incubated for an hour. Following incubation, the non-adherent cells were washed twice with 100 µl of the assay buffer and the attached cells were incubated for 5 minutes with the cell stain solution. After washing three times with deionised water, the plate was air-dried, and the stain solubilised with the extraction buffer for 10 minutes. Absorbance was measured at 560 nm using the GloMax®-Multi+Detection system microtiter plate reader. Data were expressed as percentage cell adhesion using the formula in section 2.11.

2.14 Statistical analysis

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

CHAPTER THREE

3 Results

3.1 The effect of the *Momordica balsamina* extract on the viability of HT-29 and C3A liver cells

An ideal anticancer drug must selectively inhibit cancer cell viability or proliferation and lack pronounced toxicity to normal cells (Fraczkowska *et al.*, 2018). To investigate the ability of the *M. balsamina* extract to selectively decrease the viability of HT-29 cancer cells without affecting liver cells, the MTT assay was performed. Colon HT-29 cancer cells were treated for 24 and 48 hours with increasing concentrations of the *M. balsamina* extract (0–200 µg/ml), 20 µM of curcumin or 0.2% of DMSO as vehicle control. As shown in **Figure 3.1A**, a significant ($p \leq 0.05$, $p \leq 0.01$) reduction in the viability of HT-29 cells was observed at concentrations of the *M. balsamina* extract above 50 µg/ml when compared to the vehicle control. Exposure to 20 µM of curcumin also resulted in a significant ($p \leq 0.05$) reduction in the viability of HT-29 cells. When used in higher concentrations, plant extracts tend to be toxic to organs such as the liver and kidneys. Because of this, low concentrations (40 and 80 µg/ml) of the *M. balsamina* extract were chosen and investigated for hepatotoxicity using the C3A liver cells. As shown in **Figure 3.1B**, the *M. balsamina* extract or curcumin had no statistically significant effect on the viability of C3A liver cells.

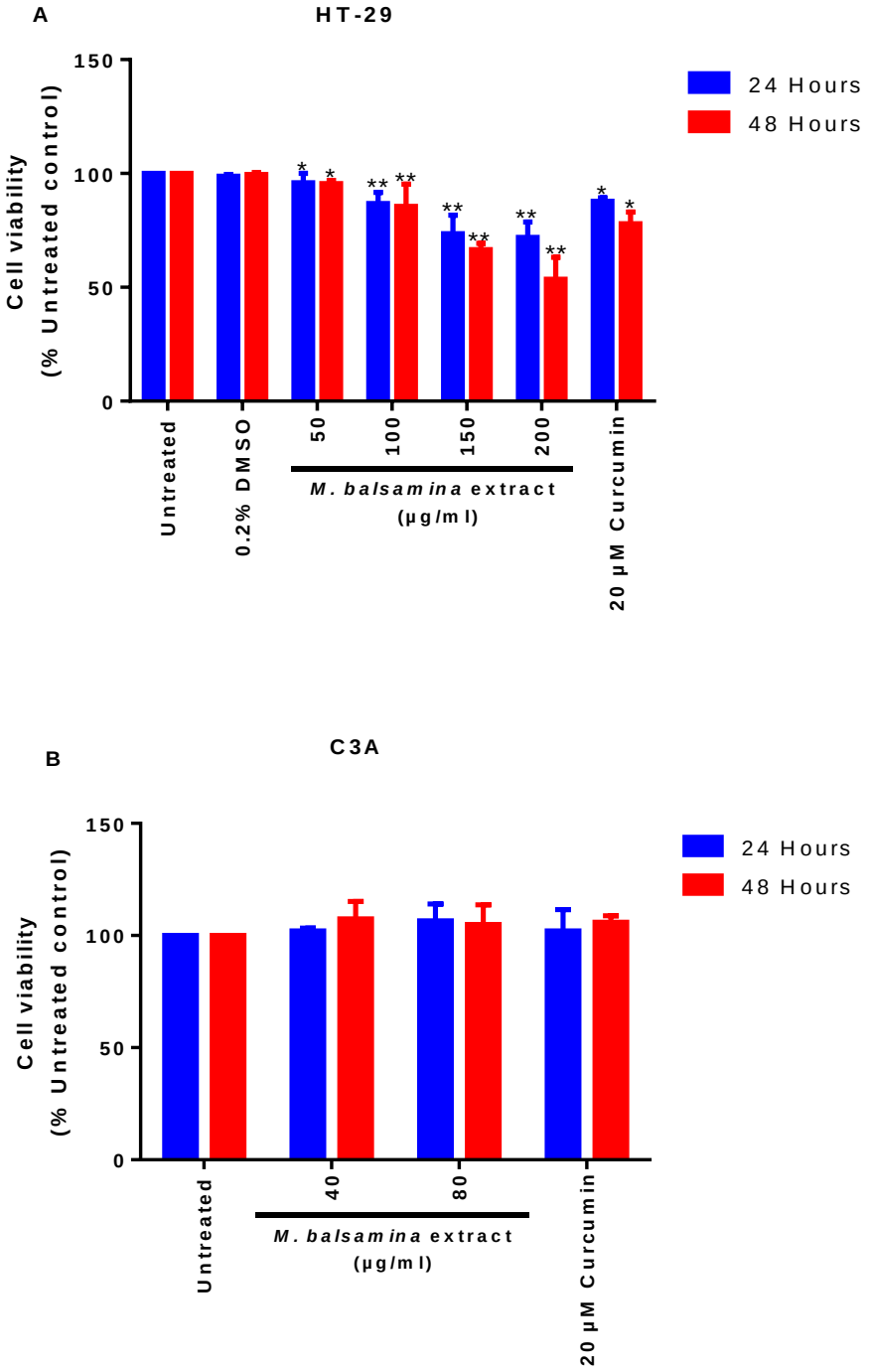

Figure 3.1: The *Momordica balsamina* extract inhibits the viability of HT-29 cells (A) without affecting the viability of C3A liver cells (B). HT-29 cells were treated for 24 and 48 hours with 0−200 µg/ml of the *M. balsamina* extract, 0.2% DMSO or 20 µM of curcumin. Liver C3A cells were treated for 24 and 48 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. Percentage cell viability was measured using the MTT assay and data expressed as percentage cell viability. Each data point represents the mean ± S.D of three independent experiments, performed in duplicate. *p ≤ 0.05, **p ≤ 0.01 indicate significant differences to the vehicle control.

3.2 The effect of the *Momordica balsamina* extract on HT-29 cell and nuclei morphology

Migrastatic drugs impede the metastatic cascade without inducing tumour shrinkage by apoptosis (Gandalovicová *et al.*, 2017). Therefore, the cell and nuclei morphological features associated with apoptosis were investigated using the AO/EB dual staining assay. HT-29 cells treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract showed no signs of pyknosis or karyokinesis, as they retained a normal spherical shaped nucleus with dim green fluorescence **(Figure 3.2)**. However, HT-29 cells treated for 48 hours with 40 µg/ml of the *M. balsamina* extract showed cell shrinkage and nuclei condensation. More apoptotic features such as loss of membrane function and formation of apoptotic bodies were observed in HT-29 cells treated for 48 hours with 80 µg/ml of the *M. balsamina* extract **(Figure 3.2)**. Furthermore, HT-29 cells treated for 24 hours with 20 µM of curcumin showed no features of apoptosis, however, when treated for 48 hours with 20 µM of curcumin, the number of cells with condensed nuclei increased **(Figure 3.2)**. The data showed that both the *M. balsamina* extract and curcumin induce apoptosis at 48 hours of treatment **(Figure 3.2)**.

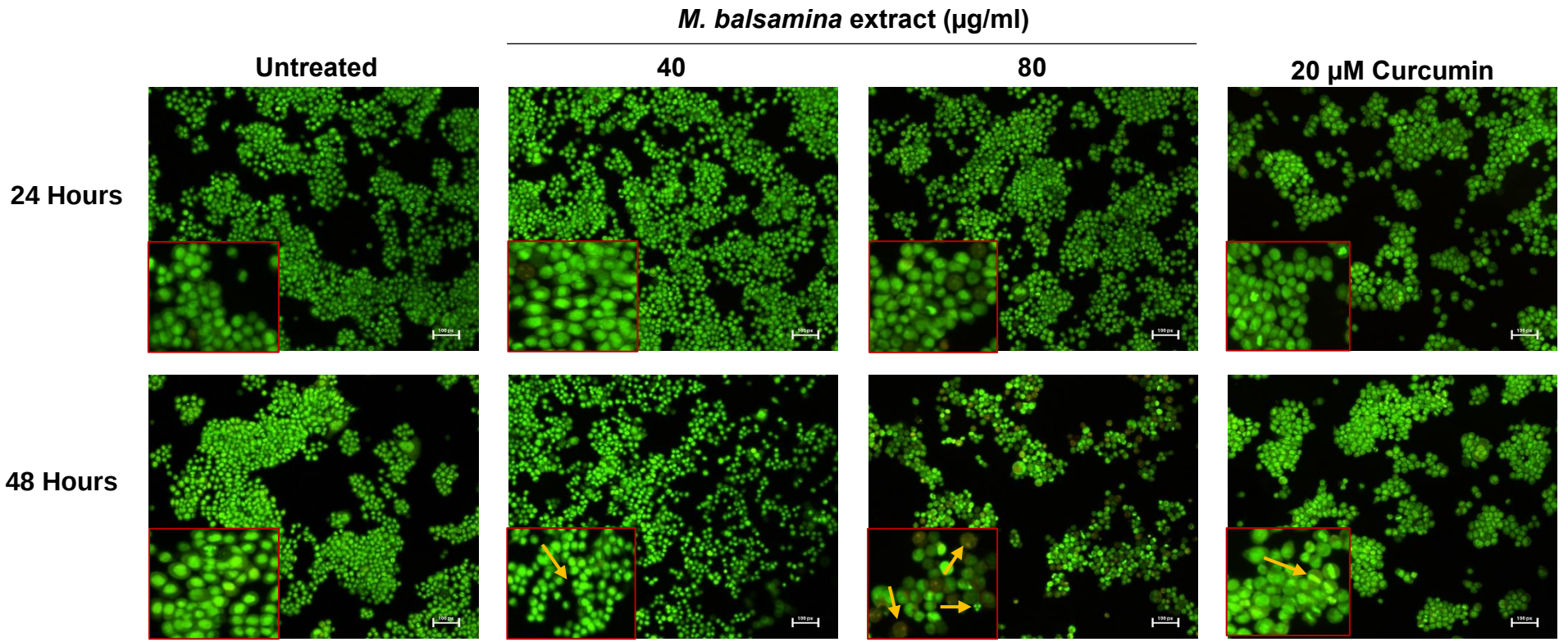

Figure 3.2: Cell and nuclei morphological changes induced by the *Momordica balsamina* extract in HT-29 cells. Cells were treated for 24 and 48 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. Cell and nuclei morphological changes were assessed by the AO/EB dual staining and images acquired under a 10× objective of a fluorescence microscope. The arrows in the magnified views on the left corners indicate apoptotic features. The pictures represent one of three independent experiments, performed in duplicate. n: 1.5×10^5 cells/well, scale: 100 px.

3.3 The effect of *Momordica balsamina* extract on intracellular ROS formation in HT-29 cells

Reactive oxygen species are key drivers of the malignant transformation that enhance the metastatic potential of primary tumours (Kumari *et al.*, 2018). To investigate the ability of the *M. balsamina* extract to scavenge the H_2O_2-induced intracellular ROS, the $DFCH_2$-DA assay was performed. As expected and evident by the number of cells with bright green fluorescence, treatment with 50 µM of H_2O_2 increased intracellular ROS production compared to untreated HT-29 cells **(Figure 3.3A)**. However, treatment with 40 or 80 µg/ml of the *M. balsamina* extract reduced the H_2O_2-induced intracellular ROS levels with an increase in concentration. Similarly, treatment with 20 µM of curcumin reduced the levels of intracellular ROS in HT-29 cells **(Figure 3.3A)**. Quantitation of total cellular fluorescence, however, revealed a non-significant reduction in H_2O_2-induced intracellular ROS formation in HT-29 cells treated with the *M. balsamina* extract or curcumin **(Figure 3.3B)**. Compared to curcumin, the *M. balsamina* extract was shown to have less effect on H_2O_2-induced intracellular ROS formation at 40 or 80 µg/ml **(Figure 3.3B)**.

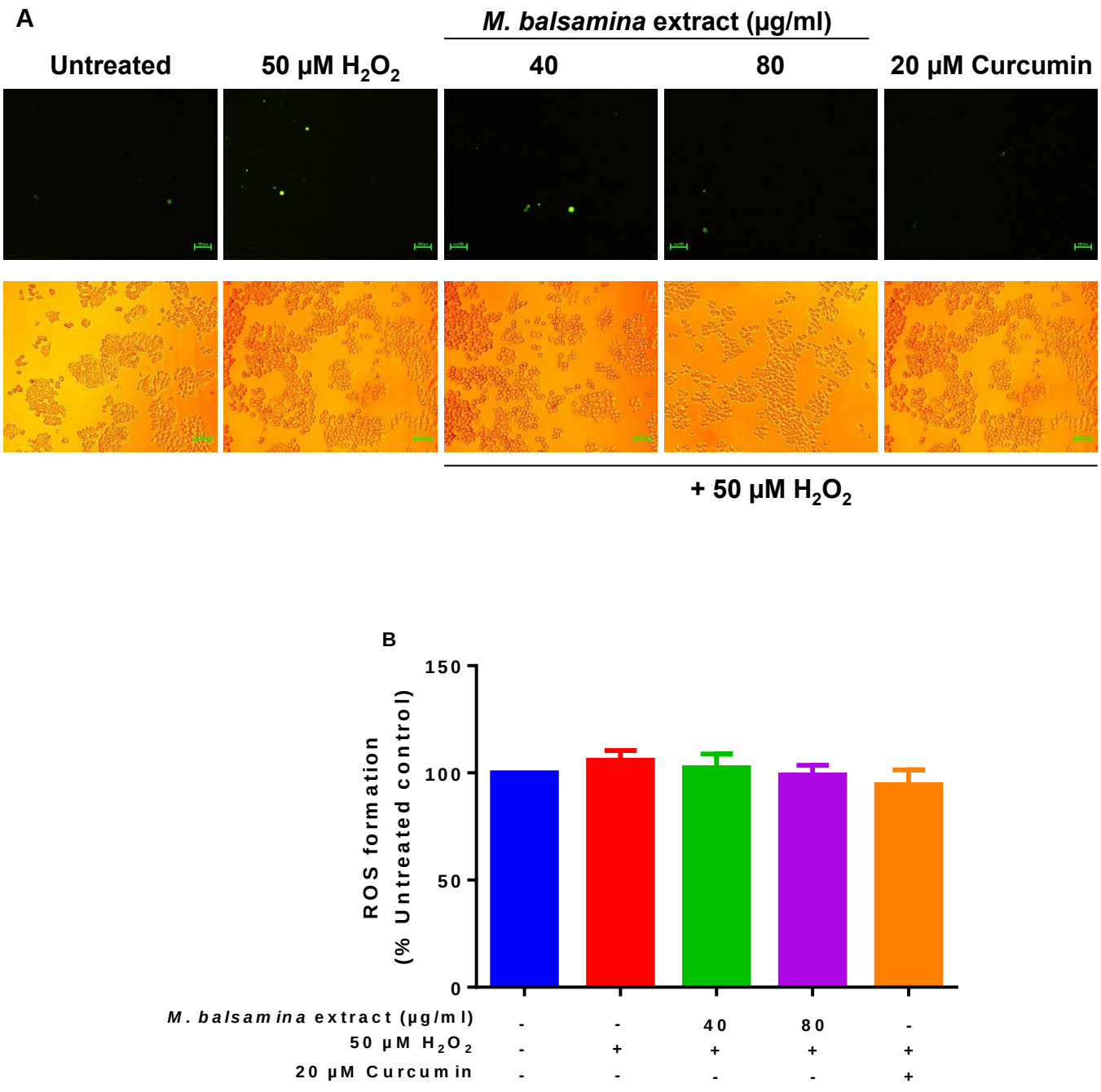

Figure 3.3: The effect of the *Momordica balsamina* extract on ROS formation in HT-29 cells. Cells were treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin in the presence of 50 µM of H_2O_2. Reactive oxygen species formation was assessed using the $DFCH_2$-DA assay. Cells were photographed under a 10× objective of a fluorescence and phase contrast inverted light microscope **(A)**. Total cellular fluorescence was measured at an excitation/emission spectrum of 495/529 nm using a GloMax®-Multi+Detection system microtiter plate reader and data expressed as percentage ROS formation **(B)**. Each data point represents the mean ± SD of three independent experiments, performed in duplicate. n: 1.5×10^4 cells/well, scale: 100 px.

3.4 The effect of *Momordica balsamina* extract on EMT-related morphological changes in HT-29 cells

Epithelial to mesenchymal transition is a vital event in the progression of cancer metastasis (Dawei *et al.*, 2018). To determine whether the *M. balsamina* extract can inhibit EMT of HT-29 cells, light microscopy was used. As shown in **Figure 3.4**, HT-29 cells treated with 40 or 80 µg/ml of the *M. balsamina* extract retained their normal epithelial morphology and cell-cell junctions. Since there was no difference in the appearance and/or morphology between untreated cells and cells treated with 40 or 80 µg/ml of the *M. balsamina* extract, it was inferred that the *M. balsamina* extract inhibits the EMT-related morphological changes in HT-29 cells. Furthermore, HT-29 cells treated with 20 µM of curcumin also retained their cell-cell junctions but showed a change from an epithelial to a fibroblastic morphology **(Figure 3.4)**.

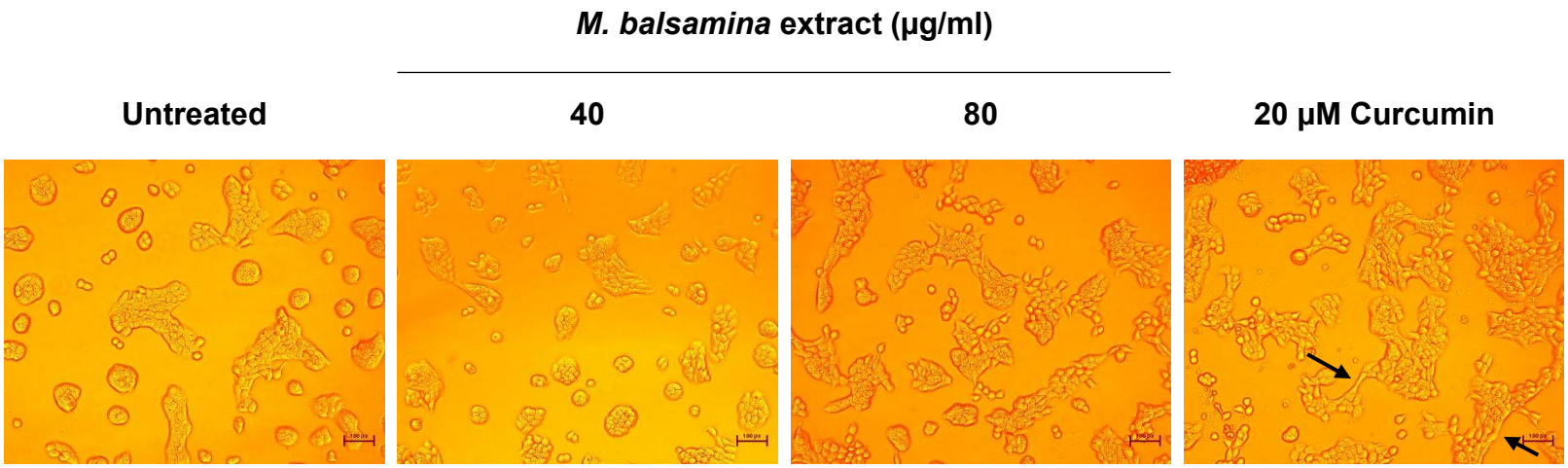

Figure 3.4: The effect of the *Momordica balsamina* extract on EMT-related morphological changes in HT-29 cells. Cells were treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin in serum-free media and images acquired under a 10× objective of a phase-contrast inverted light microscope. The arrows indicate HT-29 cells with a fibroblast morphology. The pictures represent one experiment of the three independent replicates. n: 2×10^5 cells/well, scale: 100 px.

3.5 The effect of the *Momordica balsamina* extract on HT-29 cell invasiveness

Invasion through the ECM is the most critical step in tumour growth and metastasis (Che *et al.*, 2019). The anti-invasive effects of the *M. balsamina* extract were, therefore, assessed using the QCM™ cell invasion assay kit. As evidenced by the crystal violet stained-cells, the number of HT-29 cells that invaded through the ECM to the lower chamber was reduced by treatment with 40 or 80 µg/ml of the *M. balsamina* extract **(Figure 3.5A)**. Quantitative analysis confirmed that the *M. balsamina* extract significantly ($p \leq 0.05$, $p \leq 0.01$) inhibited the invasiveness of HT-29 cells with an increase in concentration **(Figure 3.5B)**. Exposure to 20 µM of curcumin was shown to have a non-significant effect on the invasiveness of HT-29 cells **(Figure 3.5)**. At 80 µg/ml, the *M. balsamina* extract significantly ($p \leq 0.05$) inhibited the invasiveness of HT-29 cells when compared 20 µM of curcumin **(Figure 3.5)**.

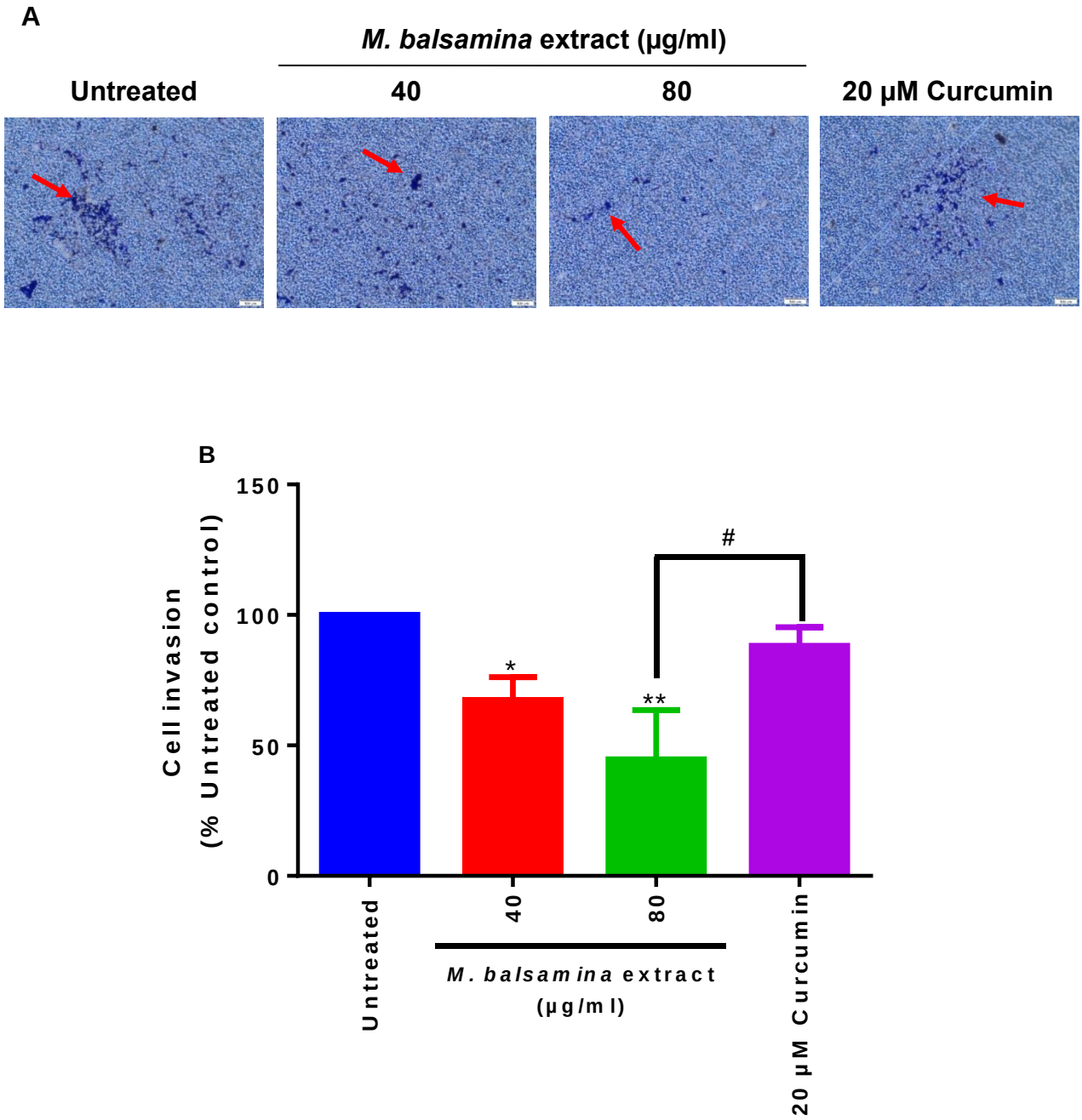

Figure 3.5: The *Momordica balsamina* extract inhibits HT-29 cell invasiveness. Cells were treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. Cell invasiveness was assessed using the QCM™ cell invasion assay kit. The invasive HT-29 cells (shown by the red arrows) were stained and images acquired under a 4× objective of an inverted light microscope **(A)** and quantified using the ImageJ software. Data were expressed as percentage cell invasion **(B)**. Each data point represents the mean ± SD of three independent experiments. #p ≤ 0.05 and *p ≤ 0.05 or **p ≤ 0.01 indicate significant differences to curcumin and the untreated control, respectively. n: 2×10^4 cells/well, scale: 500 µm.

3.6 The effect of the *Momordica balsamina* extract on the expression of MMP-2 and MMP-9 proteins in HT-29 cells

Matrix metalloproteinase-2 and -9 facilitate the remodelling and degradation of the extracellular matrix and basement membrane components during cell invasiveness (Alizadeh *et al.*, 2014). Therefore, based on the inhibitory effect of the *M. balsamina* extract on HT-29 cell invasiveness **(Figure 3.5)**, its effect on the expression of MMP-2 and MMP-9 were next assessed by western blotting. As shown in **Figure 3.6A**, the expression of MMP-2 and MMP-9 proteins was reduced by treatment with 40 or 80 µg/ml of the *M. balsamina* extract. The densitometric analysis confirmed that this reduction was significant ($p \leq 0.05$, $p \leq 0.01$) and concentration-dependent **(Figure 3.6B)**. Exposure to 20 µM of curcumin also showed a significant ($p \leq 0.01$) reduction in the expression of both MMP-2 and MMP-9 proteins. The inhibitory effect of 40 or 80 µg/ml of the *M. balsamina* extract on MMP-2 and MMP-9 protein expression, however, was not significant when compared to 20 µM of curcumin **(Figure 3.6)**.

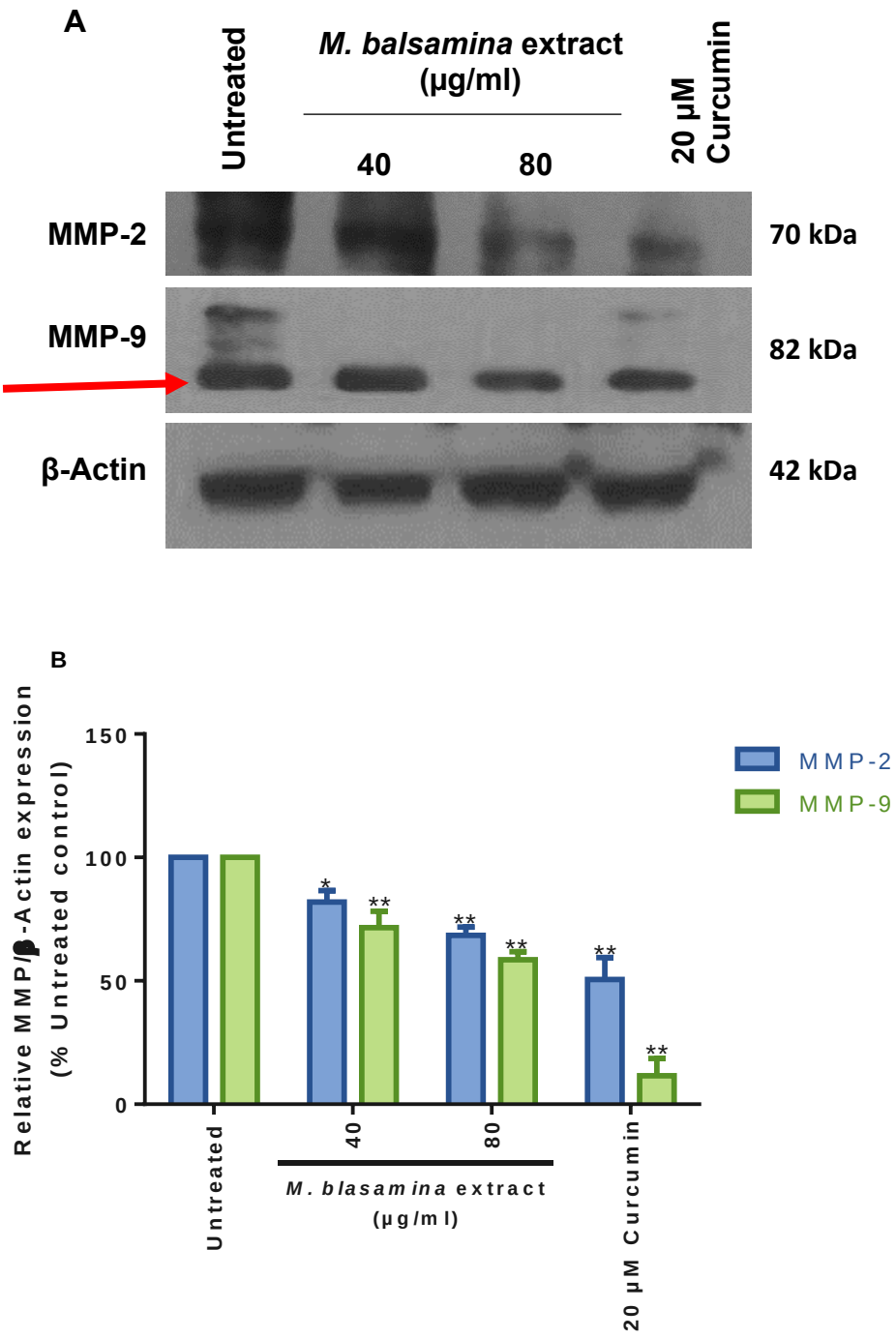

Figure 3.6: The *Momordica balsamina* extract inhibits the expression of MMP-2 and -9 proteins in HT-29 cells. Cells were treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin and the expression of MMP-2 and -9 were assessed by western blotting. Mean band densities were measured using the ImageJ software and quantitative analysis done by normalising to β-Actin. Each data point represents the mean ± SD of three independent experiments. *p ≤ 0.05, **p ≤ 0.01 indicate significant differences to the untreated control.

3.7 The effect of the *Momordica balsamina* extract on the expression of TNF-α and NF-κB proteins in HT-29 cells

The expression of MMP-2 and -9 is transcriptionally regulated by factors, such as NF-κB, which are responsive to inflammatory cytokines such as TNF-α present in the tumour microenvironment (Bigatto *et al.*, 2015; Yin *et al.*, 2009). To determine the involvement of TNF-α and NF-κB in the downregulation of MMP-2 and MMP-9 protein expression, western blotting was performed. As shown in **Figure 3.7A**, treatment with 40 or 80 µg/ml of the *M. balsamina* extract reduced the protein expression levels of TNF-α and NF-κB. The densitometric analysis confirmed a significant ($p \leq 0.01$) concentration-dependent reduction of TNF-α and NF-κB protein expression levels in HT-29 cells treated with the *M. balsamina* extract **(Figure 3.7B)**. Exposure to 20 µM of curcumin significantly ($p \leq 0.01$) downregulated the expression of TNF-α and NF-κB proteins **(Figure 3.7)**. Furthermore, the inhibitory effect of 40 or 80 µg/ml of the *M. balsamina* extract on the protein expression levels of TNF-α and NF-κB was not significant when compared to 20 µM of curcumin **(Figure 3.7)**.

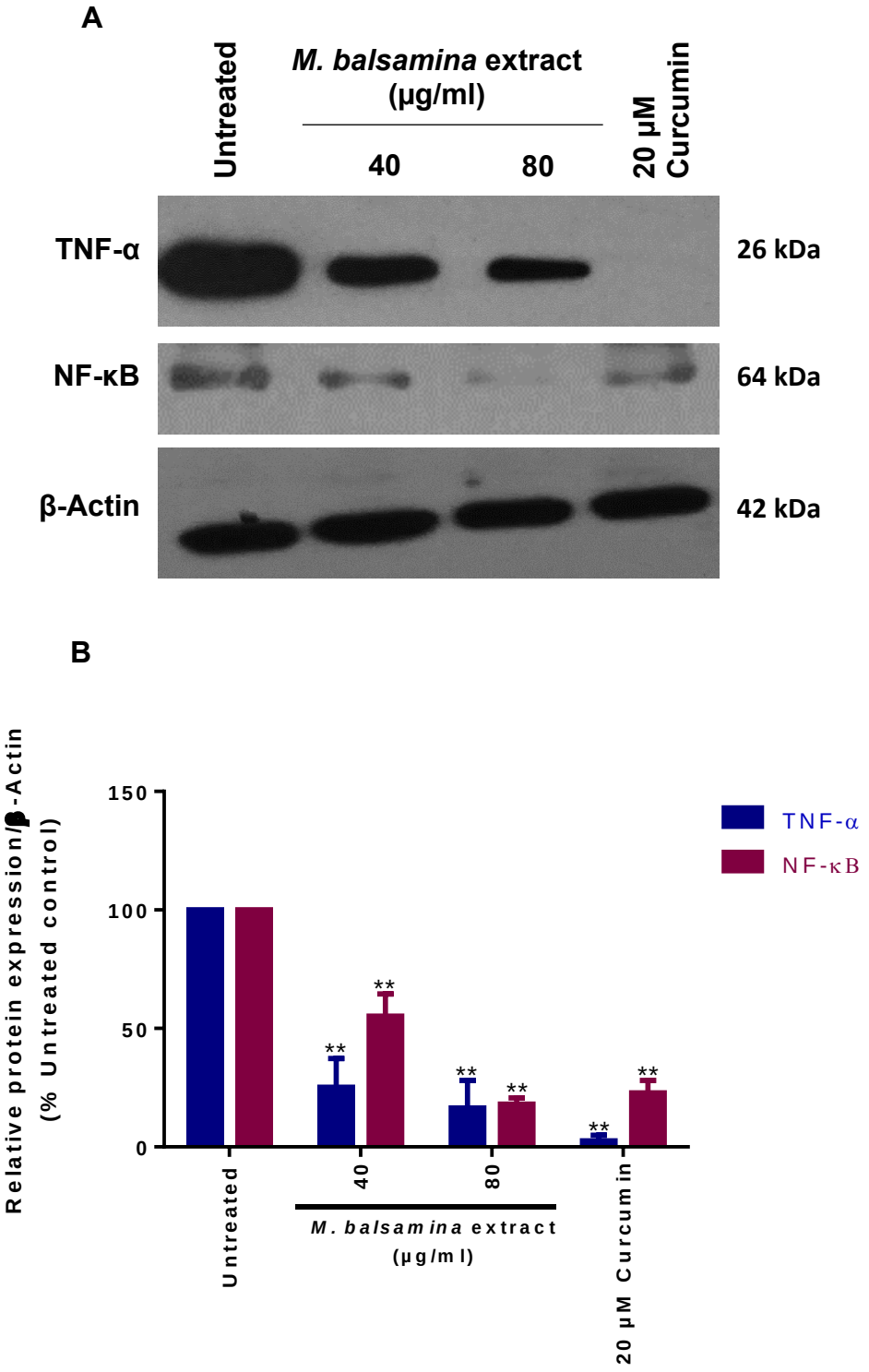

Figure 3.7: The *Momordica balsamina* extract inhibits the protein expression levels of TNF-α and NF-κB in HT-29 cells. Cells were treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. The expression levels of TNF-α and NF-κB were assessed by western blotting. Mean band densities were measured using the ImageJ software and quantitative analysis done by normalising to β-Actin. Each data point represents the mean ± SD of three independent experiments. **p ≤ 0.01 indicate significant differences to the untreated control.

3.8 The effect of the *Momordica balsamina* extract on TIMP-3 protein expression in HT-29 cells

Matrix metalloproteinase-9 is strongly involved in colon cancer migration and invasiveness (Fan *et al.*, 2019). Therefore, to determine the effect of the *M. balsamina* extract on the expression levels of TIMP-3, an MMP-9 proteolytic activity inhibitor, western blotting was performed. As shown in **Figure 3.8A**, exposure to 40 or 80 µg/ml of the *M. balsamina* extract increased the protein expression levels of TIMP-3. The densitometric analysis showed a concentration-dependent upregulation of TIMP-3. A significant ($p \leq 0.01$) increase in the protein expression levels of TIMP-3 was observed in HT-29 cells treated with 80 µg/ml of the *M. balsamina* extract **(Figure 3.8B)**. Exposure to 20 µM of curcumin showed a non-significant increase in the expression of TIMP-3 protein **(Figure 3.8)**. The data further revealed that at 80 µg/ml, the *M. balsamina* extract upregulates the expression of TIMP-3 better than 20 µM of curcumin **(Figure 3.8)**.

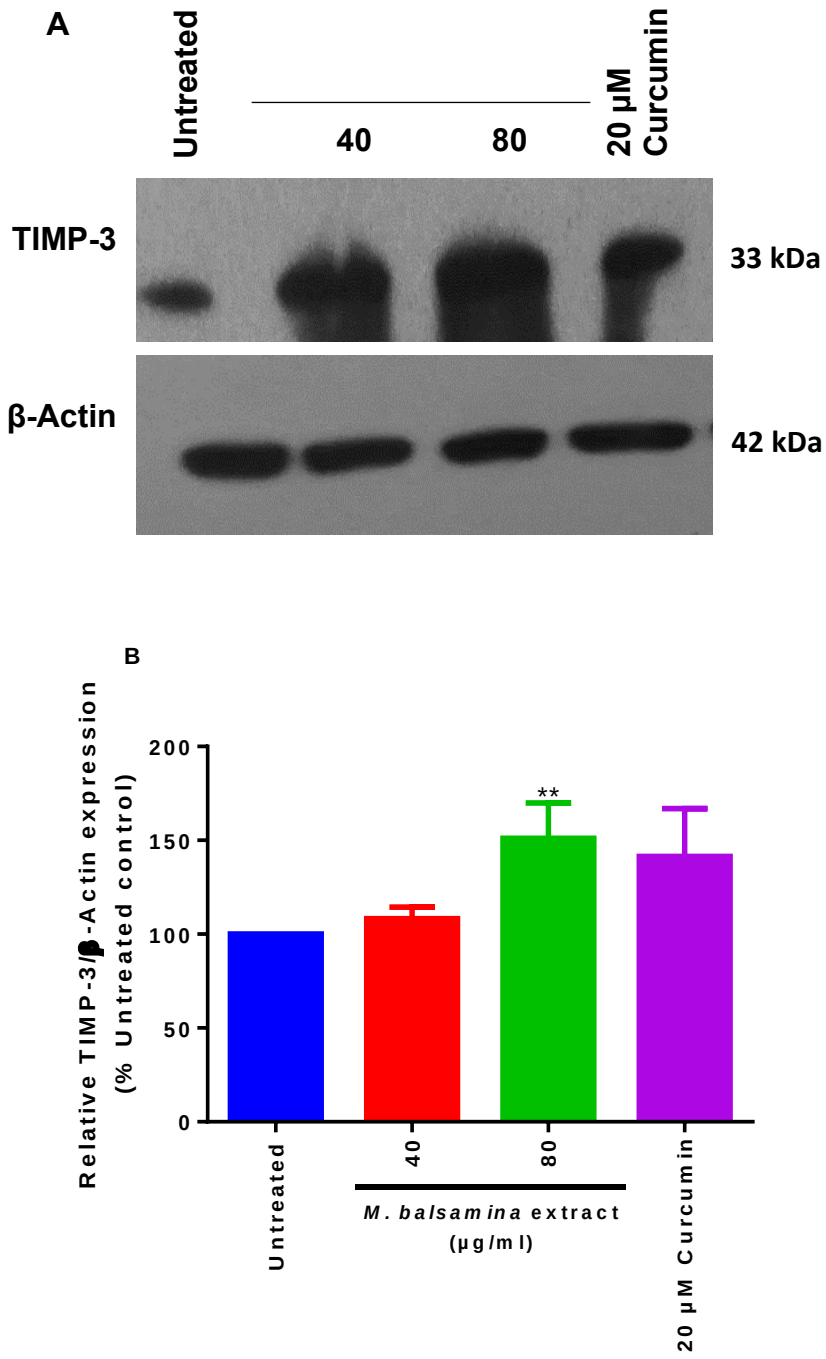

Figure 3.8: The *Momordica balsamina* extract increases the expression of TIMP-3 protein in HT-29 cells. Cells were treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin and the expression levels of TIMP-3 were assessed by western blotting. Mean band densities were measured using the ImageJ software and quantitative analysis done by normalising to β-Actin. Each data point represents the mean ± SD of three independent experiments. **p ≤ 0.01 indicates a significant difference to the untreated control.

3.9 The effect of the *Momordica balsamina* extract on HT-29 cell migration

Cell migration is a pivotal step in the metastatic cascade (Yoon *et al.*, 2019). As such, the anti-migratory effects of the *M. balsamina* extract were determined using the wound healing assay. As shown in **Figures 3.9A & B**, there was no significant changes in wound size and percentage wound area at 6 hours of treatment with 40 or 80 µg/ml of the *M. balsamina* extract. However, at 24 hours, the wound of untreated HT-29 cells was completely closed whereas the wound of HT-29 cells treated with 40 or 80 µg/ml of the *M. balsamina* extract was still open. Quantitative analysis showed this to be significant ($p \leq 0.01$) and in an *M. balsamina* extract concentration-dependent manner as compared to untreated cells **(Figure 3.9C)**. Similarly, exposure to 20 µM curcumin significantly inhibited ($p \leq 0.01$) the migration of HT-29 cells. Furthermore, when compared to 20 µM of curcumin, the *M. balsamina* extract showed a non-significant anti-migratory effect at 40 or 80 µg/ml **(Figure 3.9)**.

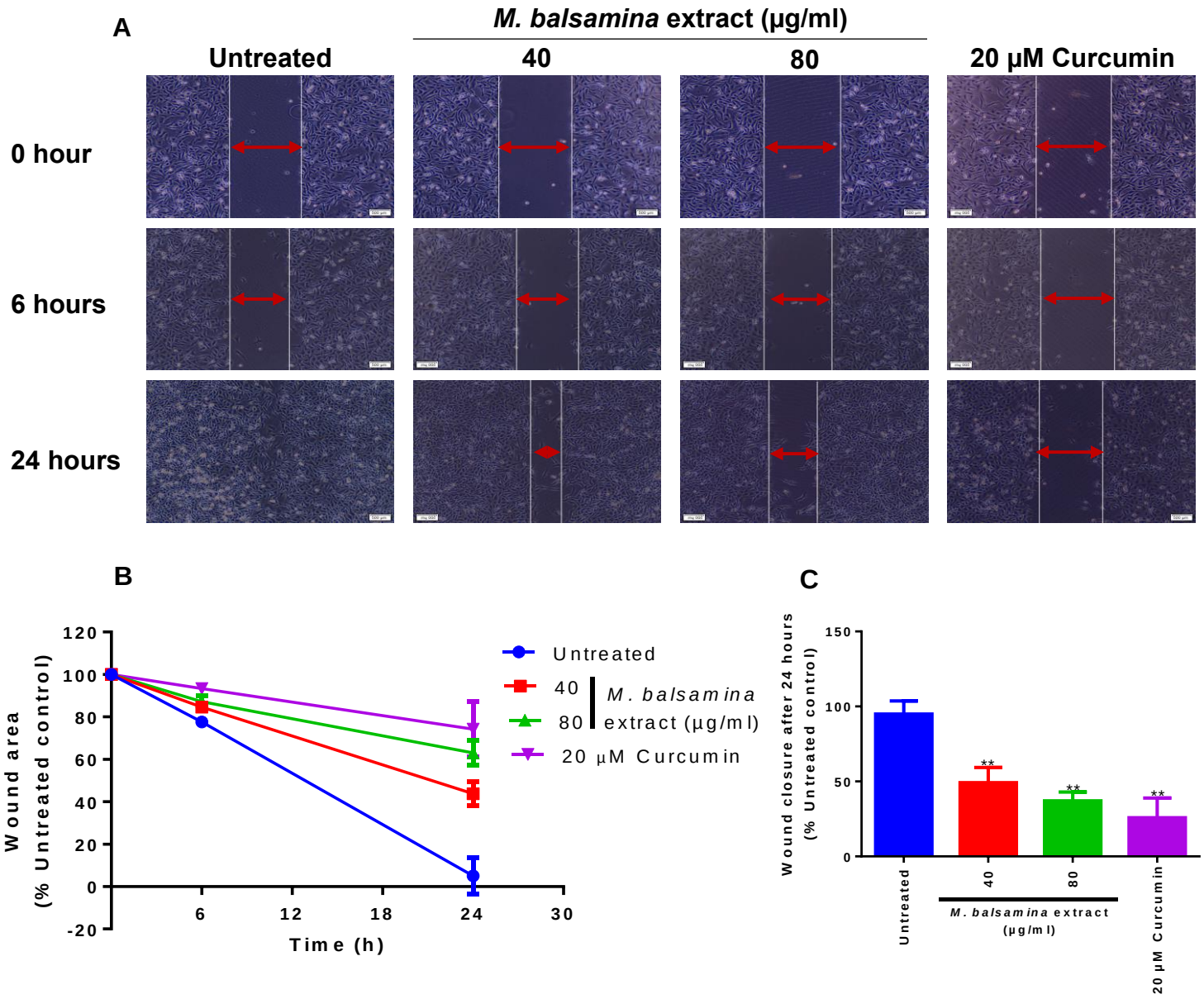

Figure 3.9: The *Momordica balsamina* extract inhibits HT-29 cell migration. A confluent HT-29 cell monolayer was wounded and treated for 6 and 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. After each incubation period, cells were photographed under a 4× objective of an inverted light microscope **(A)**. The wound sizes were measured using the LCmicro software and data expressed as percentage wound area **(B)** and wound closure **(C)**. Each data point represents the mean ± S.D of three independent experiments, performed in duplicate. **p ≤ 0.01 indicate significant differences to the untreated control. Scale: 500 µm.

3.10 The effect of the *Momordica balsamina* extract on HT-29 cell adhesion

Cell-ECM adhesion is a prerequisite for tumour formation at secondary sites (Holle *et al.*, 2016). The anti-adhesive properties of the *M. balsamina* extract were, therefore, next investigated using the cell adhesion assay. Treatment with 40 or 80 µg/ml of the *M. balsamina* extract inhibited the adhesion of HT-29 cells to matrigel-coated plates **(Figure 3.10A)**. Quantitative analysis revealed that the percentage cell adhesion of HT-29 cells treated with 40 or 80 µg/ml of the *M. balsamina* extract was significantly ($p \leq 0.05$, $p \leq 0.01$) reduced with an increase in concentration and treatment period **(Figure 3.10B)**. Treatment with 20 µM of curcumin was also shown to significantly ($p \leq 0.01$) inhibit the adhesion of HT-29 cells to Matrigel-coated plates with an increase in the treatment period. At both 6 and 24 hours, 40 or 80 µg/ml of the *M. balsamina* extract showed a non-significant anti-adhesive effect when compared to 20 µM of curcumin **(Figure 3.10)**.

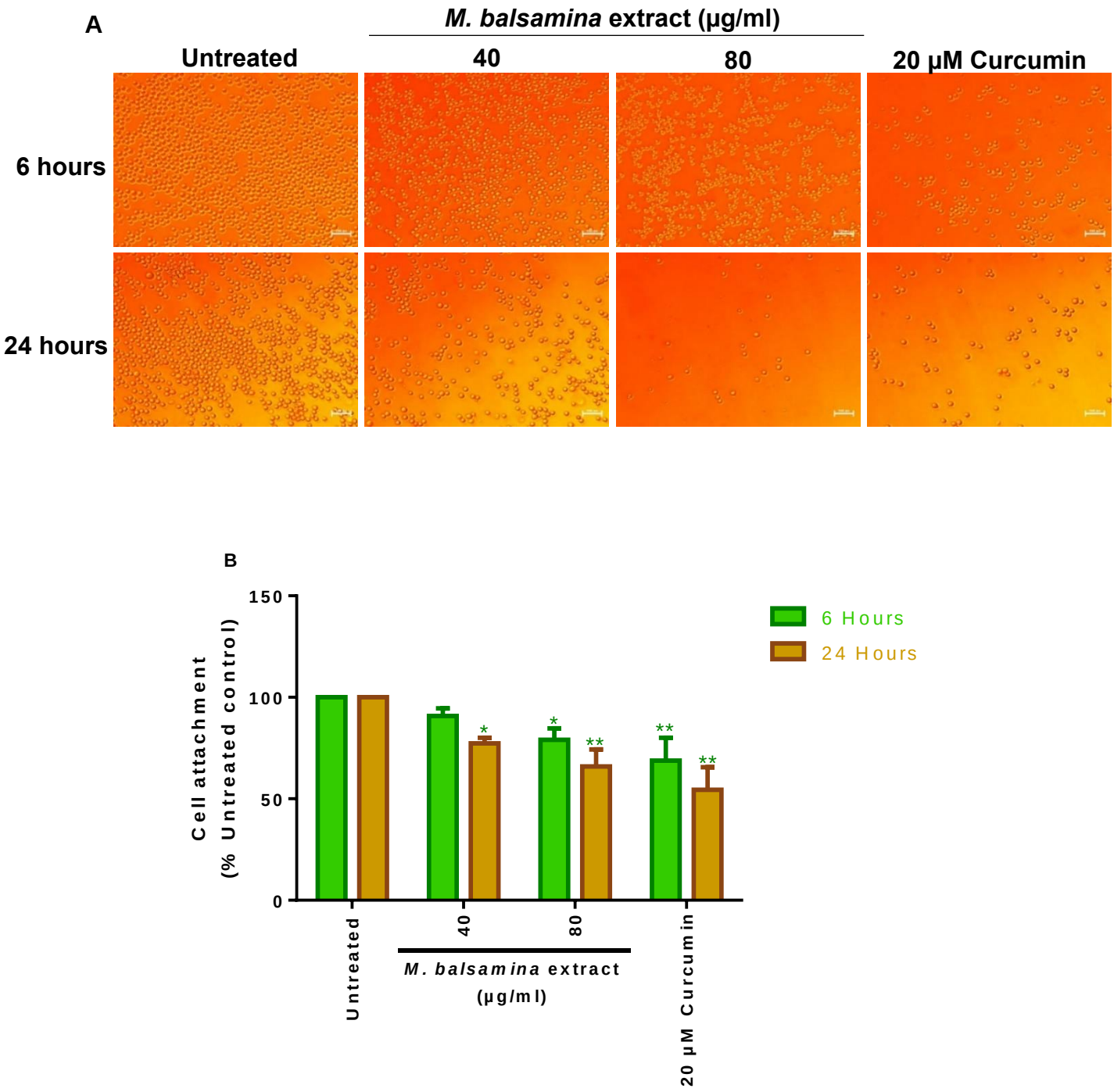

Figure 3.10: The *Momordica balsamina* extract inhibits the attachment of HT-29 cells. Cells were treated for 6 and 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. Cell attachment was assessed using the cell adhesion assay. Attached cells were photographed under a 10× objective of a phase-contrast inverted light microscope **(A)**, quantified using the MTT assay and data expressed as percentage cell attachment **(B)**. Each data point represents the mean ± S.D of three independent experiments, performed in duplicate. *p ≤ 0.05, **p ≤ 0.01 indicate significant differences to the untreated control. n: 2×10^5 cells/well, scale: 100 px.

3.11 The effect of the *Momordica balsamina* extract on the attachment of HT-29 cells to ECM proteins

The homing of cancer cells at secondary sites depends on the interaction between integrins and ECM proteins (Seguin *et al.*, 2015). Therefore, based on the inhibitory effect of the *M. balsamina* extract on HT-29 cell adhesion **(Figure 3.10)**, its effect on the adhesion of HT-29 cells to ECM proteins, collagen I, II and IV, fibronectin, laminin, tenascin C and vitronectin, was next assessed using the CHEMICON® ECM cell adhesion array kit. As shown in **Figure 3.11**, treatment with 40 or 80 µg/ml of the *M. balsamina* extract significantly ($p \leq 0.05$, $p \leq 0.01$) inhibited the adhesion of HT-29 cells to collagen IV, fibronectin, laminin, tenascin C and vitronectin with an increase in concentration. The adhesion of HT-29 cells to collagen I and II were significantly inhibited at 80 µg/ml of the *M. balsamina* extract. On the contrary, 40 µg/ml of the extract was seen to significantly promote the attachment of HT-29 cells to collagen I. The adhesion of HT-29 cells to collagen II was not significantly promoted by 40 µg/ml of the *M. balsamina* extract. Moreover, exposure to 20 µM of curcumin was seen to significantly inhibit the adhesion of HT-29 cells to collagen I and II as well as fibronectin, laminin and tenascin C. However, it's inhibitory effect to collagen IV was not significant. Furthermore, exposure to curcumin promoted the adhesion of HT-29 to vitronectin **(Figure 3.11)**.

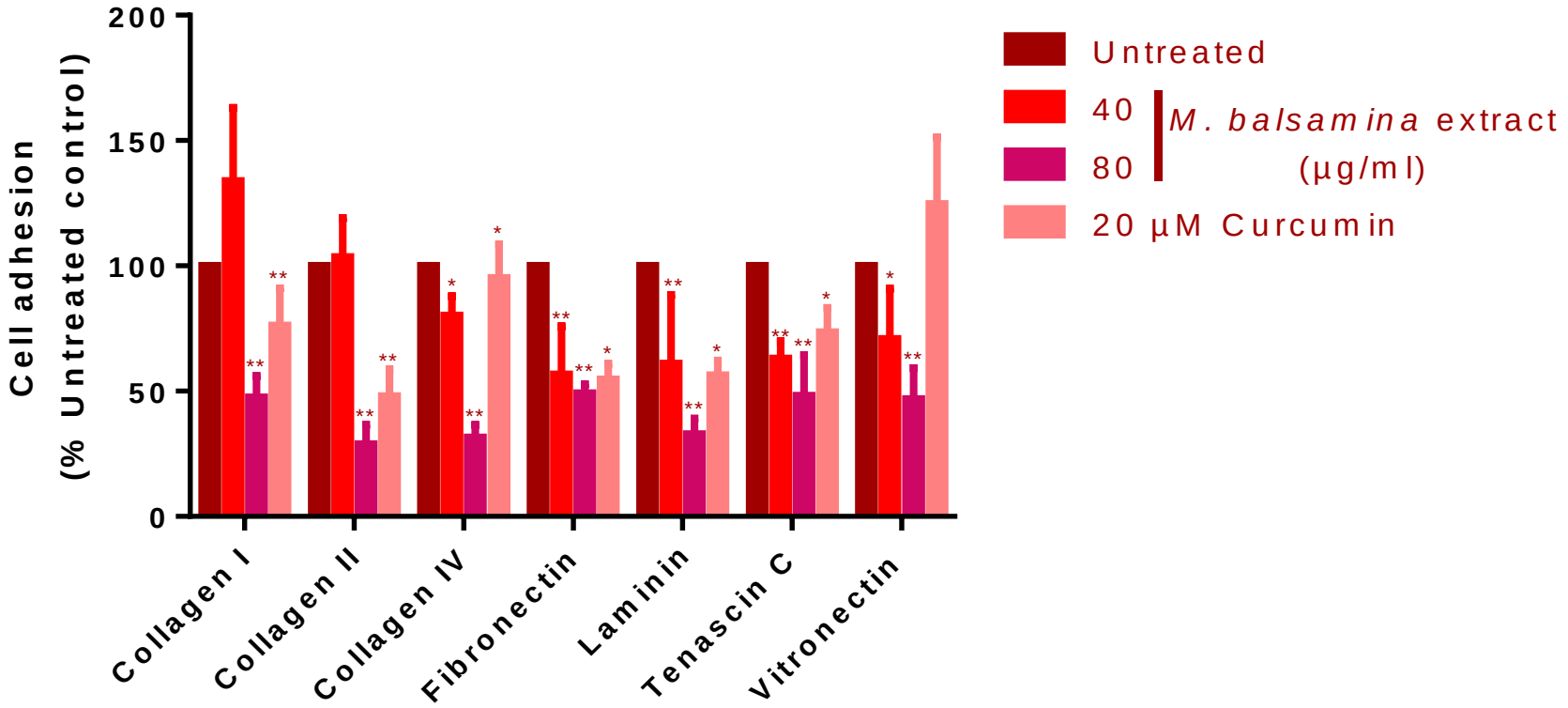

Figure 3.11: The effect of the *M. balsamina* extract on the attachment of HT-29 cells to ECM proteins. Cells were treated for 24 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. Adhesion to the indicated ECM proteins was assessed using the CHEMICON® ECM cell adhesion array kit. Data were expressed as percentage cell adhesion. Each data point represents the mean ± SD of three independent experiments, performed in duplicate. *p ≤ 0.05, **p ≤ 0.01 indicate significant differences to the untreated control.

CHAPTER FOUR

4 Discussion

Metastatic cancer remains incurable and accounts for 90% of all cancer-related deaths (He *et al.*, 2019; Tungsukruthai *et al.*, 2018). Over the past decades, metastatic cancer has been poorly treated using drugs that act by inhibiting cell proliferation and/or inducing tumour shrinkage by apoptosis (Gandalovicová *et al.*, 2017). However, tumour shrinkage is rarely sustained and is not an anti-metastatic effect (Sleeman and Steeg, 2010). These led to the development of migrastatics, drugs that interfere with cell invasiveness and all modes of metastasis (Gandalovicová *et al.*, 2017). A review by Gandalovicová *et al.* (2017) has listed several migrastatic drug candidates. However, their therapeutic use is halted due to high toxicity profiles, drug resistance and low survival rates (Hsan *et al.*, 2010). Medicinal plants have since been recognised as outstanding sources of novel compounds with greater pharmaceutical value and less or no side-effects (Manoharan *et al.*, 2014). Therefore, investigating plant-derived compounds and/or crude extracts for anti-metastatic activities may provide templates for the development of migrastatic drugs as well as identifying novel therapeutic targets. In the present study, the potential anti-metastatic effects of *Momordica balsamina* crude acetone leaf extract were investigated in human HT-29 colon cancer cells.

An ideal anticancer agent is expected to interfere with cancer cell proliferation and/or viability (Senggunprai *et al.*, 2016). Furthermore, it should selectively affect cancer cell proliferation without being toxic to highly proliferative normal cells (Fraczkowska *et al*., 2018). The cytotoxic effect of the *M. balsamina* extract was assessed using the MTT assay. The findings revealed that the *M. balsamina* extract selectively decrease the viability of HT-29 colon cancer cells without affecting C3A liver cells **(Figure 3.1)**. Apoptosis is a highly controlled and naturally programmed mode of cell death characterised by morphological and biochemical features (Grilo and Mantalaris, 2019). Nuclei fragmentation and condensation, the formation of apoptotic bodies and loss of membrane permeability are some of the features used to characterise apoptosis (Kadam and Abhang, 2016). Cellular and nuclei morphological changes associated with apoptosis were observed in several HT-29 cells treated for 48 hours with 40 or 80 µg/ml of the *M. balsamina* extract **(Figure 3.2)**. Therefore, to rule out the effect of

apoptosis on the metastasis processes that were investigated, treatment with 40 or 80 µg/ml of the *M. balsamina* was restricted to 24 hours.

Metastasis is a dissemination process during which tumour cells migrate to distal sites for secondary tumour formation (Massague *et al.*, 2017). It involves detachment from the primary tumour, cell invasiveness, intravasation, survival and migration within the circulatory systems, extravasation and colonisation at the distal organ (Guan, 2015). Tumour cell invasiveness is the most critical, rate-limiting and widely accepted hallmark of cancer metastasis (Hanahan and Weinberg, 2011). It involves the remodelling and proteolytic degradation of the ECM (Yang *et al.*, 2018). Matrix metalloproteinases, particularly, MMP-2 and -9 are strongly associated with the degradation of ECM and BM proteins, resulting in a weakened barrier and promotion of cancer invasiveness (Brown and Murray, 2015; Huang and Xin, 2018). Furthermore, MMP-2 and -9 are overexpressed in various cancer tissues (Wang *et al.*, 2019). Therefore, inhibiting the expression and/or activity of MMP-2 and -9 could serve as an essential mechanism for inhibiting cancer metastasis (Wang *et al.*, 2015). Colon HT-29 cancer cells treated with the *M. balsamina* extract showed a reduction in their invasiveness **(Figure 3.5)** which could be associated with the downregulation of MMP-2 and -9 protein expression **(Figure 3.6)**.

Matrix metalloproteinases are accurately regulated at transcriptional and/or proteolytic levels (Chaudhary *et al.*, 2013). At proteolytic level, MMP activity is tightly and specifically regulated by endogenous inhibitors known as TIMPs (Lin *et al.*, 2019). Tissue inhibitor of matrix metalloproteinases is relatively small and cysteine-rich proteins that inhibit the activity of MMPs by binding to their catalytic domains (Furtado *et al.*, 2015). Tissue inhibitor of matrix metalloproteinase-3, which preferentially inhibits the activity of MMP-9 (Hrabia *et al.*, 2019) which is strongly expressed in colon cancer tissues (Fan *et al.*, 2019) was upregulated by treatment with the *M. balsamina* extract **(Figure 3.8)**. This suggests that the observed decrease in the invasiveness of HT-29 cells treated with the *M. balsamina* extract can be attributed to the inhibition of MMP-9 activity by TIMP-3. In addition to proteolytic activation, MMPs are expressed in response to external stimuli including inflammatory cytokines such as TNF-α. Tumour necrosis factor-alpha binds to the TNF receptor 1 which then activates the translocation of NF-κB from the cytoplasm into the nucleus (Balkwill, 2009; Karin,

2006; Liu *et al.*, 2009). In the nucleus, NF-κB binds to the AP-1 sites and facilitate the expression of MMP-2 and MMP-9 (Li *et al.*, 2018). Interestingly, the *M. balsamina* extract downregulated the expression of both TNF-α and NF-κB proteins **(Figure 3.7)**, suggesting that the *M. balsamina* extract inhibits MMP-2 and MMP-9 protein expression at the transcriptional level in addition to inhibiting MMP-9 activity through TIMP-3.

In addition to its pro-invasive role, TNF-α is well-known for its ability to induce EMT (Bhat *et al.*, 2016). Epithelial to mesenchymal transition is a developmental process characterised by loss of an epithelial and a subsequent gain of a mesenchymal phenotype (Pastushenko and Blanpain, 2019). In addition to morphological changes, EMT is characterised by the loss of epithelial cell junction proteins such as E-cadherin and a concurrent gain of mesenchymal markers such as vimentin and N-cadherin (Giannelli *et al*., 2016). E-cadherin is an adherent protein responsible for the intracellular junctions between epithelial cells (Haraguchi *et al.*, 2019). At first, E-cadherin was known as a tumour suppressor, however, recent experimental data revealed its complex roles in cancer progression (Shamir and Ewald, 2015). For example, Choi *et al.* (2016) and Shamir *et al.* (2014) reported its involvement in epithelial cell dissemination and collective cell migration. In the present study, the suppressed migratory ability of HT-29 cells **(Figure 3.9)** could be attributed to the ability of the *M. balsamina* extract to inhibit EMT **(Figure 3.4)**.

The ECM is a major component of the tumour microenvironment which serves as a critical source of growth, survival, motility and angiogenic factors (Barkan, 2010). Furthermore, metastatic colonisation at the secondary site depends on the interaction between tumour cells and the ECM (Borsig *et al.*, 2019). The latter is comprised of different classes of fibrous proteins including fibronectin, laminin, collagens, vitronectin and tenascin, which are responsible for the homing of tumour cells (Alizadeh *et al.*, 2014; Seguin *et al.*, 2015). Furthermore, ECM proteins determine the fate of metastatic cells (Seguin *et al.*, 2015). For instance, laminin and tenascin C are found in the lungs and drive cells which express their respective and specific integrins to the lungs. Collagens are abundant in the bones and play a key role in bone metastasis (Borsig and Läubli, 2019). The findings of this study showed that the anti-adhesive effect of the *M. balsamina* extract is varied amongst ECM proteins **(Figure 3.11)**. Furthermore,

a significant inhibition in HT-29 cells adhesion to laminin and tenascin C as well as collagen IV suggests that the *M. balsamina* extract contains compounds with a potential to inhibit colon to lung and/or bone cancer metastasis.

Reactive oxygen species are short-lived molecules resulting from oxygen-consuming metabolic reactions (Moloney and Cotter, 2018). Abundant levels of ROS cause oxidative stress which leads to cell death while moderate levels of ROS activate signalling pathways contributing to cancer metastasis (Huang and Xin, 2018). Signalling pathways that lead to the activation of NF-κB are influenced by ROS produced in response to excessive TNF-α in the tumour microenvironment (Blaser *et al.*, 2016). Furthermore, ROS triggers the gelatinolytic activity of MMPs by oxidation of the cysteine residue in the pro-peptide domain (Rizzi *et al.*, 2019). Studies also revealed that ROS signalling induces EMT (Kim and Cho, 2014) which leads to increased cellular migration and reduced cell-cell junctions (Lu *et al.*, 2019). Phytochemical screening by Mohale (2019) showed that the *M. balsamina* extract contains various compounds including tannins, flavonoids and terpenoids which are well known for their ability to scavenge ROS. Therefore, the inhibition of EMT, cell invasiveness, migration and adhesion by the *M. balsamina* extract could be attributed to its ability to attenuate intracellular ROS **(Figure 3.4)**.

In conclusion, the *M. balsamina* extract inhibited the metastatic cascade in human HT-29 colon cancer cells by suppressing the ROS-mediated TNF-α/NF-κB/MMP-2/-9 signalling pathway *in vitro*. The findings suggest that *M. balsamina* L. may be a source of compounds with the potential to be used for the treatment of metastatic colon cancer.

RECOMMENDATIONS

Further studies may be conducted to verify and confirm the observed antimetastatic effects *in vivo.* Furthermore, studies may be conducted to fractionate and isolate the active compounds from the crude extract used in this study and investigate their antimetastatic effects *in vivo* and *in vitro*.

CHAPTER FIVE

5 References

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APPENDIX: SUPPLEMENTARY DATA

Confirmation of the pro-apoptotic effects of *Momordica balsamina* extract

Apoptotic and necrotic features can overlap depending on caspase and intracellular ATP availability, therefore, it is imperative to use more than one assay to confirm apoptosis as the mode of cell death (Elmore, 2007). In the present study, the pro-apoptotic effect of the *M. balsamina* extract was confirmed by the DAPI nuclei staining assay. As shown in **Figure S1**, HT-29 cells treated for 48 hours with 80 µg/ml of the *M. balsamina* extract resulted with an increase in the number of cells with a bright fluorescence, which is an indication of nuclei condensation. Furthermore, 48 hours exposure to 20 µM curcumin increased the number of cells with condensed nuclei **(Figure S1)**. The AO/EB assay showed apoptotic features at 48 hours treatment **(Figure 3.2)**, therefore, it can be confidently concluded that the onset of apoptosis is at 48 hours exposure to the *M. balsamina* extract.

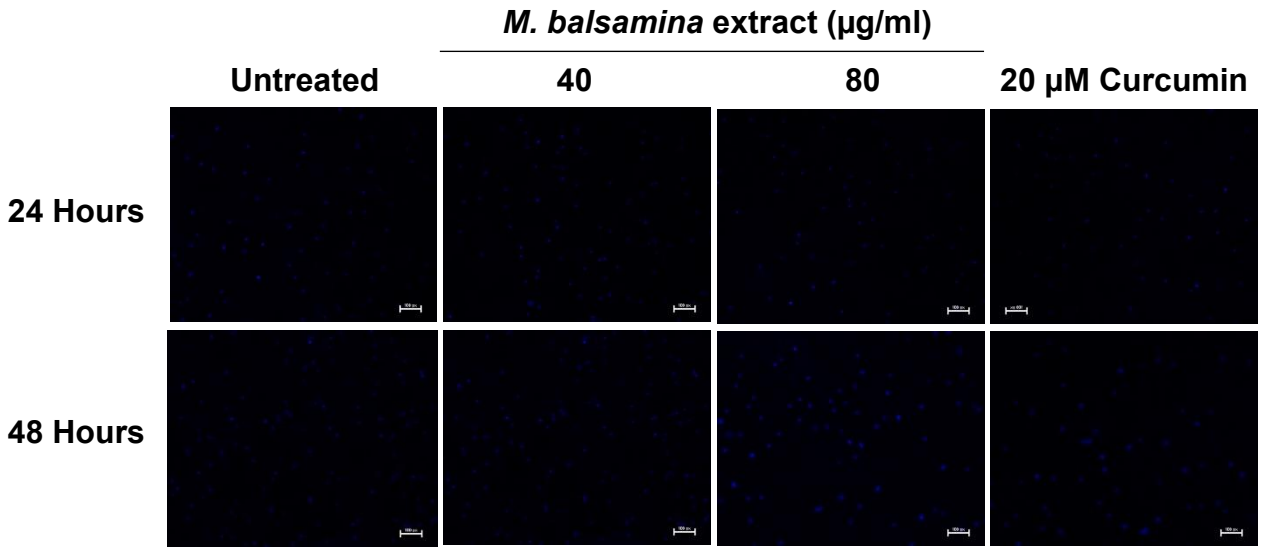

Figure S1: The effect of the *Momordica balsamina* extract on HT-29 nuclei morphology. Cells were treated for 24 and 48 hours with 40 or 80 µg/ml of the *M. balsamina* extract or 20 µM of curcumin. Nuclei morphological changes were assessed by DAPI staining and images acquired under a 10× objective of a fluorescence microscope. The pictures represent one of the three independent experiments, performed in duplicate. n: 2×10^4 cells/well, scale: 100 px.