

***IN VITRO*EVALUATION OF ANTICANCER EFFECT OF *MOMORDICA BALSAMINA*
LINN. LEAF EXTRACT IN HUMAN BREAST MCF-7 CANCER CELLS**

by

Itumeleng Tania Boshielo

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Supervisor: Dr. VG Mbazima

Co-Supervisor: Dr S Riedel van Heerden

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DECLARATION

I, **Boshielo Itumeleng Tania**, hereby declare that the work presented in this dissertation is a true reflection of my findings and all information extracted from the work of others has been cited and fully referenced.

Surname, Initials

Date

DEDICATION

This work is dedicated to the most precious gift that God has given to me, my handsome son **Onthatile Boshielo** and my caring grandmother **Asnath Moloto** for always being the pillar of my strength.

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Psalm 136:1

"Give thanks to the Lord, for he is good! His faithful love endures forever."

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LIST OF ABBREVIATIONS

❖	cIAP	Cellular inhibitor of apoptosis
❖	CXCL	Chemokine C-X-C motif ligand
❖	DMSO	Dimethylsulfoxide
❖	DMEM	Dulbecco's Modified Eagle's Medium
❖	ECM	Extracellular matrix
❖	EGF	Epidermal growth factor
❖	FADD	Fas-associated protein with death domain
❖	Fas-L	Fas-ligand
❖	FGF	Fibroblast growth factor
❖	HGF	Hepatocyte growth factor
❖	IGFBP	Insulin-like growth factors binding protein
❖	IL	Interleukin
❖	MMPs	Matrix metalloproteinases
❖	PDGF	Platelet-derived growth factor
❖	PI	Propidium Iodide
❖	Serpin	Serine proteinase inhibitors
❖	TIMPs	Tissue inhibitors of metalloproteases
❖	TNF	Tumour necrosis factor
❖	TRAIL	TNF-related apoptosis-inducing ligand
❖	TSP	Thrombospondin
❖	uPA	urokinase Plasminogen activator
❖	VEGF	Vascular endothelial growth factor
❖	XIAP	X-linked inhibitor of apoptosis

ABSTRACT

Cancer is a broad group of various diseases characterised by unregulated cell proliferation which leads to the formation of tumours (Vickers, 2004). Some tumours remain confined to their site of origin while some gain the ability to spread to other parts of the body, a process known as metastasis (Weiss, 1990). The burden of cancer continues to rise, due to inefficient prevention strategies and serious side effects, as well as the cost of cancer regimens (Sondhi et al., 2010). Medicinal plants represent a reservoir of bioactive compounds that can be useful in the management of cancer with less or no side effects (Wong et al., 2012). The aim of this study was to investigate the anti-cancer effects of *M. balsamina* leaf extract in breast MCF-7 cancer cells. In this study, *M. balsamina* leaves powder was extracted using acetone. The biological effect of the extract was assessed on the viability of MCF-7 cells using the MTT assay. The extract's ability to induce apoptosis was assessed using the Hoechst/propidium iodide dual staining method. Its anti-metastatic potential was investigated by determining its effect on MCF-7 cell migration, attachment and invasion using wound healing, adhesion, invasion assay, respectively. The human apoptosis antibody and human angiogenesis antibody array kits were used to determine the effect of the extract on the expression levels of proteins involved in apoptosis and metastasis, respectively. Treatment of MCF-7 cells with different concentrations of the extract showed a significant decrease in cell viability after 48 h incubation at 10 - 20 µg/ml. The decrease in cell viability was associated with the induction of apoptosis as seen by nuclear condensation and loss of membrane permeability in cells treated with the extract. Inhibition of migration, adhesion and invasiveness of the MCF-7 cells was seen in the treated cells. The extract also modulated proteins implicated in cell apoptosis, adhesion, migration and invasion such as Bcl-2 family of proteins, IGFBP, uPA, MMPs. In conclusion, based on the results, the extract show pro-apoptotic and anti-metastasis potential. Thus *M. balsamina* can be considered as a potential source of compounds with anti-cancer activity

CHAPTER 1

1 INTRODUCTION

Cancer is characterised by uncontrolled proliferation of abnormal cells that compromises an enormous group of associated diseases (Goel and Gude, 2014a). It is one of the most fatal diseases that pose a threat to human health worldwide, accounting for the second most leading cause of deaths each year among all ages and sexes (Jemal et al., 2011; Siegel et al., 2013). According to the recent report of World Health Organisation (February, 2014), 8.2 million patients died from cancer in 2012. It has been also estimated that the number of annual cancer cases will increase from 14 million in 2012 to 22 million within the next two decades (WHO, 2014). Normal cells, under steady state conditions, proliferate as needed to replace themselves as they age or become injured. However, failure to control proliferation can result in tumour formation. The resulting tumour can either be benign or malignant. Benign tumours are generally slow growing, enclosed within a fibrous capsule, non-invasive, and morphologically resemble their cellular precursor. If a benign tumour is not close to a critical vascular or neural tissue, prompt diagnosis and treatment frequently results in a cure. In contrast, malignant tumours rarely encapsulate, grow rapidly, invade regional tissues and have morphologic abnormalities, such that their tissue of origin may be unrecognisable (Talmadge and Fidler, 2010). Furthermore, malignant cells have the ability to metastasise, that is, the dissemination of tumour cells from primary to distant target sites. This involves several steps such intravasation, migration, adhesion, extravasation and angiogenesis (Hsieh et al., 2007; Patel et al., 2011). Metastasis is a prime cause of death in cancer patients and remains a challenge in cancer treatment. Targeting one or more processes involved in cancer metastasis holds a great promise for anticancer therapy (Goel and Gude, 2014b).

Another promising avenue for the development of novel therapeutic approaches is therapeutic targeting of apoptosis, since it is known to be disturbed in human cancers (Hanahan and Weinberg, 2011). This strategy is particularly relevant, since many currently used anticancer therapies utilise apoptosis signalling pathways to exert their anti-tumour activities (Wang, 2001). The available chemotherapeutic agents only

provide minimal survival benefits in advanced-stage cancer due to various factors, namely toxicity, serious side effects and drug resistance (David and Zimmerman, 2010). In addition, currently available cancer treatments are exclusively unaffordable to the large number of the population in the developing countries. **Yearly medical costs for cancer treatment in the United states have been estimated at \$99 billion US dollars (Jonsson and Wilking, 2007).** Alternative approaches in cancer treatment are important due to the increase in new cancer cases and limited effectiveness of the present treatments (Bandgar and Gawande, 2010). Medicinal plants have become an integral part of ethnomedical approach to cancer treatment and other life threatening diseases. Plant products, both as extracts and plant-derived compounds; have shown potential as chemo-preventive agents against various types of cancers (Aruna and Sivarama, 1990). Due to the lack of effective drugs that can successfully eradicate breast metastatic tumours, this study aims to screen for potential apoptotic and anti-metastatic activity which could lead to further drug development from traditionally used medicinal plant **with less side effects.**

1.1 Apoptosis

Apoptosis, also known as programmed cell death, is an active process of eliminating stressed cells without triggering an inflammation response or damaging the surrounding cells (Shishodia and Pundir, 2015). Kerr et al., (1972) documented characteristic morphological changes of an apoptotic cell via electron microscopy, including shrinkage of the cells, detachment from the surrounding tissue, nuclear and chromatin condensation as well as DNA cleavage. Furthermore, the formation of apoptotic bodies and loss of cytosolic membrane asymmetry leads to the translocation of phosphatidylserine to the outer surface of the cell (Kerr et al., 1972). The exposure of phosphatidylserine to the outer membrane serves as an “eat me” signal resulting in the phagocytosis of the apoptotic cells. Apoptosis is then mediated via the extrinsic pathway, which involves the activation of cell surface death receptors, and/or the intrinsic pathway mediated by the mitochondrial signalling pathway (Jin and El-Deiry, 2005; Armstrong, 2006).

1.1.1 Extrinsic Pathway

The initiation of extrinsic apoptosis signalling pathway involves the transmembrane receptors from the tumour necrosis factor (TNF) receptor gene superfamily such as TNF receptor, TNF-related apoptosis-inducing ligand (TRAIL-R1, TRAIL-R2) and Fas

(Locksley et al., 2001). This pathway is triggered when specific ligands **bind** to death receptors, such as TRAIL-R1 and TRAIL-R2, leading to receptor trimerisation and the recruitment of the adaptor protein, Fas-associated protein with death domain (FADD) (Hsu et al., 1995; Grimm et al., 1996; Wajant, 2002). FADD then associates with pro-caspase-8 via dimerisation of the death effector domain. At this point, a death-inducing signalling complex (DISC) is formed, resulting in the auto-catalytic activation of pro-caspase-8 to caspase-8 (Kischkel et al., 1995). Sufficient activation of caspase-8 in turn, directly activates the effector caspases (caspase-3, -6, -7) leading to the execution of apoptosis (Abdulghani and El-Deiry, 2010).

1.1.2 Intrinsic pathway

The intrinsic pathway is triggered in response to DNA damage, hypoxia or oncogene overexpression (Haupt et al., 2003). As a sensor of cellular stress, p53 is a critical initiator of the intrinsic pathway. It is a tumour suppressor gene, which is known to regulate cell cycle, DNA repair and apoptosis (Finlay et al., 1989; Gadea et al., 2002). In non-stressed cells, p53, is kept at very low levels via ubiquitin dependent-proteolysis triggered by its negative regulator, the oncoprotein Mouse double minute 2 homolog (Mdm2) (Haupt et al., 1997). However, under stress conditions, p53 is phosphorylated at a number of sites including Ser15, Ser20, and Ser33, which disfavours p53's interaction with Mdm2 and leads to its activation (Shieh et al., 1997). Once activated, p53 translocates from the cytoplasm and accumulates in the nuclei of cells (Appella and Anderson, 2000). In the nucleus, p53 becomes an active transcription factor where it binds to DNA inducing or repressing the expression of specific genes including the Bcl-2 family of proteins (Yonish-Rouach et al., 1994). This family of proteins is divided into pro- and anti-apoptosis proteins. Pro-apoptotic Bcl-2 members can be divided into 2 groups according to their function and the number of Bcl-2 Homology (BH) domains : (i) multidomain pro-apoptosis proteins, such as Bax, Bak and Bok, characterised by the presence of BH domains 1-3 and BH-3-only pro-apoptotic proteins such as Bik, Bid, Bad, Bim, Bmf, Noxa and Puma. Anti-apoptotic proteins include Bcl-2, Bcl-XL and Mcl-1 (Kim et al., 2000). Several hypotheses have been proposed to explain the anti-apoptotic function of Bcl-2. Bcl-2 may prevent the release of the mitochondria activators of the cytosolic caspases, which eventually mediates apoptosis specific intracellular proteolysis (Chao and Korsmeyer, 1998). The association of Bcl-2 with the apoptosis activating factor 1 (Apaf-1) may prevent the cytochrome c release and the activation of the two initiator

caspases, caspase-9 and 2. Also, Bcl-2 might act by modulating the collapse of the mitochondrial transmembrane potential that occurs during apoptosis (Hockenbery et al., 1990). The relative amounts or equilibrium between these pro- and anti-apoptotic proteins influence the susceptibility of cells to a death signal. An important feature of the members of Bcl-2 family is their ability to form homo- as well as heterodimers, suggesting neutralizing competition between these proteins. Thus, Bcl-2 (a 239 amino acids protein, 26 kDa) forms heterodimers with Bax, a pro-apoptotic protein with ~ 21% amino acid identity with Bcl-2 (Adams and Cory, 1998). In addition to Bax, several other genes have been reported to encode proteins having sequence homology with Bcl-2 and capacity to form heterodimers with it. The Bad protein (pro-apoptotic) can also bind to anti-apoptotic proteins, including Bcl-2 and Bcl-XL to neutralise their anti-apoptotic function (Yang et al., 1995).

Neutralisation of the anti-apoptotic proteins result in the formation of pores on the outer mitochondrial membrane causing the release of cytochrome c and other apoptogenic factors such as SMAC/DIABLO into the cytoplasm (Adams and Cory, 2007) which promotes apoptosis by binding and neutralising members of the family of inhibitor of apoptosis proteins (IAPs). Inhibitors of apoptosis including c-IAP-1, c-IAP-2 and XIAP can block caspase-3 activity through their baculovirus inhibitor of apoptosis repeat (BIR) domains (Nachmias et al., 2004). In the cytosol, cytochrome c along with apoptosis protease activating factor-1 (APAF-1) and pro-caspase-9 form the apoptosome. Within the apoptosome, pro-caspase-9 is activated and cleaves downstream effector caspases-3- or -7, leading to apoptosis (Youle and Strasser, 2008; Brunelle and Letai, 2009).

Although the extrinsic and intrinsic pathways are activated by different mechanisms, these two pathways are interconnected (Figure 1.1). Dependency of cells on the mitochondrial pathway to undergo apoptosis following a death receptor stimulus, defines two cell types, namely type I and type II cells (Kantari and Walczak, 2011). In type II cells, activated caspase-8 cleaves pro-apoptotic Bcl-2 family member Bid to form truncated Bid (tBid), which can then interact with Bax/Bak. This interaction increases the release of cytochrome c from the mitochondria (Zhang and Fang, 2005). Whereas type I cells do not require Bid cleavage and activation of the extrinsic pathway is sufficient to induce TRAIL- and CD95-induced apoptosis (Kantari and

Walczak, 2011). Thus, Bid provides a connection between extrinsic and intrinsic pathways.

1.1.3 p53

The tumour suppressor p53 is a transcription factor that activates vital damage containment procedures to restrict aberrant cell growth in response to DNA damage, oncogene activation, hypoxia and the loss of normal cell contacts (Lohrum and Vousden, 1999; Vogelstein et al., 2000). p53 is activated by external and internal stress signals that promote its nuclear accumulation in an active form. In turn, p53 induces either viable cell growth arrest or apoptosis. The latter activity is crucial for tumour suppression. In addition, p53 contributes to cellular processes such as differentiation, DNA repair and angiogenesis, which also appear to be vital for tumour suppression (Vogt Sionov and Haupt, 1999). Indeed, approximately 50% of human cancers bear p53 gene mutations; in the majority of the remaining cancer cases, p53 activity is compromised by alternative mechanisms (Vogelstein et al., 2000). These involve elevation in the expression levels of p53 inhibitors, such as Mdm2 or the E6 protein of Human Papillomavirus (HPV), or silencing of key p53 co-activators, such as Alternate Reading Frame (ARF) (Vogelstein et al., 2000; Vogt Sionov et al., 2001). Under normal conditions p53 is a short-lived protein. The p53 inhibitor Mdm2 is largely responsible for keeping p53 in this state. Mdm2 inhibits the transcriptional activity of p53 and, more importantly, promotes its degradation by the proteasome. However, the status of p53 is drastically altered when cells are exposed to stress, including DNA damage, untimely expression of oncogenes, hypoxia and nucleotide depletion (Giaccia and Kastan, 1998).

In response to stress, the p53-Mdm2 interaction is disrupted to enable p53 to associate with factors needed for activation of its target genes. Stress-induced p53 activation involves post-translational modification of p53 on multiple sites by phosphorylation, acetylation, and sumoylation (Vogelstein et al., 2000; Brooks and Gu, 2003) and modifications to Mdm2 that can enhance Mdm2 autoubiquitination and degradation (Stommel and Wahl, 2004). With regard to p53 modifications, phosphorylation has been studied most extensively and has been proposed to play a critical role in the stabilisation and activation of the tumour suppressor. These studies have been greatly facilitated by the availability of antibodies that recognise p53 modified on specific phosphoserine or phosphothreonine residues (Apella and

Anderson, 2001). Multiple serine (6, 9, 15, 20, 33, 37, 46, 315, 371, 376, 378, and 392) and three threonine residues (18, 55, and 81) have been reported to undergo phosphorylation in response to diverse stresses (Jimenez et al., 1999; Ljungman, 2000).

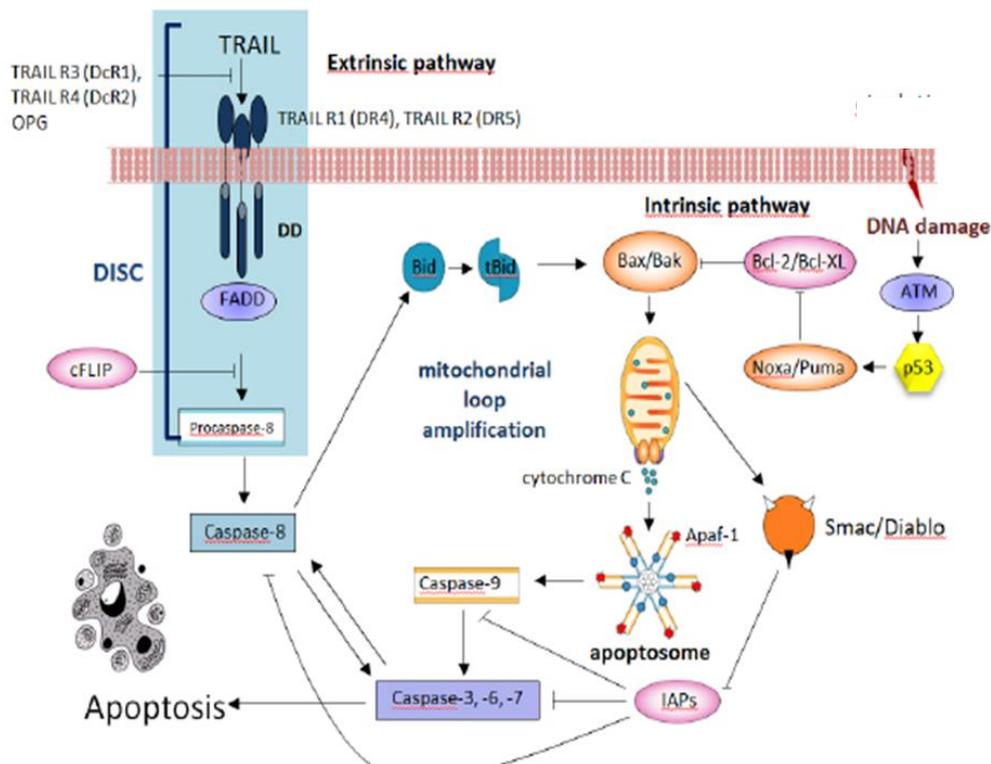


Figure 1.1: Intrinsic and extrinsic apoptosis pathways. The extrinsic apoptosis pathway involves the binding of a ligand to a death receptor which then leads to the recruitment of the adaptor molecule, FADD. This then allows Pro-caspase-8 to bind to FADD leading to DISC formation and resulting in its activation. Once activated, caspase-8 directly activates executioner caspases (caspase-3, -6, and -7) or cleaves Bid to truncated Bid (tBid). Truncated Bid (tBid) then translocate to the mitochondria to promote the assembly of Bax-Bak oligomers which results in the outer mitochondrial membrane permeability and initiates the intrinsic pathway. The pores in the mitochondrial membrane lead to the release of Cytochrome c into the cytosol, resulting in apoptosome assembly. Active caspase-9 then promotes a proteolytic cascade of effector caspases activation that leads to morphological changes associated with apoptosis. Further cleavage of pro-caspase-8 by effector caspases generates a mitochondrial amplification loop that further enhances apoptosis (Khaider et al., 2012).

1.2 Cancer metastasis

Metastasis is the general term used to describe the spread of cancer cells from the primary tumour to surrounding tissues (Chambers et al., 2002). This results from a complex molecular cascade consisting of many steps (Figure 1.2), each of which can be rate-limiting since a failure at any step may halt the process (Poste and Fidler, 1972). Metastasis involves cancer cells detaching from the primary tumour, invading local surroundings and entering the bloodstream or lymph vessels (intravasation) and migrate within the circulatory system to distant tissues. At the distant site(s), the cancer cells adhere to the vascular endothelium then exit the bloodstream and/or lymph vessels (extravasation) colonise new sites in the body and divide to form tumour nodules (micrometastasis). This is followed by further cell proliferation and formation of blood vessels (angiogenesis) that provide the tumour nodules with sufficient oxygen and nutrients to develop into large tumours (macrometastasis) (Divoli et al., 2011).

1.2.1 Cell dissociation and intravasation

In order to metastasise, the cells in the primary tumour must permeate and migrate to adjacent tissues but they must first detach from adjoining tumour cells and expand migratory as well as invasive capacities. The capability of tumour cells to dissociate and travel depends on their differentiation state which results in the detachment of single tumour cells from the primary tumour (Zijlstra et al., 2008; Tsai and Yang, 2013). Loss of cell-cell adhesion within the primary tumour mass allows disaggregation of tumour cells and hence helps in the initial dissemination (Cavallaro and Christofori, 2004). There are two main groups of cell–cell adhesion molecules that mediate tumour cell dissociation: (i) calcium-dependent (cadherin, E-cadherin, N-cadherin, selectin, and integrin) and (ii) calcium-independent (immunoglobulin and lymphocyte homing receptors) adhesion molecules (Joyce, 2005; Friedl and Alexander, 2011). As cancer cells become metastatic, affinity and avidity for their extracellular matrix (ECM) become altered. The degradation of the ECM components and basement membrane is facilitated by the proteolytic activity of several proteases such as matrix metalloproteinases (MMPs) and Urokinase-type plasminogen activator (uPA). Matrix metalloproteinases have been particularly implicated in ECM degradation associated with tumour growth (Roy et al., 2009). Urokinase-type plasminogen activator (uPA) is a 411 residues serine protease originally identified for its ability to activate plasminogen and generate plasmin, a broad-spectrum matrix-

and fibrin-degrading enzyme (Gong et al., 2000; Carriero et al., 2011). Degradation of the ECM eventually permits cancer cells to permeate the basal membrane and invade nearby tissues.

1.2.1.1 Matrix metalloproteinases

The MMPs are a large family of proteases which include the collagenases, gelatinases, stromelysins, and matrilysins (Blood and Zetter, 1990). They act by degrading almost all components of the basement membrane and ECM. Under normal conditions MMP expression is maintained at low levels except during periods of physiological processes such as of tissue remodelling, wound repair and organ development (Nagase et al., 2006) as well as in pathological processes including regulation of **inflammatory series of events**, osteoarthritis, rheumatoid arthritis and cancer (Egeblad and Werb, 2002; Kessenbrock et al., 2010).

Over-expression of MMPs, such as MMP-2 and -9, associated with metastasis has been reported in breast, colon and lung cancers (Freije et al., 1994; Newell et al., 1994; Wolf and Rovyer, 1993). MMP-2 is a gelatinase that can degrade type IV collagen, fibronectin and all basement membrane components. It is mainly involved in ECM remodeling and is intensively expressed by tumour and stromal components (Galis et al., 1994; Kamel et al., 2010; Łukaszewicz-Zajac et al., 2011). It promotes endothelial cell migration but reduces cell viability under some conditions such as hypoxia (Ben-Yosef et al., 2005).

MMP-9 is a member of metzincin enzyme family that degrades type IV collagen. It plays an important role in normal physiological responses such as wound healing and bone formation (Stuelten et al., 2005; Hallett et al., 2013). It also promotes cell cycle progression, cell growth and is associated with pro-oncogenic events such as neo-angiogenesis, tumour cell proliferation and metastasis (Egeblad and Werb, 2002). Under normal physiological conditions few of normal cell types express MMP-9; however, a majority of human metastatic tumour cells show increased MMP-9 activity as compared to benign cells (Morini et al., 2000). There are several potential therapeutic agents based on MMPs and their inhibitors under investigation for their ability to decrease the invasive capacity of cancer cells and thus down-regulate any subsequent positive feedback loops generated by their action. For example, potent and selective inhibitors of MMPs such as s-3304 and MMI-166 have been synthesised and are undergoing phase clinical trial (Chiappori et al., 2007;

Nakabayashi et al., 2010). Therefore, prevention of cancer cell dissemination by inhibiting the activity of both MMP-2 and 9 could be a major goal of cancer therapy.

Matrix metalloproteinase 8 (MMP-8), another member of the MMP family has emerged as a candidate to play a protective role during tumour progression. MMP-8 is mainly produced by neutrophils, and it has been implicated in a variety of tissue remodeling processes associated with inflammatory conditions (Van Lint and Libert, 2006). MMP-8 is very efficient in the degradation of fibrillar collagens (Khatwa et al., 2010), but may also target additional extracellular matrix proteins, as well as other proteases, cell adhesion proteins, protease inhibitors, growth factors, and chemokines (Sorsa et al., 2011). The relevance of MMP-8 as a protective factor in cancer has been further extended by the finding that experimental modulation of the expression levels of this enzyme alters the metastatic behaviour of human breast cancer cells (Montel et al., 2004). Montel et al (2004) used genetic engineering to study the effects on the behaviour of the tumours that they generated in athymic mice and found out that the knock-down of expression in NM-2C5 cells by transduction with a sequence encoding a specific ribozyme and overexpression of MMP-8 in M-4A4 cells by retroviral transduction both strikingly changed metastatic performance in opposite directions, indicating that this gene plays a role in the regulation of tumour metastasis. However, information about the molecular mechanisms underlying the putative role of MMP-8 in the regulation of the metastatic process is not yet documented.

The inhibition of the proteolytic action by the tissue inhibitors of the matrix metalloproteinases (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) family is an important regulatory point in the regulation of the MMPs (Rawlings et al., 2004; Boy et al., 2008; Roy et al., 2009). Inhibition of the MMPs by TIMPs is either directly inhibiting the activity of MMPs by forming tight, non-covalent inhibitory complexes with them or controlling the activation process itself (Duffy, 1996). The balance between active proteases and their inhibitor(s) is controlled for normal function in the body but during metastasis it is disrupted resulting in the over-expression of the active proteases.

1.2.2 Cell migration and attachment

The ability of tumour cells to detach from an established solid tumour by secretion of MMPs enables the cells to migrate and enter the lumen of the vessel. There are two major ways in which cells are reported to migrate; individually, when cell-cell

junctions are absent or collectively when cell–cell adhesions are still present (Friedl and Wolf, 2010). Single tumour cells can either migrate using a fibroblast- or a leukocyte-like strategy for passage via ECM as compared with collective cells which forms a monolayer and can invade two dimensionally or build up cell strands for three-dimensional invasion (Gray et al., 2010). Force generation, which is provided by substrate binding integrins, is required in leading cells to pull cells which migrate as clusters from the front or push them from the rear. Single tumour cells are more likely to be destroyed than cells travelling in clumps where the cells at the centre are shielded (Nash et al., 2002). Tumours cells come into contact with platelets, leukocytes and the vascular endothelium thus this interaction shields and protects them from immune destruction as well as assist in the metastatic process (Blood and Zetter, 1990). The migration of the cells to the vessels is facilitated with the help of several chemokines such as CXCL16, CXCL12 and CXCR4 as well as altered expression of several adhesion molecules. Once in the bloodstream and/or in the lymphatic system, the tumour cells are subjected to events which threatens their survival such as the shear forces generated by the blood flow, physical constraints and immune response attack (Molloy and van' Veer, 2008). In order to survive the harsh and changing environment of the blood and lymphatic systems, cells dynamically change their physical properties. The cells that manage to survive the pressures of the circulatory systems arrest in capillary beds at distant organs (Quigley and Armstrong, 1998). The preferred sites of metastasis for a given type of cancer often include the first capillary beds downstream of the primary tumour. Examples are metastasis of colon cancer cells to the liver and of breast cancer cells to the lungs, where the initial arrest of tumour cells may be mainly caused by physical restriction in capillaries of small diameter (Orr and Wang, 2001; Chambers et al., 2002). In such cases, the formation of aggregates comprising circulating tumour cells and host cells may enhance passive trapping in capillaries by increasing the diameter of tumour cell emboli. However, during metastasis to either the liver or the lung, tumour cells can also arrest in vessels of larger diameter than capillaries (Al-Mehdi et al., 2000), demonstrating that active adhesion to the vasculature via specific proteins, such as selectins, integrins and metadherin, can also contribute to initial arrest (Brown and Ruoslahti, 2004; Wang et al., 2004; Hu et al., 2009).

1.2.3 Extravasation and attachment

Extravasation is a process in which circulating tumour cells leave the blood or lymphatic system and colonise the targeted metastatic site. In this process, cancer cells first attach to the vascular endothelium and migrate along the endothelial cell lining and enter the surrounding tissue (Yilmaz et al., 2007). Extravasation can take place in two different ways. One possibility is that cancer cells start to proliferate in the lumen of a vessel. Due to the growth of the tumour, the vessel wall is destroyed and the cancer cell's way into the tissue of the organ is paved (Leber and Efferth, 2009). The second possibility for the cancer cells to penetrate an organ is similar to the intravasation which includes the degradation of endothelium and basement membrane through proteolysis (Mathias and Thomas, 2009). Tumour cells migrate passively or actively within the vessel lumen (Stoletov et al., 2010) and respond to certain signals delivered by the vascular endothelium (Strell and Entschladen, 2008). Balkwill and Mantovani (2001) reported the assumption that tumour cells might use chemokine gradients to spread around the body, therefore, tumour cells might use similar localization signals as leukocytes. The stromal cell-derived factor (SDF)-1 α is such a chemokine, which is expressed in organs that are the first destination of breast cancer metastases (Muller et al., 2001). Both the vascular endothelium permeability and movement of cancer cells are necessary for the process of extravasation. Extravasation may also be triggered by hypoxia response factors such as VEGF, MMP-1, and MMP-2. Once the cells have colonised their new sites they may be destroyed as most of them undergo apoptosis within 24 h (Chambers et al., 2002). Some of the cells may proliferate and form macrometastasis or enter a state of micrometastasis dormancy (Uhr and Pantel, 2011). Tumour dormancy is an extended stage in tumour progression in which tumours remain asymptomatic for a prolonged period of time and do not increase its burden. In the micrometastasis dormancy, there is a state of balance between apoptosis and cell proliferation resulting in no increase of tumour load. The dormant tumours are often only a few millimetres in diameter and highly prevalent in the general population and even could be left after primary tumour removal or chemotherapy (Almog, 2010). Only a small number of dormant cells can initiate growth as micrometastases, and an even smaller number grow into macroscopic tumours (Gelao et al., 2013). On the other hand, tumour cells need to receive enough supply of oxygen and nutrients through formation of new blood vessels (angiogenesis) in their new environment in order to grow and form successful secondary tumours (Hanahan and Weinberg, 2011).

1.2.4 Angiogenesis

Angiogenesis is the process of formation of new blood vessels from the host vasculature (Hanahan and Weinberg, 2011). Tumours cannot grow beyond a certain size, generally 1 – 2 mm, due to the lack of oxygen and other essential nutrients (McDougall et al., 2006). Therefore, angiogenesis is a required step for the supply of nutrients and oxygen to the tumour nodule. This leads to the transition of tumours from a dormant state to an actively growing tumour (Hogan and Kolodziej, 2002). Angiogenic stimuli activate endothelial cells that subsequently undergo phenotypical changes that trigger the release of proteases allowing them to degrade the ECM, migrate, proliferate, avoid apoptosis and finally differentiate into new vessels (Carmeliet and Jain, 2011). A variety of angiogenic growth factors, such as fibroblast growth factors (FGF), angiogenin, and VEGF, a molecular marker of angiogenesis (most often up-regulated in cancer) and platelet-derived growth factor (PDGF), induce blood vessel growth in tumours and are categorised as angiogenic activators (Martinez-Poveda et al., 2005).

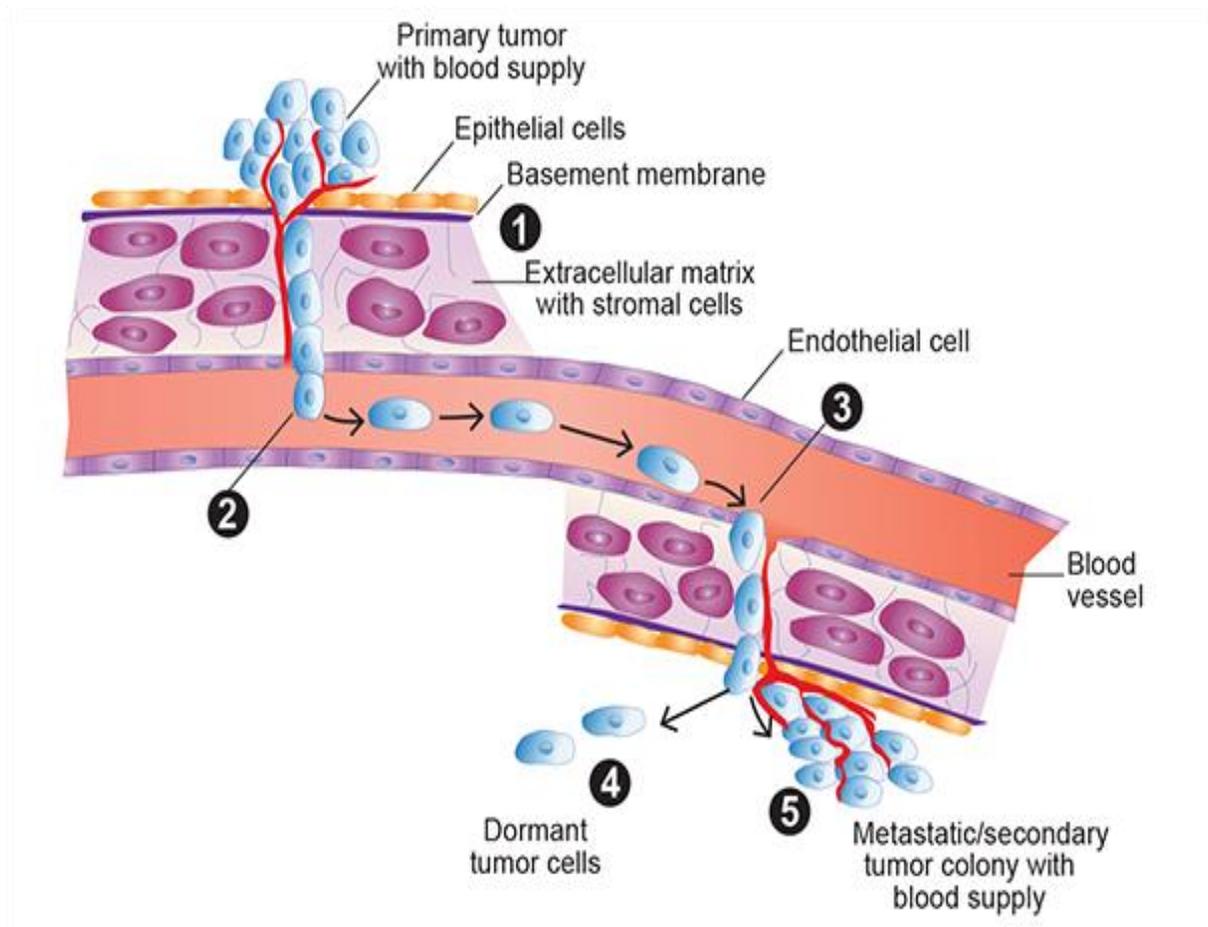


Figure 1.2: **(1)** Some of the cells in the primary tumour invade into the surrounding normal tissue by passing through the basement membrane and extracellular matrix (ECM). The invasion of the basement membrane is by the degrading ability of enzymes called matrix metalloproteinases (MMP). **(2)** The escaped cells then enter the blood circulatory system either directly by actively passing through endothelial cells that line the blood vessels or passively through the lymphatic system. **(3)** Cells in the circulatory system invade and home in the tissues at the secondary site. **(4)** Once at the secondary site, the tumour cells can rest dormant for a certain time period, or **(5)** re-establish growth to form secondary metastatic tumour colonies and form a new blood supply to nourish the metastatic tumour in the process known as angiogenesis. Dormant cells also can proliferate and eventually establish a new metastatic tumour at a later stage (Meadows and Zhang, 2015).

1.3 Medicinal plants

Plants have been employed for many generations to help manage different conditions including cancer with relatively less side effects than alternative non-natural drugs. The traditional herbal medicines are often criticised due to the absence of scientific proof. Nevertheless, recognition of the potential of natural products is increasing nowadays (Wong et al., 2012). Plant products, however, also play an important secondary role in the health care sectors of developed countries, with 70 – 80% of populations of developed countries having used some form of alternative or complementary medicine (Gopal et al., 2014). Herbal treatments are the most popular form of traditional medicine and are highly lucrative in the international marketplace. The global market for herbal products is expected to reach \$5 Trillion US dollars by 2050 (Anand and Neetu, 2011). Natural compounds are good sources for the development of new remedies for different diseases. Experimentally, several medicinal plants and herbal ingredients have been reported to have anticancer effects (Sharma et al., 2011; Tan et al., 2011; Teiten et al., 2013). In many cases, the structures of the active compounds in the plant extracts have been determined, and in some cases the potential mechanisms of action have been elucidated (Kratz et al., 2008). Also, a number of phytochemicals isolated from medicinal plants have been shown to decrease cell proliferation, induce apoptosis, delay metastasis and inhibit angiogenesis (Hajzadeh et al., 2006 ; Tavakkol-Afshari et al., 2006; Shu et al., 2010; Tan et al., 2011; Mortazavian et al., 2012; Mortazavian and Ghorbani, 2012; Sadeghnia et al., 2014). Currently, some of these plant-derived compounds are widely used for chemotherapy of cancerous patients. For example, Taxol analogues, vinca alkaloids (vincristine, vinblastine), and podophyllotoxin analogues have played an important role in treatment of such patients (Saklani and Kutty, 2008).

Momordica balsamina L. (Figure 1.3) commonly known as African pumpkin, African cucumber, Balsam apple and Balsam pear as well as “nku” in Northern Sotho belongs to the family Cucurbitaceae (Welman, 2004; Shai et al., 2010). The plant is a perennial herb native to tropical regions of Africa with soft stems and tendrils which climb up shrubs, boundary fields and fences (Thakur et al., 2011). The green leaves are deeply palmately 5 - 7 lobed, about 12 cm long, margin toothed and stalked. The plant produces spindle shaped fruits (dark green when unripe and bright to deep orange when ripe). The seeds are embedded into a sweet edible red fleshy pulp tasting like watermelon (Welman, 2004). *Momordica balsamina* is fairly common and

widespread in Namibia, Botswana, Swaziland and all the provinces of South Africa except the Western Cape. It is also indigenous to tropical Africa, Asia, Arabia, India and Australia. It has been cultivated in gardens in Europe. This species is closely related to *Momordica charantia* (Bitter melon) which shows various medicinal properties. The leaves, fruits, seeds, and bark of this plant are reported to have various medicinal and nutritional properties (Benoit-Vical et al., 2006; Bot et al., 2007).

Momordica balsamina is also known to possess a wide spectrum of medicinal and nutritional properties. It is used as a traditional folk medicine in many countries (Hassan and Umar, 2006). In Hausa land of Nigeria and Republic of Niger, the leaves are cooked as part of green vegetables soup for lactating mothers, where it is believed to help the mother to regenerate her lost blood during labour and to purify her breast milk (Hassan and Umar, 2006).

Phytochemical screening of *M. balsamina* Linn has revealed the presence of tannins, saponins, lectins and triterpenes (Akinniyi, 1986; Rocha E Silva et al., 2015). *M. balsamina* led to the isolation of several cucurbitane triterpenoids (Ramalhete et al., 2009a; Ramalhete et al., 2009b; Ramalhete et al., 2010; Ramalhete et al., 2011a; Ramalhete 2011b). The leaves, fruits, seeds, and bark of these plants are reported to have various medicinal and nutritional properties (Tommasi et al., 1995; Karumi et al., 2003; Matawalli et al., 2004; Benoit-Vical et al., 2006; Hassan and Umar, 2006; Bot et al., 2007). The fruit pulp extract of *M. balsamina* shows anti-HIV property (Bot et al., 2007). The leaves and fruit extracts of this plant shows antiplasmodial activity and is being used against malaria as African traditional medicine (Benoit-Vical et al., 2004). The extract of various parts of this plant shows shigelloidal, anti-diarrhoeal, antiseptic, antibacterial, antiviral, antiinflammatory and antimicrobial properties (Iwalokun et al., 2001; Banderia et al., 2001; Tommasi et al., 1995; Karumi et al., 2003; Kainyemi et al., 2005; Thakur et al. 2009; Jigam et al., 2004). The leaves are also important source of nutrients having 17 amino acids with adequate mineral composition like potassium, magnesium, phosphorus, calcium, sodium, zinc, manganese and iron (Hassan and Umar, 2006). Although *M. balsamina* has been reported to have the above mentioned medicinal uses, its pro-apoptotic and anti-metastatic effect on MCF-7 breast cancer cells has not been documented.

Mormodica charantia which belongs to the family of Cucurbitaceae is another plant that has showed anti-cancer activities. It is commonly known as bitter gourd (BG) and has been traditionally used as medicine to treat malaria, peptic ulcers, kidney stones, eczema (Dandawate et al., 2016) as well as in the treatment of type II diabetes (Rahman et al., 2015). Previous studies have showed that extracts from different parts of *M. charantia* possess anticancer properties. MAP30, Momordica anti-HIV protein, inhibited proliferation MDA-MB-231 breast cancer cells and increased survival in MDA-MB-231 breast cancer mice xenografts (Lee-Huang, 2000); Lectin from BG has also been shown to induce G2/M cell cycle arrest, autophagy and apoptosis in hepatocellular cancer (Zhang et al., 2015). (Dia and Krishnan 2016), showed that a peptide (BG-4) isolated from *M. charantia* induce apoptosis in HCT-116 and HT-29 human colon cancer cells. Other previous studies showed that MAP30, *Momordica* anti-HIV protein, inhibited proliferation MDA-MB-231 breast cancer cells and increased survival in MDA-MB-231 breast cancer mice xenografts, Lectin from BG has also been shown to induce G2/M cell cycle arrest, autophagy and apoptosis in hepatocellular cancer.



Figure 1.3: Plant of *Momordica balsamina* bearing fruits.

1.4 Aim

The aim of this study was to investigate the anti-cancer effects of *M. balsamina* leaf acetone extract in breast MCF-7 cancer cells.

1.5 Objectives

The objectives of the study were to:

- i) Collect and extract *M. balsamina* leaves using acetone.
- ii) Determine the pro-apoptotic effects of *M. balsamina* leaf extract:**
 - a. On the viability of MCF-7 breast cancer cells using the MTT assay.
 - b. Assess features associated with apoptosis in MCF-7 breast cancer cells exposed to the *M. balsamina* extract using Hoechst/PI double staining assay.
 - c. Assess the effect of the extract on the protein expression levels of apoptosis genes using proteome profiler antibody arrays.
- iii) Assess the anti-metastatic potential of *M. balsamina* by:**
 - a. Investigating the effect of the extract on cell migration using the scratch wound healing assay.
 - b. Determine the effect of the extract on cell adhesion using the attachment assay.
 - c. Investigate the effect of the extract on the invasiveness of MCF-7 cancer cells using the Boyden chamber invasion assay.
 - d. Assess the effect of the extract on the protein expression levels of metastasis regulatory genes using proteome profiler antibody arrays

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Equipment

- ❖ Waring Commercial Blendor (Model 32BL79, Dynamics Corporation, New Hartford, Connecticut, USA)
- ❖ GloMax®-Multi+ Detection System (Promega, Madison, USA)
- ❖ CO₂ incubator (NAPCO model, Instrulab cc, Johannesburg, SA)
- ❖ Centrifuge (Model GS-15R, Beckman Coulter, Germany)
- ❖ Inverted light and fluorescence microscope (Nikon, Japan)

2.2 Cells, culture media and chemicals

- ❖ 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, Curcumin, Hoechst, Propidium Iodide and Crystal violet (Sigma-Aldrich, Saint Louis, Missouri, USA)
- ❖ Dimethylsulfoxide (DMSO) (Merck Chemicals, (PTY) LTD, Darmstadt, Germany)
- ❖ Foetal bovine serum (FBS), DMEM medium and Phosphate-buffered saline (PBS) (HyClone, Logan, USA)
- ❖ MCF-7 cell culture (ATCC HTB-22, Rockville, USA)
- ❖ Proteome profiler (R&D systems, Minneapolis, MN, USA)
- ❖ Organic solvent Acetone (Rochelle Chemicals, Johannesburg, SA)
- ❖ PSN (penicillin, streptomycin and neomycin cocktail) and Trypsin (Gibco, Auckland, New Zealand)
- ❖ Taxol (Bioscience, Bristol, UK)

2.3 Plant material collection and extraction

Momordica balsamina plant leaves were collected from Letsitele, Donn village in the Limpopo province. The plant leaves were washed then dried at room temperature and powdered using a commercial blender. The powdered leaves (2 g) were extracted by soaking in 200 ml of acetone for 3 days at room temperature. After extraction, the extract was filtered with filter paper (Whatman no.1) and the filtrate air-dried. The dried filtrate was dissolved in DMSO (>99.9%) to prepare a stock solution (10 mg/ml) and stored at -20°C.

2.4 Cell culture and treatment

The MCF-7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). Cells were grown in DMEM (HyClone, USA) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (HyClone, USA) and 1% antibiotic mixture, Penicillin, Streptomycin and Neomycin, (PSN) (Gibco, New Zealand) and maintained in a tissue culture incubator at 37°C in a humidified air containing 5% CO₂. The cells were treated with various concentrations of the *M. balsamina* extract prepared by diluting the stock solution with culture media. Stock solutions of Curcumin (200 µM) and Taxol (1mM) dissolved in DMSO served as positive controls for metastasis and apoptosis assays, respectively.

2.5 Cytotoxic assay

To determine the effects of the extract on the viability of MCF-7 cells, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used as described by Karimian *et al.* (2014). Cells (5 x 10⁴ /well) were seeded in 96-well plates and allowed to attach overnight. The cells were then treated with 0 - 200 µg/ml of the extract, 20 µM of Curcumin, 50 nM Taxol and incubated in a humidified atmosphere of 5% CO₂ at 37°C for 48 h. **Treatment with 0 - 200 µg/ml included 16 replicates per concentration, whereas treatment with 20 µM of Curcumin, 50 nM Taxol had 8 replicates each.** After 44 h incubation, 20 µL of MTT (5 mg/ml in media) solution was added and further incubated for 4 h. The medium containing MTT was then replaced with DMSO to dissolve the formazan crystals formed within the cells. The dissolved formazan crystals were quantified by spectrophotometry at 560 nm using the GloMax®-Multi+ Detection System (Promega, Madison, USA). Percentage cell viability was determined using the formula:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control}} \times 100\%$$

2.6 Analyses of pro-apoptotic effects

2.6.1 Hoechst 33258/propidium iodide dual staining

The changes in nuclear morphology associated with apoptosis, in MCF-7 cells treated with the extract, were assessed using the Hoechst/propidium iodide dual staining method (Liu et al. 2012). Cells (5×10^5 /well) were seeded in 6-well plates and treated as described in section 2.5 for 48 h. Cells were then washed once with phosphate buffered saline (PBS) and stained with fluorescent DNA binding dyes Hoechst 33258 (25 $\mu\text{g/ml}$) and propidium iodide (PI) (25 $\mu\text{g/ml}$) dissolved in PBS, for 20 min in the dark at room temperature. Nuclei were visualised and photographed at 200x magnification using a fluorescence microscope (Nikon, Japan).

2.7 Analyses of anti-metastatic effects

2.7.1 Scratch wound healing assay

The effect of the extract on the ability of MCF-7 cells to migrate was determined as described by Boukerche et al. (2007) with some modifications. Cells (5×10^5 /well) were plated into 6-well culture plates for overnight. A wound was created by scratching the monolayer using a pipette-tip. Cell debris was removed by rinsing the scratched monolayer twice with PBS. The wounded cell monolayer was then exposed to 0, 80 and 100 $\mu\text{g/ml}$ of the extract and 20 μM of Curcumin for 6 and 24 h. To monitor wound closure, cell monolayers were visualised and photographed using 100x magnification at 0, 6 and 24 h under an inverted light microscope (Nikon, Japan).

2.7.2 Boyden chamber invasion assay

The effect of the extract on the ability of MCF-7 cells to invade and pass through a matrigel-coated membrane filter was determined using the Boyden chamber assay kit (Millipore, USA). The invasion assay was conducted in trans-well filters coated with matrigel matrix (8 μm pore size) following the manufacturer's protocol. Briefly, cells (1×10^6 /well) were seeded in the upper chamber of the trans-well filters and exposed to 0, 80 and 100 $\mu\text{g/ml}$ of the extract in serum free media. The inserts were

then placed in 24-well plates containing a chemoattractant (10% FBS in media) and incubated for 24 h. After incubation, non-invading cells were removed with a cotton swab and invasive cells were stained and photographed under an inverted light microscope (Nikon, Japan). Cells stained with 0.5% crystal violet were quantified by dissolving in 10% acetic acid and absorbance read at 560 nm using a microtiter plate reader (Promega, USA).

2.7.3 Cell adhesion assay

The ability of the extract to impede the adhesiveness of MCF-7 cells was assessed as described previously by Maria et al. (2014) with minor modifications. Cells (5×10^5 /well) were detached, washed with serum-free medium, incubated at 37°C with concentrations mentioned in section 2.7.1 for 3 h. Cells were then seeded into 6-well plates and allowed to adhere at 37°C in a humidified atmosphere of 5% CO₂ for 6 and 24 h. After washing twice with PBS, to remove the non-adherent cells, the adherent cells were stained with 0.5% crystal violet for 30 min. Following staining, cells were washed, air-dried and photographed under an inverted microscope (Nikon, Japan). The stained cells were further dissolved in 10% acetic acid and absorbance read at 560 nm using a microtiter plate reader (Promega, USA).

2.8 Analysis of protein expression

The human apoptosis antibody and human angiogenesis antibody array kits were used to determine the effect of *M. balsamina* on the expression of proteins involved in apoptosis and metastasis, respectively, as described by the manufacturer (R&D systems, Minneapolis, MN, USA). Briefly, 218 and 300 µg of protein from each sample were incubated with the human apoptosis and angiogenesis array membranes, respectively, at 4°C for overnight. The membranes were then washed with array wash buffer for 5 min at room temperature. Streptavidin-HRP solution was added to the membranes then incubated for 30 min at room temperature on an orbital shaker. Pictures of the arrays were captured using the ChemiDoc XRS and the signal density of each spot representing the protein of interest was determined using imager Quantity One software (BioRad Laboratories, California, USA). The background signal was subtracted from total signal value of each spot and each pair of duplicate spots was averaged for each protein in each treatment group.

2.9 Statistical analysis

Statistical differences between treatments and controls were calculated using the Graphpad Instat 3 software by one-way ANOVA followed by Dunnett`s comparison test. The data were expressed as mean \pm standard deviation (SD) of three independent experiments. P value \leq 0.05 was considered significant

CHAPTER 3

3 RESULTS

3.1 The effect of *M. balsamina* leaf acetone extract on MCF-7 cell viability

To determine the effect of the extract on MCF-7 cell viability, the MTT assay was used to measure the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to purple formazan in living cells. As shown in Fig. 3.1, the extract, significantly reduced MCF-7 cell viability from 10 $\mu\text{g/ml}$ as compared to the untreated cells. For the subsequent assays, the concentrations of the extract investigated were 0, 80 and 100 $\mu\text{g/ml}$ for metastasis assays, these concentrations were deemed to be non-toxic to the cells as more than 90% of cell viability was still retained at 100 $\mu\text{g/ml}$. The concentrations (125 and 140 $\mu\text{g/ml}$) were used for apoptotic assays since they reduced cell viability by 50 % or more. Taxol and Curcumin treatment retained more that 90% cell viability.

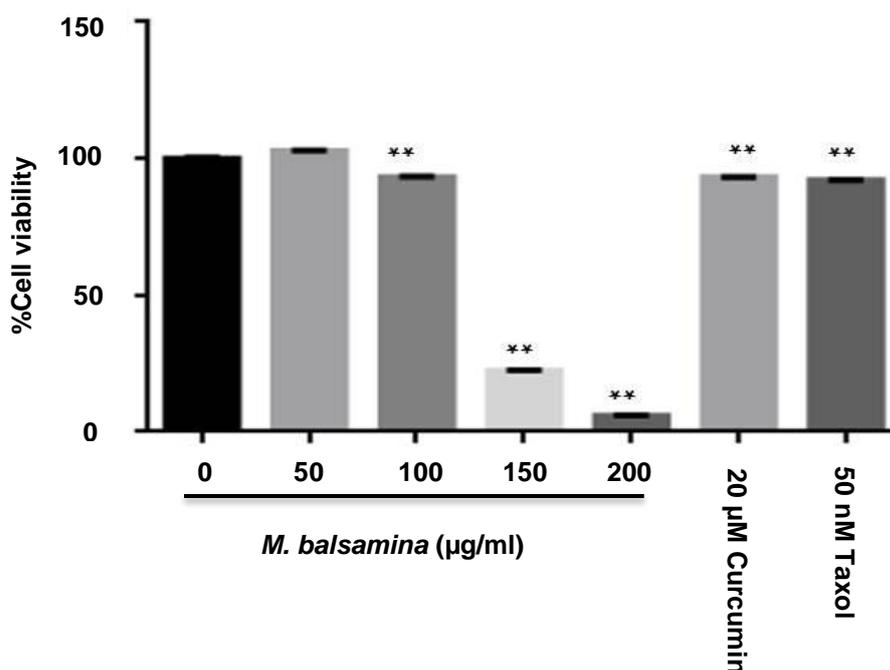


Figure 3.1: Effect of the *M. balsamina* leaf acetone extract on MCF-7 cell viability. Cells were treated with 0 – 200 $\mu\text{g/ml}$ of the extract, 20 μM of Curcumin and 50 nM of Taxol for 48 h. The % cell viability was assessed by the MTT assay. The data shows the mean \pm S.D of three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, indicate a significant difference to the control.

3.2 The effect of *M. balsamina* leaf acetone extract on the nuclear morphology of MCF-7 cells

The nuclear morphological changes associated with apoptosis in MCF-7 cells exposed to the extract were assessed by Hoechst 33258/PI dual staining. The blue fluorescent Hoechst 33258 is a cell permeable nucleic acid dye used to identify chromatin condensation and fragmentation. Condensed chromatin in apoptotic cells fluoresces more brightly than the chromatin that is not condensed. The red-fluorescent propidium iodide is a cell membrane impermeable DNA-binding dye, which can only stain the DNA when there is a loss of plasma membrane integrity, which occurs only in dead cells. As shown in Fig. 3.2, the nuclei of untreated cells appeared to have an intact oval shape and fluoresced blue. Furthermore, only a nuclei of the untreated cells were stained by PI. On the other hand, nuclei of cells treated with extract and Taxol exhibited a bright-blue fluorescence indicative of condensed chromatin. A concentration-dependent response is seen for the PI as the amount of fluorescent cells increased from 100 µg/ml and above. The cells were also photographed under normal light shown in the first row of the figure below. The untreated cells retained their normal angular or polygonal shape and most of the cells treated with the extract showed loss of the normal sheet-like growth. Cells treated with the positive control also showed to have lost their normal morphology.

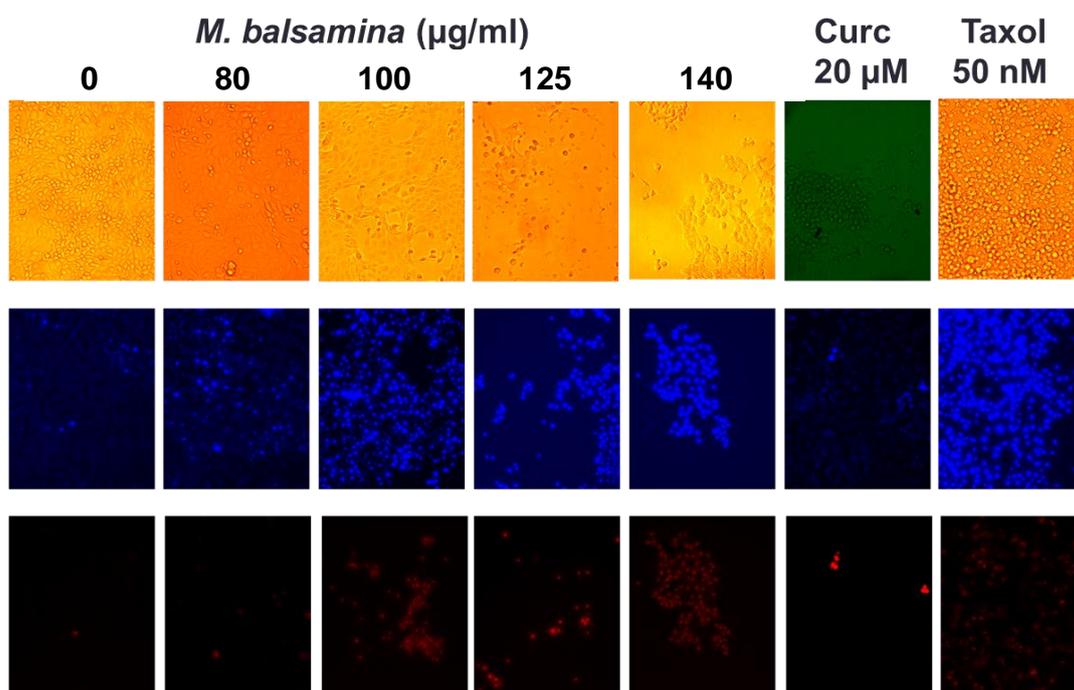


Figure 3.2: Hoechst 33258/ PI staining of nuclear DNA in treated and untreated MCF-7 cells. Cells were exposed to 0, 80 and 100 µg/ml of the extract, 20 µM of Curcumin and 50 nM of Taxol as positive controls for 48 h. Cell nuclei were then stained with Hoechst 33258 and PI then examined under 20x magnification using a fluorescence microscope. Brightly blue fluorescent stained nuclei within the cells indicated apoptotic morphological changes such as chromatin condensation and bright red fluorescent stained cells with PI indicated cells at a later stage of apoptosis.

3.3 The effect of *M. balsamina* leaf acetone extract on expression levels of the Bcl-2 family of proteins in MCF-7 cells

The Bcl-2 family of proteins controls a critical step in the commitment to apoptosis by regulating permeabilisation of the mitochondrial outer membrane. To determine the expression profile of the Bcl-2 family of proteins in MCF-7 cells in response to the extract, the human apoptosis proteome profiler array kit was used. As shown in Fig 3.3, the expression of Bad, Bax, Bcl-2, Bcl-x, was up-regulated upon treatment with the extract and Taxol. Significant increase in the expression of Bad was seen in cells treated with extract. Bax expression was seen to be significantly increased at 125 µg/ml of the extract. The anti-apoptotic protein, Bcl-x, expression was also significantly increased upon treatment with the extract and Taxol.

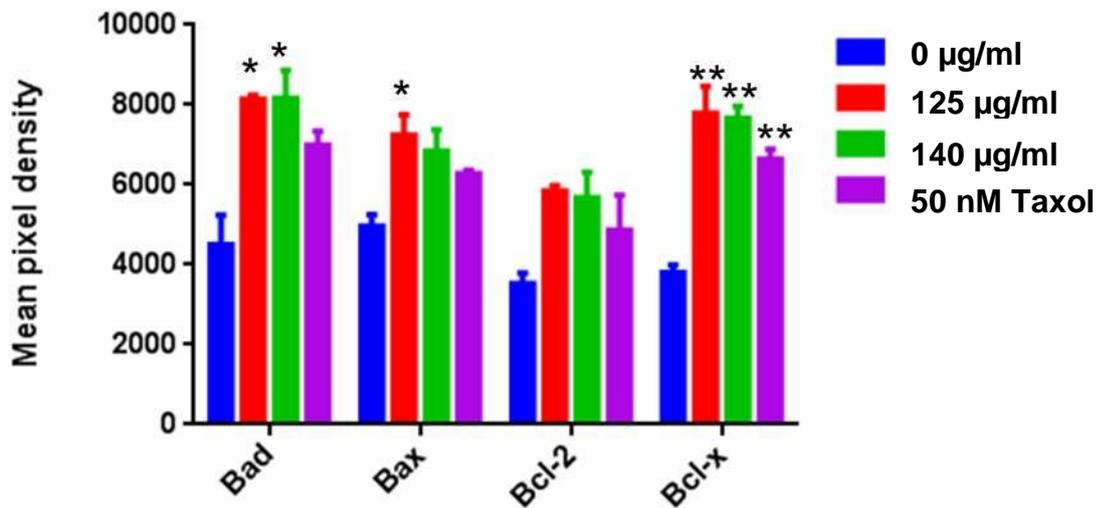


Figure 3.3: Effect of *M. balsamina* leaf acetone extract on expression levels of pro-apoptotic and anti-apoptotic proteins in MCF-7 cells. MCF-7 cancer cells were treated with (0, 125 and 140 µg/ml) of the extract and 50 nM of Taxol for 24 h. Protein expression levels were determined using the human apoptosis antibody array kit. Data shows the mean pixel density of each representative pair of duplicate spots on the array determined by quantity one software and the mean±S.D of duplicate values. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ indicate a significant difference to the control.

3.4 The effect of *M. balsamina* leaf acetone extract on expression levels of pro-apoptotic markers

Changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition pore can lead to apoptosis. Loss of the mitochondrial transmembrane potential leads to the release of pro-apoptotic proteins (cytochrome c, SMAC/DIABLO) from the intermembrane space into the cytosol. These proteins activate the caspase-dependent mitochondrial pathway. The effect of the extract on the expression of pro-apoptotic markers was determined using the proteome profiler kit. Treatment of MCF-7 cells with the extract and Taxol significantly up-regulated the expression of cytochrome c, SMAC/DIABLO, pro-caspase-3 and cleaved caspase-3. However, pro-caspase-3 was not significantly up-regulated when the cells were treated with Taxol as seen in Fig 3.4.

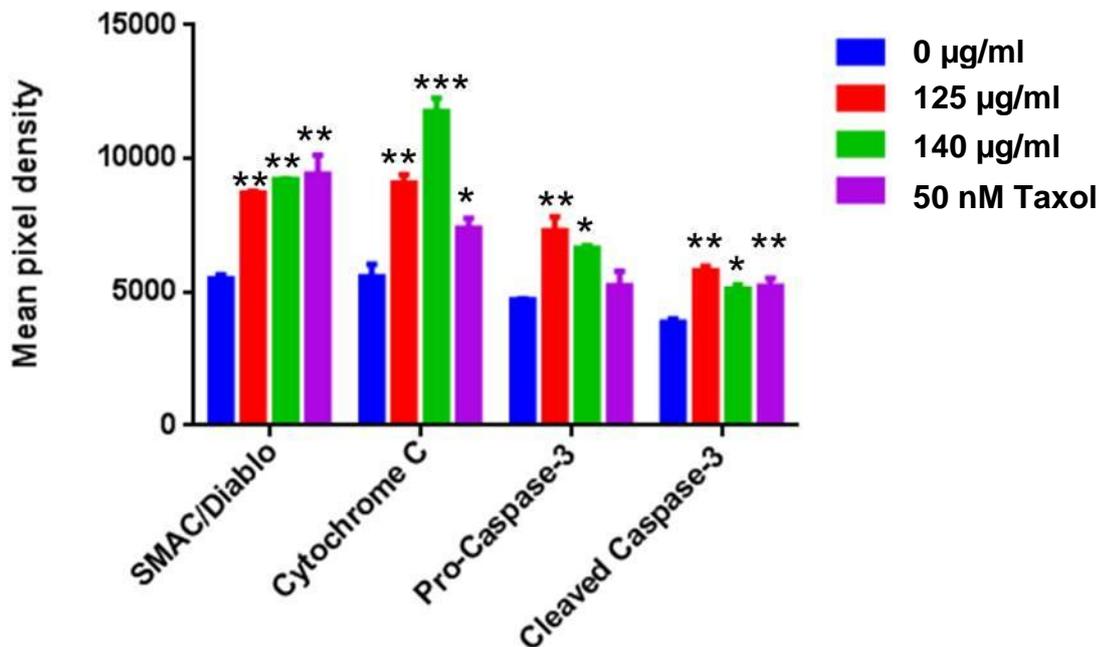


Figure 3.4: Effect of *M. balsamina* leaf acetone extract on the expression levels of pro-apoptotic proteins. MCF-7 cancer cells were treated with (0, 125 and 140 µg/ml) of the extract and 50 nM of Taxol for 24 h. Protein expression levels were determined using the human apoptosis antibody array kit. Data shows the mean signal density of each representative pair of duplicate spots on the array determined by quantity one software and the mean±S.D of duplicates values. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 indicate a significant difference to the control.

3.5 The effect of *M. balsamina* leaf acetone extract on the expression levels of apoptotic death receptors

Death receptors are cell surface receptors that transmit apoptotic signals initiated by specific ligands such as Fas, TNF alpha and TRAIL and in response, rapidly ignite the cell's extrinsic apoptosis machinery. The proteome profiler human apoptotic array kit was applied to determine the relative levels of apoptosis receptors/ligands involved in the extrinsic apoptosis pathway. The expression of specific ligands of the tumour necrosis factor (TNF) family and death receptors, such as TNF receptor (R) 1, TNF-related apoptosis-inducing ligand (TRAIL) R1, TRAIL R2, Fas and FADD was significantly increased by treatment with the extract and Taxol (Fig 3.5).

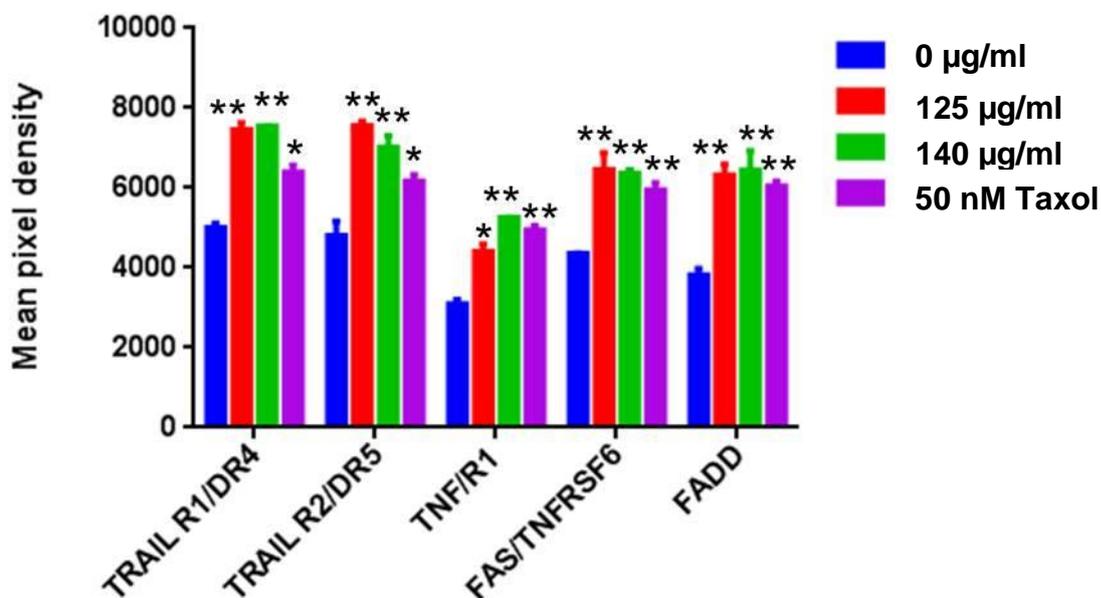


Figure 3.5: Effect of *M. balsamina* leaf acetone extract on the expression levels of apoptotic death receptors involved during the extrinsic pathway. MCF-7 cancer cells were treated with (0, 125 and 140 µg/ml) of the extract and 50 nM of Taxol for 24 h. Protein expression levels were determined using the human apoptosis antibody array kit. Data shows the mean signal density of each representative pair of duplicate spots on the array determined by quantity one software and the mean±S.D of duplicates values. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ indicate a significant difference to the control.

3.6 The effect of *M. balsamina* leaf acetone extract on the expression levels of apoptosis-inhibitory proteins in MCF-7 cells

Inhibitor of apoptosis (IAP) proteins are implicated in multiple ways in cell death regulation including inhibition of apoptosis. The human apoptosis array kit was used to examine the effect of the extract on apoptosis-inhibitory proteins expression profiles. Interestingly, the protein expression levels of negative regulators of apoptosis such as cIAP-1/2 and XIAP were significantly increased in cells treated with the extract and Taxol (Fig. 3.6).

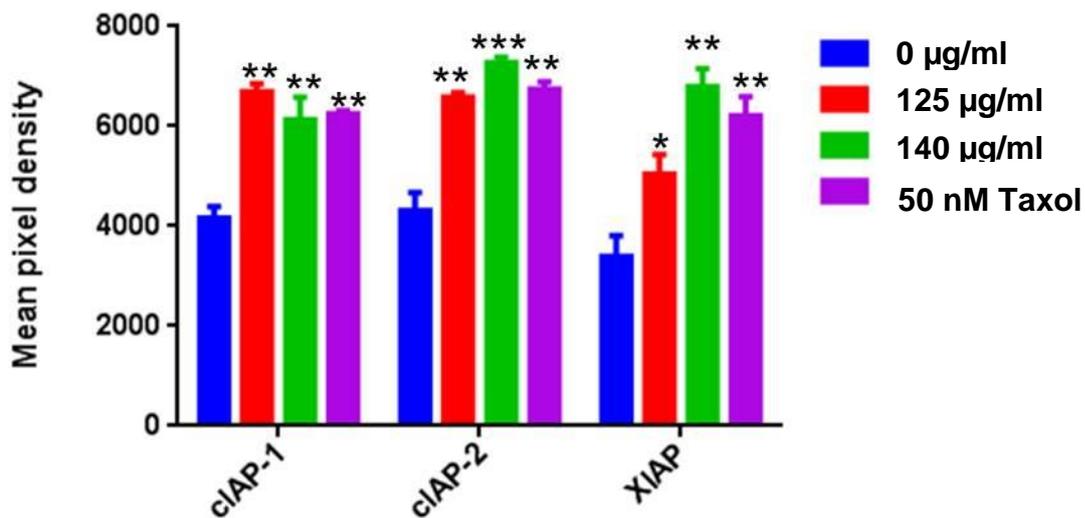


Figure 3.6: Effect of *M. balsamina* leaf acetone extract on the expression levels of apoptosis-inhibitory proteins. MCF-7 cells were treated with (0, 125 and 140 µg/ml) of the extract and 50 nM of Taxol for 24 h. Protein expression levels were determined using the human apoptosis antibody array kit. Data shows the mean signal density of each representative pair of duplicate spots on the array determined by quantity one software and the mean±S.D of duplicates values. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 indicate a significant difference to the control.

3.7 The effect of *M. balsamina* leaf acetone extract on p53 phosphorylation in MCF-7 cells

The tumor suppressor protein, p53, is a transcription factor that is kept at low levels in healthy cells. Phosphorylation at specific sites is regarded as the first crucial step of p53 stabilisation. Once p53 is stabilised then its interaction with Mdm2 is inhibited. To assess the effect of the extract on the expression of phosphorylated p53, the protein array profiler kit was used. Figure 3.7 shows that the extract significantly up-regulated the phosphorylation of p53, at Ser15, Ser46, Ser392 involved in promoting cell cycle arrest and activating apoptosis. Cells treated with Taxol only showed a significant up-regulation of p53 only at the Ser392 site.

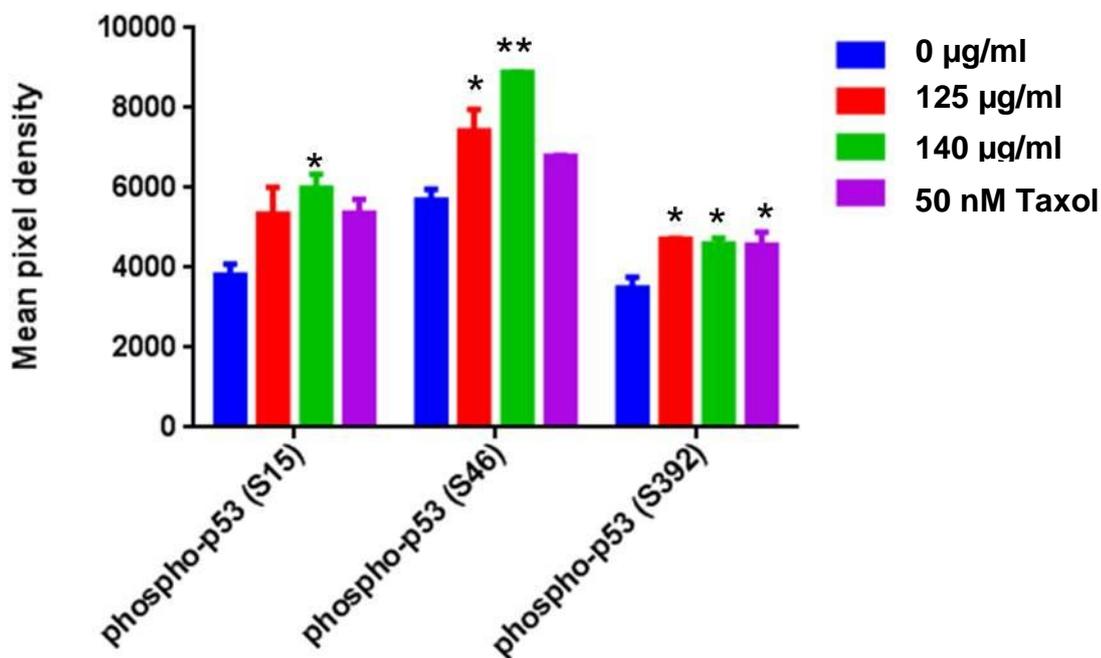
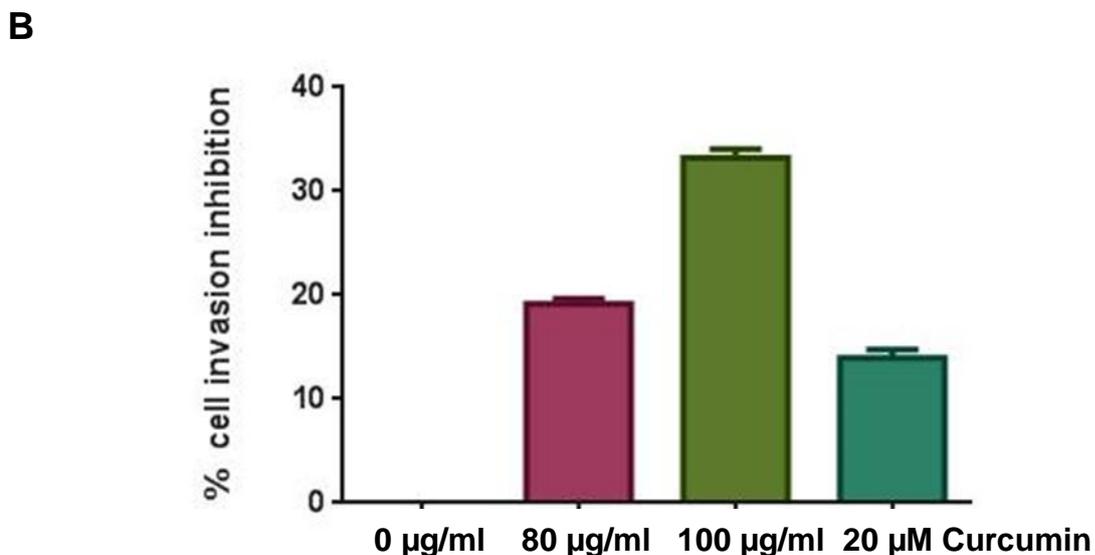
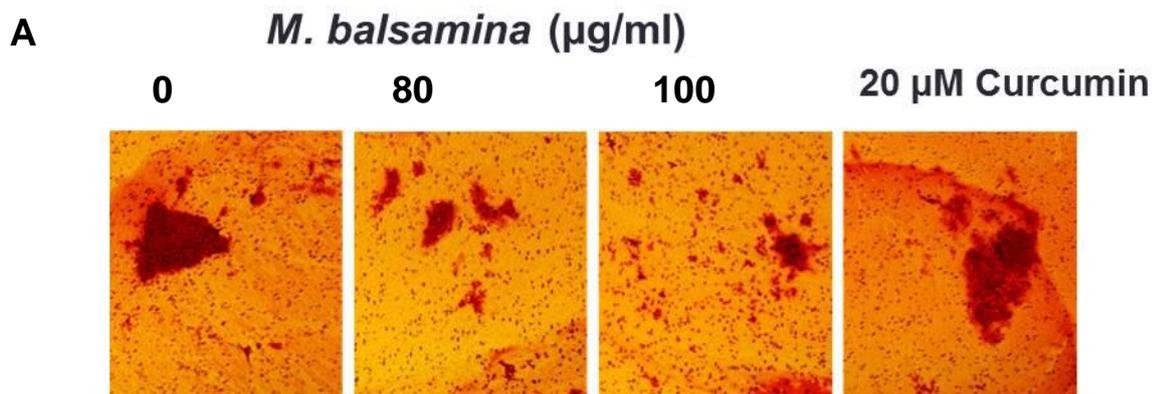


Figure 3.7: Effect of *M. balsamina* leaf acetone extract on p53 phosphorylation in MCF-7 cells. The cells were treated with (0, 125 and 140 µg/ml) of the extract and 50 nM of Taxol for 24 h. Protein expression levels were determined using the human apoptosis antibody array kit. Data shows the mean signal density of each representative pair of duplicate spots on the array determined by quantity one software and the mean±S.D of duplicates values. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ indicate a significant difference to the control.

3.8 The effect of *M. balsamina* leaf acetone extract on the invasiveness of MCF-7 cells

To determine whether the extract has an effect on the invasiveness of MCF-7 cancer cells, the invasion assay was conducted. After 24 h of treatment with the extract, the number of invasive cancer cells was decreased in a dose-dependent manner as shown in Fig. 3.8A & B. Furthermore, the expression levels of proteins, such as the TSP -1 and uPA, which influence the structure of the ECM and promote cell invasiveness during metastasis, were significantly down-regulated at 100 $\mu\text{g/ml}$ of the extract. MMP-9 was shown to also be down-regulated, although not significantly, in both the cells treated with the extract and Curcumin (Fig. 3.8C). In contrast, MMP-8 protein expression was significantly up-regulated in the treatment group as well as the positive control when compared to the control.



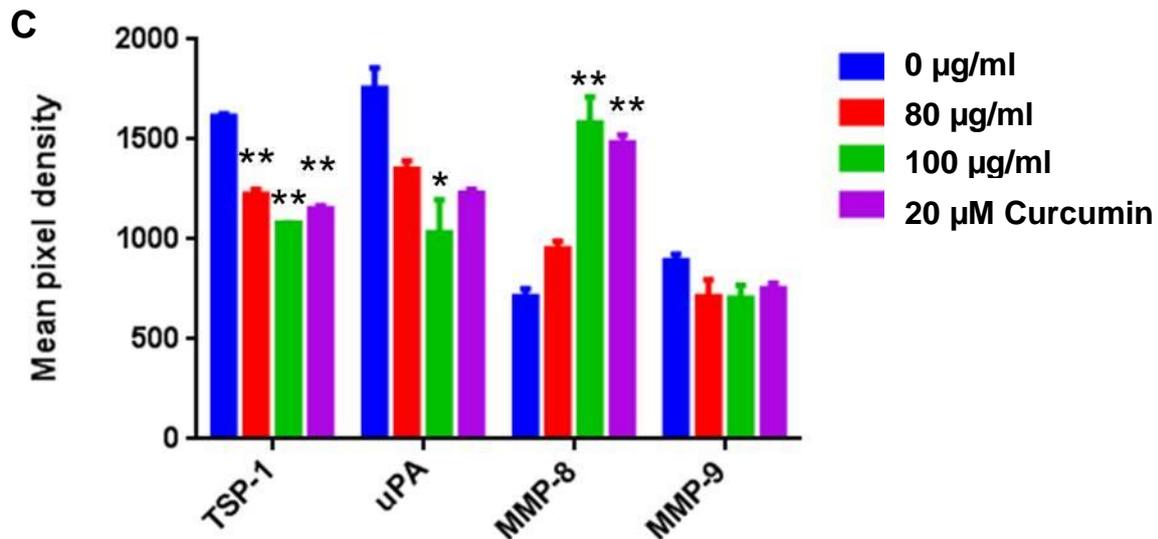


Figure 3.8: Effect of *M. balsamina* leaf acetone extracts on MCF-7 cell invasiveness. Cells were plated in trans-well inserts coated with matrigel matrix and treated with 0, 80 and 100 µg/ml of the extract and 20 µM of Curcumin, as a positive control for 24 h. Cells that invaded the matrigel matrix and migrated to the bottom of the membrane were fixed and stained with 0.5% crystal violet solution. Photographs of the stained cells were captured under (10X) magnification of an inverted light microscope (A). Stained cells were dissolved in 10% acetic acid and the absorbance measured at 560 nm using a microtiter plate reader (B). Protein expression levels were determined using the human angiogenesis antibody array kit. Data shows the mean signal density of each representative pair of duplicate spots on the array determined by the Quantity One software and the mean±S.D of duplicates values. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ indicate a significant difference to the control (C).

3.9 The effect of *M. balsamina* leaf acetone extract on the expression levels of angiogenesis and tumour cell invasion inhibitors

A variety of protease inhibitors have been shown to inhibit metastasis by inhibiting cell invasiveness. The angiogenesis array kit was used to determine the expression levels of angiogenesis and tumour cell invasion inhibitors in treated and untreated MCF-7 cells. A significant increase in the expression level of inhibitor of MMP-9, (TIMP-4), as well as plasminogen activator inhibitor-1 (Serpin E1) was observed in MCF-7 cells treated with 100 µg/ml of the extract. TIMP-1 (Inhibitor of MMP-9) level was significantly increased in cells treated with the positive control only (Fig 3.9).

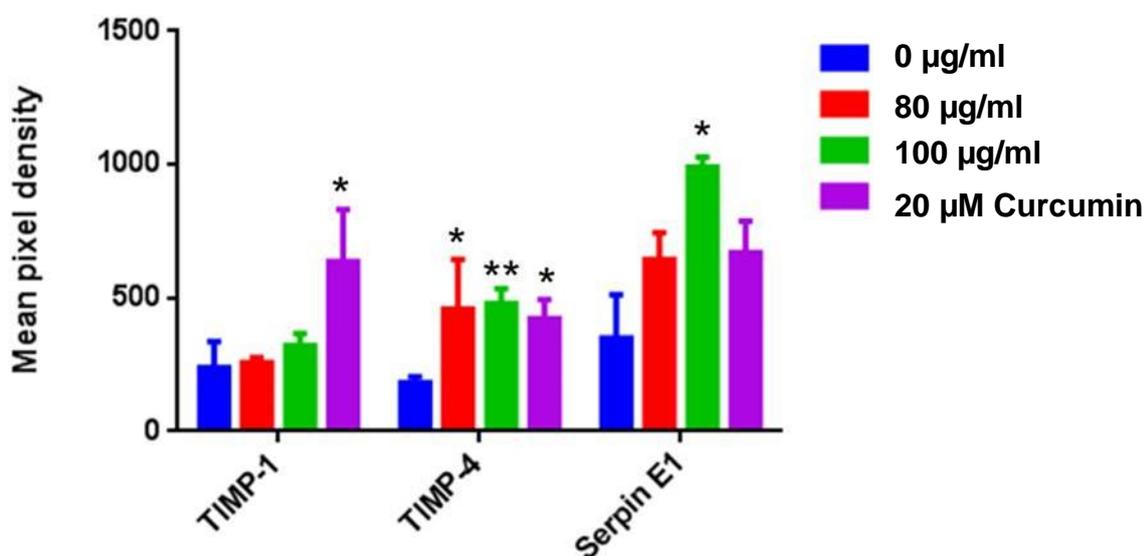
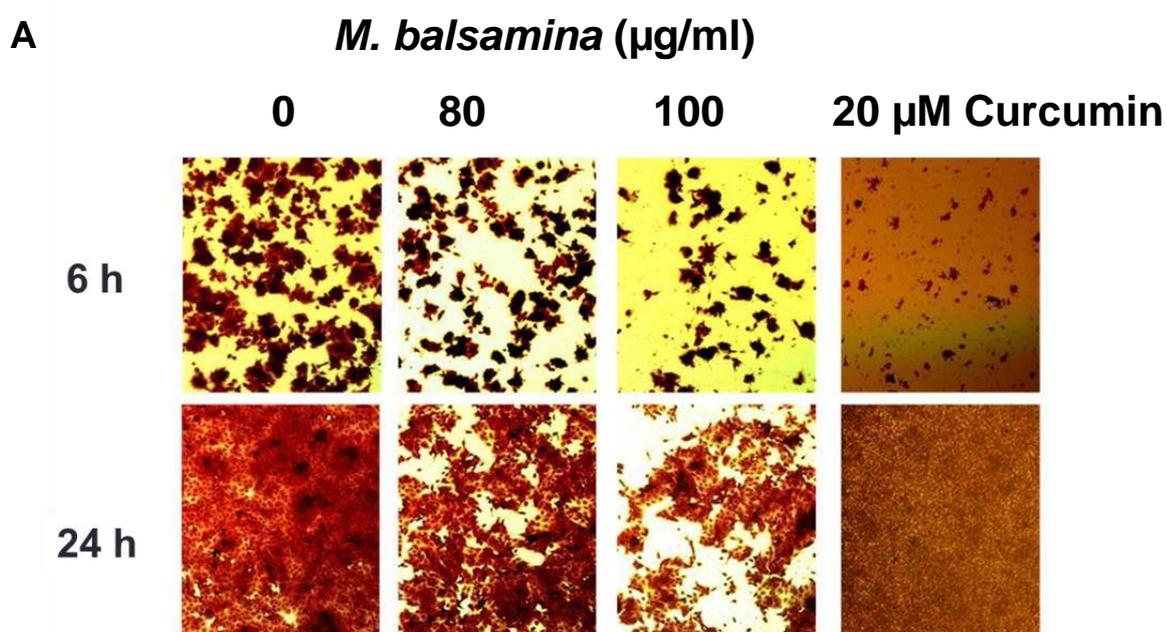


Figure 3.9: Effect of *M. balsamina* leaf acetone extract on expression levels of the inhibitors of MMPs and uPA. MCF-7 cancer cells were treated with (0, 80 and 100 µg/ml) of the extract and 20 µM of Curcumin for 24 h. Protein expression levels were determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised using the ChemiDoc XRS imager and the signal density of each spot representing the proteins of interest was determined using Quantity One software. Data shows the mean signal density of each representative pair of duplicate spots on the array and the mean±S.D of duplicates values. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 indicate a significant difference to the control.

3.10 The effect of *M. balsamina* leaf acetone extract on the adhesion of MCF-7 cells

The ability of MCF-7 cells to adhere after exposure to the extract was determined using the attachment assay. The results revealed that 8 and 10 $\mu\text{g/ml}$ of the extract were able to suppress the adhesiveness of the cells to the cell culture plate surface in a concentration-dependent fashion (Fig. 3.10A). The percentage inhibition of cell adhesion 10 $\mu\text{g/ml}$ was estimated at 20 and 15% after 6 and 24 hours, respectively (Fig. 3.10B). The positive control showed to have inhibited the adhesion ability of MCF-7 cells more as compared to the cells treated with the extract.



B

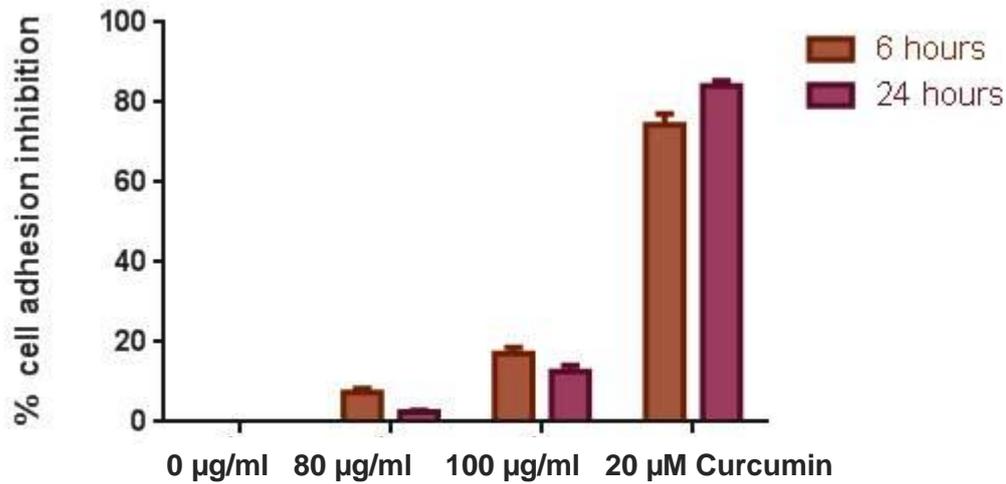
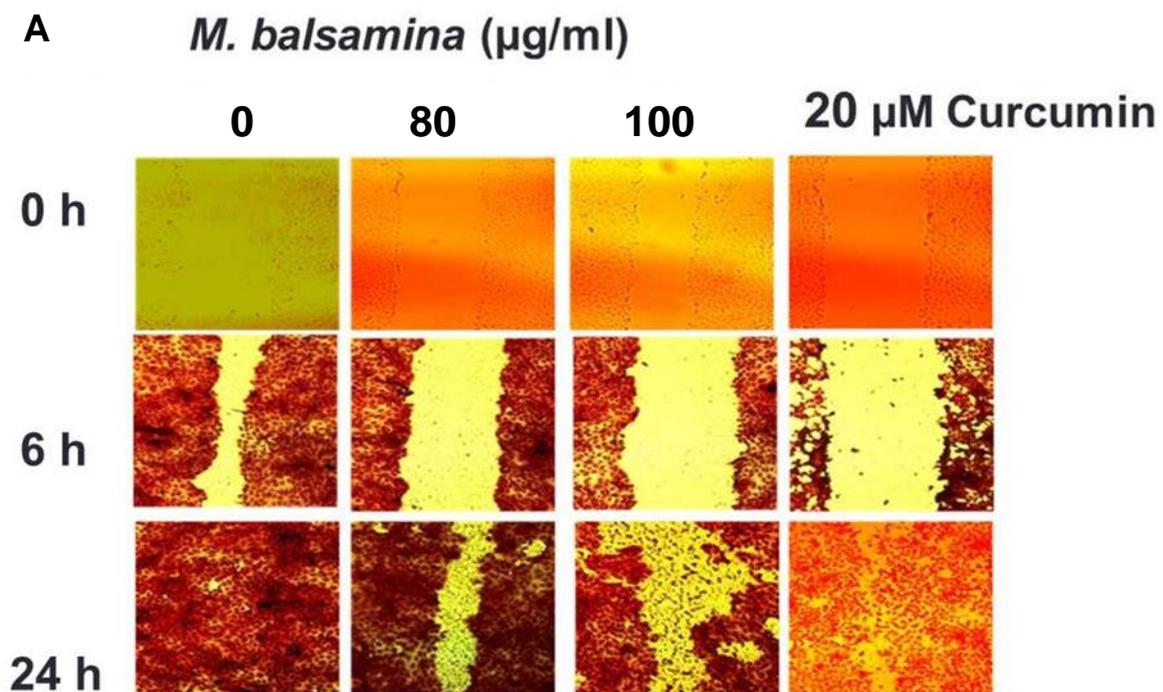


Figure 3.10: Effect of *M. balsamina* leaf acetone extract on MCF-7 cell adhesion. Cells were pre-treated with 0, 80 and 100 µg/ml of the extract and 20 µM of Curcumin, as a positive control for 3 h and then allowed to attach on a 6-well plate for 6 and 24 h. **The 80 and 100 µg/ml treatment had two replicates each.** Attached cells were fixed and stained with 0.5 % crystal violet solution. Photographs of attached cells were captured under 10X) magnification of an inverted light microscope (A). Percentage inhibition of cell adhesion was estimated by solubilising the crystal violet stained-cells with 10% acetic acid and the absorbance was read at 560 nm using a microtiter plate reader (B).

3.11 The effect of *M. balsamina* leaf acetone extract on the migration of MCF-7 cells

The scratch wound healing assay was used to determine the effect of the extract on the migration of MCF-7 cells. The results demonstrated a concentration-dependent inhibition of the rate of wound-healing of MCF-7 cells exposed to 80 and 100 µg/ml of the extract as compared to untreated cells. The wound of untreated cells was almost closed and completely closed after 6 and 24 h, respectively. In contrast, MCF-7 cells treated with the extract showed a greater wound area at 6 and 24 h. (Fig. 3.11A). Cell motility factors that stimulate migration of tumour cells during metastasis such as IGFBP-1, IGFBP-3 and CXCL16 were markedly but not significantly down-regulated in extract-treated groups compared to the untreated control, while Curcumin had no effect (Fig 3.11B).



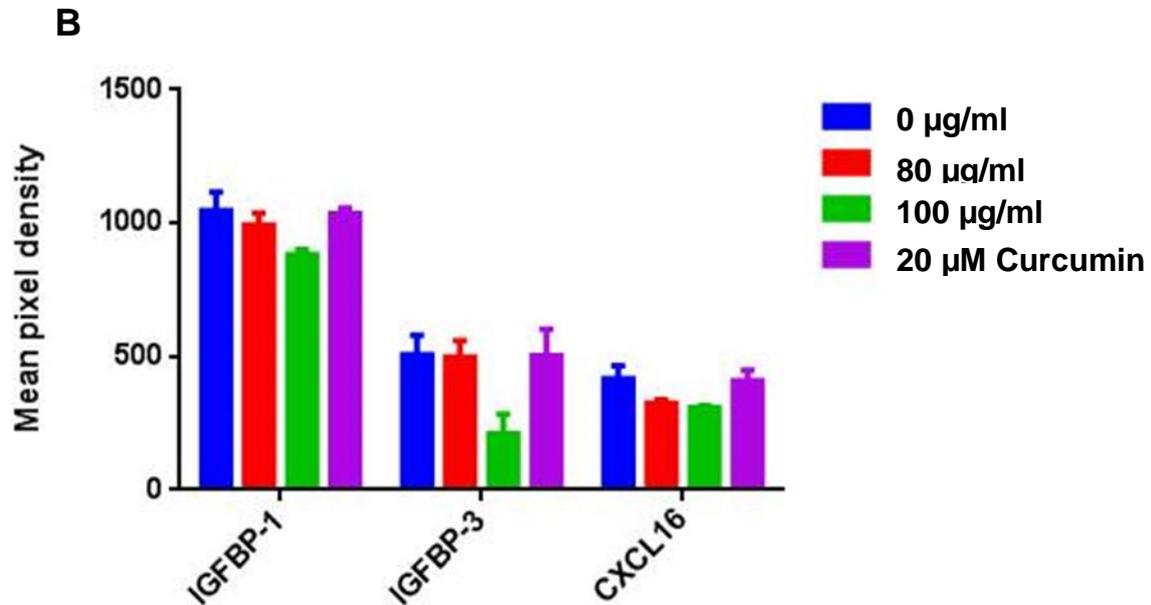


Figure 3.11: Effect of the *M. balsamina* leaf acetone extract on MCF-7 cell migration. A wound was created on MCF-7 cell monolayers then exposed to 0, 80 and 100 µg/ml of the extract and 20 µM of Curcumin, as a positive control. Photographs of the wound closure were captured under (10X) magnification of an inverted light microscope at 0, 6 and 24 of incubation (A). Protein expression levels were determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised using the ChemiDoc XRS imager and the signal density of each spot representing the proteins of interest was determined using Quantity One software. Data shows the mean signal density of each representative pair of duplicate spots on the array and the mean±S.D of duplicates values. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ indicate a significant difference to the control (B).

3.12 The effect of *M. balsamina* leaf acetone extract on the expression levels of angiogenic/metastasis growth factors involved in metastasis of MCF-7 cells.

Many different proteins have been identified as angiogenic activators and play an important role in neovascularisation. Human angiogenesis array kit was utilised to monitor the angiogenesis-related protein expression profiles. The expression of metastasis growth factors such as HGF, FGF-1 and IL-8 was down-regulated in the extract-treated groups as compared to the untreated control. Conversely, EGF expression showed to be up-regulated in cells treated with 100 µg/ml of the extract and Curcumin (Fig 3.12).

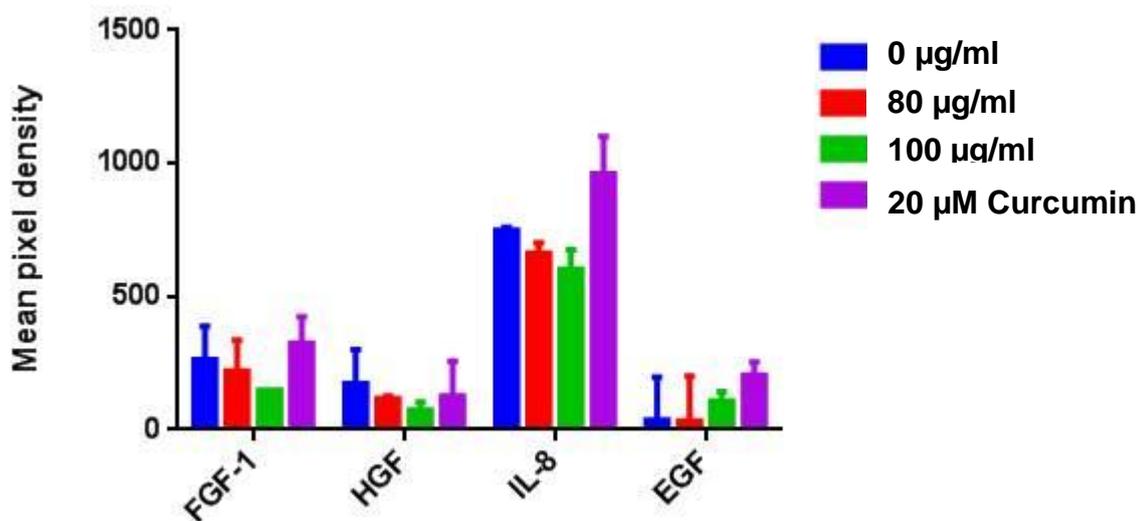


Figure 3.12: Effect of *M. balsamina* leaf acetone extract on the expression levels of angiogenic growth factors involved in proliferation and regulation of angiogenesis in tumour cells during metastasis. MCF-7 cancer cells were treated with (0, 80 and 100 µg/ml) of the extract and 20 µM of Curcumin for 24 h. Protein expression levels were determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised using the ChemiDoc XRS imager and the signal density of each spot representing the proteins of interest was determined using Quantity One software. Data shows the mean signal density of each representative pair of duplicate spots on the array and the mean±S.D of duplicates values. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 indicate a significant difference to the control.

3.13 The effect of *M. balsamina* leaf acetone extract on the expression levels of angiogenic and angiostatic factors

Up-regulation of the activity of angiogenic factors such as VEGF is in itself not enough to initiate blood vessel growth, and the functions of negative regulators or inhibitors of vessel growth may need to be down-regulated. There are many naturally occurring proteins that can inhibit angiogenesis, including angiostatin, endostatin, platelet factor 4 and thrombospondin. The expression levels of angiogenic and angiostatic factors in response to treatment with the extract were determined using the human angiogenesis array kit. Expression levels of the angiogenic factor, Angiogenin, VEGF, was decreased in extract-treated cells. VEGF was also seen to be down-regulated at 100 µg/ml of the extract. However, a significant increase in VEGF expression was observed in cells exposed to 80 µg/ml of the extract. Furthermore, the expression levels of angiostatic proteins such as Thrombospondin-2 and vasohibin were increased in the treatment groups as compared to the untreated cells. A significant increase in the expression of Vasohibin was observed in cells treated with 100 µg/ml of the extract and Curcumin (Fig 3.13).

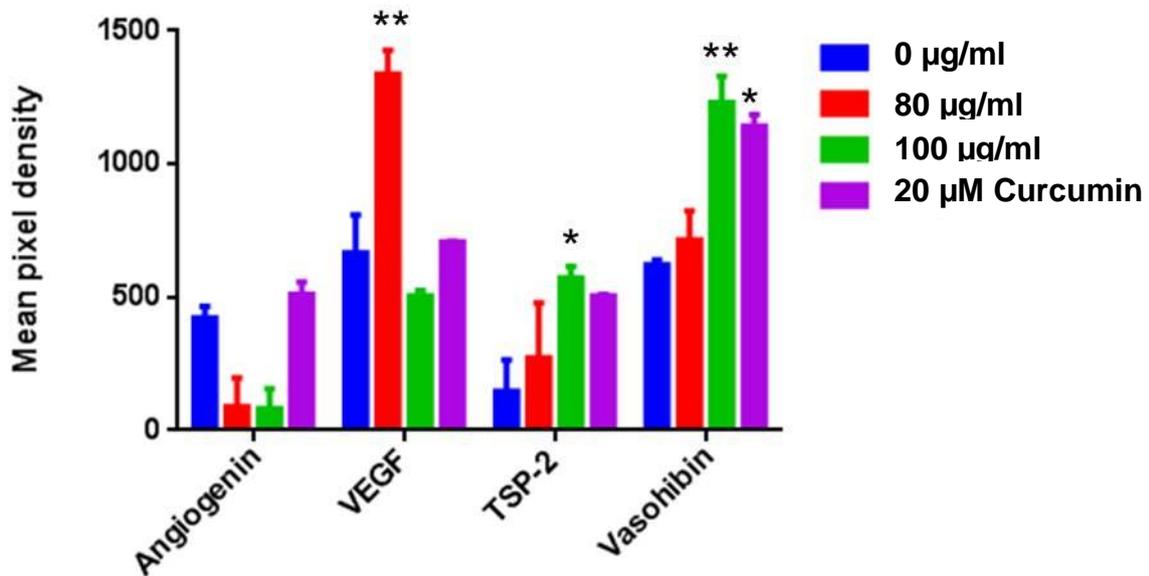


Figure 3.13: Effect of *M. balsamina* leaf acetone extract on expression levels of angiogenic and angiostatic factors involved in regulation of angiogenesis in tumour cells during metastasis. MCF-7 cancer cells were treated with (0, 80 and 100 µg/ml) of the extract and 20 µM of Curcumin for 24 h. Protein expression levels were determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised using the ChemiDoc XRS imager and the signal density of each spot representing the proteins of interest was determined using Quantity One software. Data shows the mean signal density of each representative pair of duplicate spots on the array and the mean±S.D of duplicates values. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 indicate a significant difference to the control.

CHAPTER 4

4 DISCUSSION

Cancer is a key health issue across the world, causing substantial patient morbidity and mortality. Patient prognosis is tightly linked with metastatic dissemination of the disease to distant sites, which accounts for a vast percentage of cancer patient mortality (Sporn, 1997; Talmadge and Fidler, 2010). Approximately 10 to 15% of breast cancer patients develop distant metastasis (Lee, 1983). Therefore, there is a need for a multipronged approach to prevent the primary tumour from spreading. Two essential properties of new anticancer drugs are to stop cancer cell proliferation by inducing apoptosis as well as inhibit the dissemination of tumour cells to other body organs. Plants have always served as a good source of medicinal remedies and are continuing to play a crucial role in the management of many health conditions. There are many existing natural products that have been reported to have potential anti-neoplastic and/or tumour prevention properties (Bhattaram et al., 2002). Although there has been considerable attention focused on identifying phytochemical compounds within *Momordica balsamina*, research on the effect of *M. balsamina* as an adjunct to cancer treatment is still very minimal. In this study, the leaf acetone extract of this plant was evaluated for its anticancer effect on breast MCF-7 cancer cells.

The extract was evaluated for its effect on the viability of MCF-7 cells. It was observed that the extract had significant cytotoxic activity on MCF-7 cells at concentrations between 100 - 200 µg/ml (Fig 3.1). Apoptosis is characterised by biochemical events that lead to changes in cell morphology which include membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation (Kerr et al., 1972). Apoptosis was investigated by examining the nuclear morphology of the cells by Hoechst 33342/PI staining. Our results as seen by staining of the nuclear DNA by PI and condensed chromatin in treated cells (Fig 3.2), suggest that the observed decrease in cell viability was associated with apoptosis. Based on these morphologic changes, the extract appeared to cause apoptosis of breast MCF-7 cancer cells.

To gain an insight in the molecular mechanism(s) involved in the *M. balsamina* extract-induced apoptosis in MCF-7 cells, the expression levels proteins that are critical in the intrinsic and extrinsic pathways of apoptosis was carried out using the human antibody apoptosis array kit. The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins, which governs the release of cytochrome c from the mitochondria (Cory and Adams, 2002; Kuwana et al., 2002). The Bcl-2 family comprises anti-apoptotic (Bcl-2, Bcl-X_L) and pro-apoptotic members (Bax and Bak). In response to stress activation, Bax forms a homodimer and releases cytochrome c from the mitochondria (Skulachev, 1998), which results in caspase-9 activation (Adams and Cory, 1998). In the extrinsic pathway, plasma membrane receptors are activated by pro-inflammatory ligands, including FAS, TNF- α and TRAIL. These ligands bind to death domain-containing receptors at the plasma membrane that become activated and recruit the adapter proteins FADD and TRADD to form a death-inducing signalling complex (DISC). DISC formation initiates the proteolytic cleavage of pro-caspases 8, 10 and 2, leading to the activation of caspases 7, 6 and 3 then cell death (Dorn et al., 2009; Konstantinidis et al., 2012).

We found that the expression of the pro-apoptotic proteins of the Bcl-2 family, Bax and Bad, were highly expressed after treatment with the extract (Fig 3.3). Changes in the expression of the Bcl-2 family proteins led to the overexpression of cytochrome c and SMAC/DIABLO, which resulted in the translocation of these proteins from the mitochondria to the cytosol. This in turn led to the activation of effector caspases such as cleavage pro-caspase 3 to cleaved caspase 3, which were up-regulated in treated cells as shown in Fig 3.4. These data suggests evidence that the intrinsic pathway is activated.

Overexpression of the anti-apoptotic proteins has been demonstrated to inhibit cell death induced by many stimuli, including growth factor deprivation, hypoxia and oxidative stress (Reed, 2008). However, in this study, anti-apoptotic proteins (Bcl-2 and Bcl-x) were found to be up-regulated (Fig 3.3). This could be due to the cancer cells defence mechanism to try and evade apoptosis. The intrinsic mitochondrial pathway is triggered in response to various forms of cellular stress which provokes the activation of one or more pro-apoptotic BH3-only proteins. BH3-only proteins act as sensors for various death stimuli and relieve the inhibitory action of anti-apoptotic Bcl-2 proteins. The activation of BH3-proteins results in the oligomerisation of Bax and Bak. There is evidence interaction of pro-apoptotic BH3-only protein with Bcl-2

allows Bax and Bak to fulfil their death-inducing function (Zong et al., 2001). In healthy cells, BH3-only proteins are kept inactive (Cory and Adams, 2002). In response to pro-apoptotic signals, they become transcriptionally up-regulated and/or posttranslationally modified (or relocalised) to gain their full pro-apoptotic potential (Oda et al., 2000; Yu et al., 2001). This could be the explanation of induction of apoptosis through the intrinsic pathway seen in this study, even though anti-apoptotic proteins were overexpressed. Increase in the expression of death receptors (TNFR1, TRAIL R1, TRAIL R2, Fas and FADD) (Fig 3.5) suggests the involvement of the extrinsic apoptotic pathway induced by the treatment of the extract.

Mitochondria release multiple pro-apoptotic molecules, such as SMAC/DIABLO; in addition to cytochrome c. SMAC/DIABLO binds to XIAP, preventing it from inhibiting caspases (Pradelli et al., 2010; Kaufmann et al., 2012). Both proteins can promote apoptosis by counteracting the inhibitor-of-apoptosis proteins (IAPs). It is assumed that inhibitors of apoptosis, such as XIAP, would be down-regulated if the cells were attempting to undergo apoptosis. However, in this study, an increase in the inhibitors of apoptosis proteins, (c-IAP1, c-IAP2, XIAP) was observed in cells treated with the extract (Fig 3.6). This increase might be as a result of a last ditch effort by the IAP proteins to protect the cells from death (Deveraux and Reed, 1999).

A major mechanism of p53 stabilisation and activation is triggered by DNA damage, which induces protein phosphorylation. DNA damage induces p53 phosphorylation of multiple serine (6, 9, 15, 20, 33, 37, 46, 315, 371, 376, 378, and 392) and three threonine residues (18, 55 and 81) at its N- and C- termini. Phosphorylation of the N-terminus of p53 affects its affinity for Mdm2 and subsequent degradation (Shieh et al., 1997; Giaccia and Kastan, 1998). Once activated via phosphorylation, p53 can trigger apoptosis by activating both the intrinsic and extrinsic pathway of apoptosis (Fridman & Lowe, 2003). In this study, phosphorylation of p53 at Serine 15, Serine 46 and Serine 392, was observed. This suggests that the extract-induced apoptosis is p53-dependent. In this study p53 could be directly inducing Bax transcription. Studies have shown that Bax induction by stress-activated p53 can overcome the anti-apoptotic effects of Bcl-2 thus promoting p53-dependent apoptosis. Another possibility that could explain what was seen in the study is the ability of p53 to bind to pro-apoptotic Bcl-2-family proteins, leading to permeabilisation of mitochondria and apoptosis (Tomita et al. 2006). p53 can also activate the extrinsic apoptotic pathway through the induction of genes encoding three transmembrane proteins: Fas, DR5

and PERP (Nagata and Golstein, 1995). In this study, p53 may be enhancing levels of Fas at the cell surface by promoting trafficking of the Fas receptor from the Golgi as shown in a study done by Bennett et al., (1998). Another possibility is the induction of DR5, the death-domain-containing receptor for TNF-related apoptosis-inducing ligand (TRAIL) by p53. DR5 is induced by p53 in response to DNA damage (Wu et al., 1997) and in turn promotes cell death through caspase-8 (Ashkenazi and Dixit, 1998).

Inhibition of metastasis offers another avenue for the treatment of cancer. In the metastatic process, primary tumour cells break away from their neighbouring cells and invade through the basement membrane. Subsequently, metastasising cells enter the circulation via lymphatic system and migrate to distant organs where they exit the vasculature. Eventually, tumour cells that successfully adapt to the new microenvironment proliferate from micrometastases into clinically detectable metastatic tumours (Fig 1.2) (Fidler, 2003; Geiger and Peeper, 2009).

Since, interruption of one or more of these steps is a potential approach for anti-metastatic therapy (Palmer et al., 2011). Our study investigated the potential anti-metastatic effect of the plant extract in MCF-7 cells by determining its effect on cell invasiveness, migration and adhesion. In investigating the effect of the extract on the invasiveness of MCF-7, the data (Fig. 3.8A & B) demonstrated a decrease in the invasiveness of MCF-7 cancer cells after 24 h of treatment. Tumour cell invasiveness is dependent on the ability of tumour cells to produce ECM-degrading enzymes such as MMPs and uPA. Matrix metalloproteinase-9 (MMP-9) is a member of matrix degrading enzymes involved in cancer development, invasiveness and metastasis. Urokinase plasminogen activator is an extracellular matrix-degrading protease involved in cancer invasiveness and metastasis, interacting with plasminogen activator inhibitor-1 (Duffy, 2004). In vitro evidence suggests that uPA can cleave proteins other than plasminogen, such as fibronectin, alpha6 integrin, hepatocyte growth factor (HGF), urokinase plasminogen activator receptor (uPAR), and uPA itself (Danø et al., 2005; Schmitt et al., 2010). The inhibition of uPA was reported to prevent metastasis, tumour growth and angiogenesis in different animal models (Kariko et al., 1993). Cancer patients have been reported to have higher circulating levels of TSP-1 compared with normal subjects (Tuszynski et al., 1992). Albo et al., (1999) showed that TSP-1 up-regulates the plasminogen/plasmin system and promotes breast tumour cell invasion. In this study it was observed that the extract, at

100 µg/ml, down-regulated the protein expression levels of TSP-1, uPA and MMP-9 at (Fig. 3.8C).

Matrix metalloproteinase-8 (MMP-8), also known as collagenase-2 or neutrophil collagenase, is reported to function in the recovery processes and tumour suppression. Matrix metalloproteinase-8 is reported to exert anti-metastatic effects by decreasing tumour cell invasiveness and migration by enhancing their adhesion to the ECM. Additionally, MMP-8 expression in breast cancer is an indicator of reduced risk of axillary lymph node metastasis (Gutierrez-Fernandez, 2008). Our results demonstrated an increase in MMP-8 expression in MCF-7 treated with the extract (Fig. 3.8C). This suggests that in addition to down-regulating the expression of metastasis-promoting proteins such as MMP-9 and uPA, the extract impede MCF-7 cell invasiveness through the up-regulation of MMP-8.

Although there are several mechanisms that regulate MMP expression, the ultimate control is achieved through their interaction with the TIMPs. This leads to the inhibition cancer cell invasiveness (Page-McCaw et al., 2007). Another protein known to play a role in the inhibition of cancer cell invasiveness is Serpin-E1 (Plasminogen Activator Inhibitor-1). Possible mechanisms by which Serpin-E1 prevent cancer cell dissemination include prevention of excessive degradation of the extracellular matrix, modulation of cell adhesion, angiogenesis and cell proliferation (Czekay et al., 2003; Roca et al., 2003). Serpin E1 inhibits uPA, which is an enzyme responsible for the cleavage of plasminogen to form plasmin. Plasmin mediates the degradation of the extracellular matrix either by itself or in conjunction with matrix metalloproteinases (Carter and Church, 2009). The protein expression levels of Serpin E1, TIMP-1 and TIMP-4 were seen to be up-regulated after treatment with the extract (Fig 3.9). These results suggest that the inhibition of MCF-7 cell invasiveness by the extract is through the down-regulation of MMP-9, TSP-1 and uPA mediated by their inhibitors, such as TIMP-1, TIMP-4 and Serpin E1.

The formation of secondary tumours at distant sites is dependent on the migratory ability of tumour cells. The migration of the cells, following local tumour invasion and degradation of the ECM, to the vessels is facilitated by several proteins. These include chemokines, such as CCL21, which is secreted in order to attract tumour cells to lymphatic vessels through (Alitalo et al., 2005; Molloy and van' Veer, 2008). CXCL16, a trans-membranous CXCL16 chemokine reduces proliferation while

soluble CXCL16 chemokine enhances proliferation and migration (Abel et al., 2004). Insulin-like growth factor binding proteins (IGFBPs) are circulating transport proteins for (Insulin-like growth factor) IGF-I and IGF-II. IGFBPs also interact with many other molecules, which not only influence their modulation of IGF action but also mediate IGF-independent activities that regulate processes such as cell migration. IGFBP-3 is the predominant IGFBP in circulation (Firth and Baxter, 2002; LeRoith and Roberts, 2003). IGFBP-1 binds to cell surfaces integrin receptors and increase cell migration (Jones et al., 1993). Results in a study done by Torng et al., (2008) showed evidence that IGFBP-3 plays a direct role in the regulation of endometrioid carcinoma cell migration, invasion and metastasis. Our results (Fig 3.11) indicated that the extract inhibited the ability of MCF-7 cells to migrate. This might be attributed to the down-regulation of motility factors that stimulate migration of tumour cells during metastasis such as IGFBP-1, IGFBP-3 and CXCL16.

The adhesion of escaped cancer cells from primary cancer mass to remote tissues and organs constitutes another critical event in cancer metastasis. Cell adhesion is crucial in the establishment of a secondary cancer mass at the distant organ (Divoli et al., 2011). Another approach in trying to impede metastasis would be preventing cells from detaching from the primary tumour or inhibiting them from attaching at the secondary site. If cancer cells fail to detach, they will not be able to invade the basement membrane and enter the circulatory system. Adhesion of cancer cells to the secondary site allows cells to divide and form secondary tumours (Divoli et al., 2011). Observations in this study showed a reduction in the number of cells that were able to attach in the treated groups compared to the untreated control (Fig. 3.10A & B).

For a secondary tumour to develop and grow, new blood vessels must be formed via vasculogenesis and angiogenesis. Tumour growth and progression require angiogenesis because in its absence tumour growth is restricted to a few millimeters in diameter due to the physical constraint set by simple diffusion of nutrients and oxygen. In addition, angiogenesis and vascularisation allows metastatic tumour cells escape into the circulation and lodge in other organs (Carmeliet and Jain, 2000; Folkman, 2002). The process of angiogenesis is regulated by a balance between pro- and anti-angiogenic molecules and is usually derailed in cancer (Folkman, 2002). Among several angiogenic factors, vascular endothelial growth factor (VEGF), has been reported to be a critical mediator of angiogenesis and is usually up-

regulated in cancer (Ferrara, 2002). Another pro-angiogenic factor, IL-8, is reported to recruit immune cells to cancer tissues and form an inflammatory microenvironment that promotes cancer progression (McLean et al., 2011). In this study, angiogenesis growth factors such as HGF, FGF-1, and IL-8 were seen to be down-regulated in MCF-7 cells treated with the extract. On the contrary, EGF which functions to block angiogenesis growth signals showed to be up-regulated (Fig 3.12). Expression of major pro-angiogenic factors such as Angiogenin, VEGF, was decreased while the angiostatic proteins such as TSP-2 and Vasohibin were increased in the treatment group as compared to the control (Fig 3.13). The blocking of pro- angiogenic proteins expression in tumour cells can thus lead to inhibition of neovessel formation in tumours. These data suggests that the extract should be able to block angiogenesis through up-regulation of the activity of inhibitors of vessel growth proteins and down-regulation of angiogenic factors that promote the development of new blood vessels.

Conclusion: The results of this study have showed anti-cancer effects of *M. balsamina* leaf acetone extract in breast MCF-7 cancer cells through induction of apoptosis and inhibition of metastasis. The study has also identified multiple biological pathways as potential targets of the anti-tumour activities of the extract. It will be of interest in the future to identify the active chemical compounds in the extract that target these pathways. It would also be important to determine whether the anti-metastatic and pro-apoptotic activities of this extract can be attributed to active chemical compounds identified from the extract. The extract also showed to be more effective than both positive controls (Curcumin and Taxol) used in this study.

CHAPTER 5

5 REFERENCES

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