Determination of the molecular mechanism(s) involved in the pro-apoptotic activity of *Momordica balsamina* acetone extract in lung A549 cancer cells

by

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DISSERTATION

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DECLARATION

I Maedza Mudalahothe declare that the dissertation titled “Determination of the molecular mechanism(s) involved in pro-apoptotic activity of *Momordica balsamina* acetone extract in lung A549 cancer cells” is my own work in design and execution. It has not been submitted previously to this or any University to obtain an academic qualification. All the sources I have quoted or used have been properly acknowledged.

Mudalahothe M (Miss) .....

Surname, Initials (title) **Date**

DEDICATION

I would like to dedicate this work to God almighty for his endless mercies and my family for their constant support.

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LIST OF CONFERENCES AND PRESENTATIONS

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DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle's medium
RPMI	Roswell park memorial institute medium
CO_2	Carbon dioxide
AO	Acridine orange
EB	ethidium bromide
FBS	Foetal Bovine Serum
PBS	Phosphate saline buffer
Pen/strep	Penicillin/streptomycin
7-AAD	7-aminoactinomycin D
PI	Propidium iodide
Bax	Bcl-2-associated X protein
BH	Bcl-2 homology domain
Bid	BH3 interacting-domain
BIM	Bcl-2 intercalating mediator
PI3k/Akt	Phosphoinositide 3-kinase/protein kinase B
Caspases	Cysteine-aspartic proteases
CARD	Caspase recruitment domain
CDKs	Cyclin-dependent kinases
TNF	Tumour Necrosis Factor
TNF-α	Tumour Necrosis Factor-α
TRAF1/2	TNF-receptor-associated factor 1/2

TNFRSF	TNF-Receptor Superfamily
TRAIL-R1/2	TRAIL-receptor 1/2
Fas	First apoptosis signal
FasR	Fas receptor
FasL	Fas ligand
TRAIL	TNF-related apoptosis inducing ligand
sTRAIL	soluble TRAIL
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signal complex
DNA	Deoxyribonucleic acid
OMM	Outer Mitochondrial Membrane
MOMP	Mitochondrial outer membrane permeabilisation
PVDF	Polyvinylidene difluoride
SAC	Spindle assembly checkpoints
AIF	Apoptosis inducing factor
DDR	DNA damage response
DR	Death receptor
Apaf-1	Apoptosis activating factor-1
EndoG	endonuclease G
FADD	Fas-associated death domain
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related

MDM2	Murine double minute 2
P21	protein p21
P53	protein 53
pRb	Retinoblastoma
INK4	Inhibitors of CDK4
CIP/KIP	CDK Interacting protein/Kinase Inhibitory Protein
PUMA	p53 upregulated modulator of apoptosis
RNA	Ribonucleic acid
IAPs	Inhibitors of Apoptosis Proteins
cIAP1/2	cellular Inhibitors of Apoptosis Proteins 1/2
FLICE	FADD-like IL-1b-converting enzyme
cFLIP	cellular FLICE inhibitory protein
XIAP	X-linked Inhibitors of Apoptosis Proteins
RING	really interesting gene
BIR	Baculovirus Inhibitor of Apoptosis repeats

ABSTRACT

Plant-derived products have been used for years in the treatment of various ailments with low or no side effects. Thus, screening of medicinal plants for potential anticancer activity, *in vitro*, could help identify plant extracts or compounds that can be developed for use as anticancer agents with less or no side effects. The aim of this study was to investigate the probable anticancer effects and induced mechanism of action of *Momordica balsamina* crude leaf acetone extract in lung A549 cancer cells. The effect of the extract on cell viability, proliferation and cell division cycle were determined using Muse count & viability, Ki67 proliferation and cell cycle assay kits, respectively. The presence of biochemical and morphological features associated with apoptosis were analysed by Muse annexin-V & dead cell assay kit and Acridine orange/Ethidium bromide dual staining. The effect of the extract on the mRNA expression levels of cell cycle regulatory genes was determined using RT-PCR. Proteome profiler antibody array was used to determine the effect of the extract on the protein expression levels of apoptosis regulatory genes. The findings revealed that the crude leaf acetone extract of *M. balsamina* decreased the percentage viability of lung A549 cells with less effect on the percentage viability of normal cells (KMST-6). Furthermore, a significant anti-proliferative effect in extract-treated A549 cells was observed. Characteristic nuclear and morphological features of apoptosis such as chromatin and nuclear condensation, externalisation of phosphatidylserine and loss of cell membrane function were observed in A549 cells treated with the extract. Although there was no relative upregulation of Bax and Bad protein expression, a downregulation of the Bcl-xl and Bcl-2 protein expression was observed in extract-treated cells. This led to the release of Cytochrome c and HTRA2/Omi leading to pro-caspase-3 cleavage. Furthermore, presence of HTRA2/Omi in the cytosol inhibited the functions of IAPs such as XIAP and cIAP1/2. Phosphorylation of p53 at different serine residues led to upregulated protein expression levels of p27/Kip1 protein which resulted in the cell division cycle arrest at G0/G1-phase. Reverse transcriptase polymerase chain reaction results showed that the extract modulated mRNA expression levels of *p53*, *p21*, *cyclin B* and *cdc2* genes. In summary, *M. balsamina* extract induced cell division cycle arrest and apoptosis in A549 cells through intrinsic apoptosis pathway via p53-mediated mechanism.

CHAPTER 1

1. Introduction

Cancer is a group of diseases characterised by the uncontrolled proliferation of cells and their dissemination to other body tissues through blood and lymphatic vascular systems (Ferreira *et al.*, 2014). It is a worldwide health concern and one of the leading cause of death with an estimated 11.5 million victims by 2030 (Kuete *et al.*, 2016). According to the National Cancer Registry (NCR), more than 100,000 South Africans are annually diagnosed with cancer (Van-Vuuren *et al.*, 2015). Lung cancer is reported to be the most commonly diagnosed cancer type and a leading cause of cancer-related deaths in South African males and the second leading cause of cancer-related deaths in females (Moodley *et al.*, 2016, Xin *et al.*, 2016). Between the two known lung cancer types, Non-Small Cell lung cancer (NSCLC) dominates over the Small-Cell lung cancer (SCLC) type due to its insensitivity towards current treatments (Nie *et al.*, 2015, Wu *et al.*, 2016).

Early-stage of NSCLC can be managed with the use of surgery, however, 30% to 55% of patients still develop recurrence and die (Uramoto and Tanaka, 2014). Furthermore, surgery itself possesses a certain amount of risk including a high rate of infections and respiratory complications (Moyer, 2014, Yu *et al.*, 2014). On the other hand, radiation therapy has been reported to ease the disease than surgery. Despite this, radiation is not suitable for treating late stage/widespread cancer thus, it is used in combination with other treatments options resulting in high cost of treatment (Nagata *et al.*, 2015). Chemotherapy is associated with the development of primary and secondary multidrug/chemo-resistance by tumour cells which further undermine effectiveness of the treatment (Kaur *et al.*, 2016, Ren *et al.*, 2016). Furthermore, most anticancer treatment strategies are not site-specific and thus become toxic to normal cells causing serious side-effects and exhibit high toxicity to benefit ratios (Shah *et al.*, 2016). It is estimated that more than 70% of the world's population cannot afford modern cancer treatment strategies particularly in developing countries including South Africa (Tavakoli *et al.*, 2012).

Plant-derived medicines have been in use for centuries for the treatment of various ailments (Jiang *et al.*, 2016a). To date several plant species such as *Cannabis sativa*

(Cannabinoid), *Curcuma longa* (Curcumin), *Combretum caffrum* (Combretastatins) and *C. roseus* (Vindesine) have been shown to suppress the progression and development of tumours in cancer patients (Iqbal *et al.*, 2017, Umadevi *et al.*, 2013). Many phytochemicals have been identified as active constituents in plant species used for medicinal purposes (Koduru *et al.*, 2007, Plackal *et al.*, 2015). These include polyphenols (e.g. resveratrol, gallocatechins) and flavonoids (e.g. methoxy licoflavanone, alpinumisoflavone) (Wen *et al.*, 2014). Phytochemicals have been reported to exert anti-tumour effects via distinct mechanisms; they selectively kill rapidly dividing cells, target abnormally expressed molecular factors, reduce oxidative stress, modulate cell growth factors, inhibit angiogenesis of cancerous tissue and induce apoptosis (Heo *et al.*, 2014). Plants are seen as a potential alternative source for anticancer drug discovery and development since they are easily available, evident to be efficient (Patil *et al.*, 2013) and rarely have severe side effects (Agyare *et al.*, 2018, Biswas *et al.*, 2015). Thus, screening of plant derived extracts and/or compounds could lead to the identification of anticancer drugs that are more effective, selective and that will produce lesser systemic toxicity, encourage appropriate cell death and not generate resistance (Sreekala *et al.*, 2017).

1.1 *Momordica balsamina*

Balsam apple, African cucumber or Southern balsam pear botanically known as *Momordica balsamina*, is a curious, tendril-bearing high-climbing annual vine native to the tropical regions of Africa (Shai *et al.*, 2010). *Momordica balsamina* is characterised by pale yellow, deeply veined flowers and its round, bright orange fruits (figure 1). When ripe, the fruits burst apart, revealing numerous seeds covered with a brilliant scarlet and extremely sticky coating. This plant belongs to the Cucurbitaceae family which consist of about 110 genera and 640 species found abundantly in tropics and subtropics (Behera *et al.*, 2011, Dey and De, 2015, Diaz, 2016).

Figure 1: *Momordica balsamina* plant

Several species of *Momordica* including *Momordica foetida, Momordica dioica*., *Momordica cochinchinensis* Spreng., and *Momordica charantia* were reported to contain potential antioxidant, anticancer and anti-inflammatory activities (Bharathi *et al.*, 2014, Kubola and Siriamornpun, 2011, Nagarani *et al.*, 2014, Raina *et al.*, 2016, Thiruvengadam *et al.*, 2016). Like other *Momordica* species, the leaves, seeds, bark and fruits of *M. balsamina* contain several bioactive compounds, such as alkaloids, phenolics, saponins, flavonoids, glycosides and terpenes (Ramalhete *et al.*, 2016). These compounds contribute toward the medicinal and nutritional properties of the plant (Ajji *et al.*, 2017). Including anti-plasmodial, shigellocidal, anti-diarrheal, anti-bacterial, anti-viral (Kushwaha *et al.*, 2012), anti-inflammatory, anti-microbial, anti-oxidant, analgesic and hepato-protective properties (Rathee and Kamboj, 2017). Furthermore, the genera and species of the Cucurbitaceae family are reported to contain cucurbitacin (a class of a highly oxidised tetracyclic triterpenoids). A variety of cucurbitane-type triterpenoids were reported to possess a wide range of potent biological activities (Madala *et al.*, 2016). In addition, natural and semisynthetic cucurbitacins have been reported to show promising anticancer activities including

anti-proliferation and cell division cycle arrest leading to the induction of apoptosis (Alghasham, 2013).

Although the medicinal and nutraceutical properties of *M. balsamina* is advocated in health care management, it has not received major international attention (Ajji *et al.*, 2017). A recent study from our laboratory showed that *M. balsamina* crude acetone leaf extract has anti-metastatic and pro-apoptotic effects in breast MCF-7 cancer cells (Boshielo, 2016). Given the differences in the sensitivity of different cancer cell lines towards treatment; the current study was therefore undertaken to study the probable anticancer effects and induced mechanism of action of *M. balsamina* crude leaf acetone extract in lung A549 cancer cells. An ideal anticancer agent should be selective and produce no or less systemic toxicity, encourage programmed cell death known as apoptosis.

1.2 Apoptosis

Apoptosis, also known as programmed cell death, is a precise physiological process controlled by a network of genes resulting in the removal of undesirable, old and damaged cells in multicellular organisms (Yee *et al.*, 2014). It is regarded as an ideal mode of cell death as it does not elicit damage to surrounding tissues due to phagocytosis by macrophages (Fallahian *et al.*, 2017). Several morphological and biochemical features can be used to distinguish apoptosis from other modes of cell death (Tait *et al.*, 2014). Morphological features include: cell shrinkage, loss of cell membrane function, formation of membrane blebs, chromatin condensation and apoptotic bodies (Nagappan *et al.*, 2016). Biochemical features of apoptosis include: chromosomal DNA cleavage into inter-nucleosomal fragments, phosphatidylserine externalisation and activation of caspases cascade (Byrd, 2016, Radogna *et al.*, 2015). Activation of caspases result in the cleavage of key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes (Li and Dewson, 2015).

1.2.1 Caspases

Caspases are a family of cysteinyl aspartate-specific proteases traditionally classified as inflammatory or apoptotic (Rodriguez-Berriguete *et al.*, 2015, Soriano and Scorrano, 2011). Inflammatory caspases (caspase-1, -4, -5 and -12 in humans)

mediate innate immune responses by cleaving precursors of pro-inflammatory cytokines such as IL-1β and IL-18, thereby facilitating their secretion. The apoptotic caspases (caspase-3, -6, -7, -8, -9 and -10) play a role in the initiation and execution of programmed cell death (White *et al.*, 2014). Caspases are ubiquitously expressed as inactive precursors (zymogens) with little or no protease activity which comprise four distinct domains; an amino-terminal domain of variable size (termed N-terminal polypeptide or pro-domain), a large and small subunit together with a linker region between the large and small domains flanked by Asp residues catalysis (McIlwain *et al.*, 2013, Rongvaux *et al.*, 2014). The mammalian cell death proteases are classified as either initiator or effector caspases based on their sites of action during the proteolytic caspase cascade with different pro-domains and thus, orchestrating the apoptotic cell death programme (Bouchier-Hayes and Green, 2012).

Initiator caspases have long pro-domains containing structurally related protein modules that physically link these proteases to their specific activators (Zamaraev *et al.*, 2015). Two types of interaction modules in the pro-domains of initiator caspases-8, -9 and -10 include death effector domain (DED) or caspase recruitment domain (CARD). These pro-domains contain specific protein–protein interaction sites that are crucial for initiator caspase activation (Dickens *et al.*, 2012). For instance, the pro-domains of caspase-9 contain a CARD motif, whereas the pro-domains of caspases-8 and -10 contain a pair of DED motifs. Through these motifs, the initiator caspases are recruited and activated at multi-protein platforms specific for the respective initiator caspases (Shrivastava *et al.*, 2015).

Domains such as DEDs and CARDs in certain pro-caspases physically connect the initiator caspases with critical regulatory molecules via homophilic interactions (Pennarun *et al.*, 2010). Initiator caspases have substrate specificities that are similar to caspase recognition sites present in their own sequence, implying that these caspases can utilise autocatalysis for activation. Moreover, optimal caspase recognition sites for initiator caspases are present in the sequences of several effector pro-enzymes with short pro-domains including pro-caspase-3 and -7, suggesting that these enzymes act downstream of initiator caspases in the proteolytic cascade activation (Favaloro *et al.*, 2012).

Activation of each caspase is induced by proteolytic cleavage between domains, resulting in the removal of the pro-domain and linker region. This leads to the assembly of the large (20 kDa) α subunit and small (10–12 kDa) β subunit into an active enzyme complex (Wurstle *et al.*, 2012). Activation of caspases does not always lead to the morphological features associated with apoptosis. This is because of the abundant expression of inhibitor of apoptosis proteins (IAPs) (Osterman *et al.*, 2016). Some of the IAPs include, cellular IAP1/2 (cIAP1/2), cellular FADD-like IL-1b-converting enzyme (FLICE)-inhibitory protein (cFLIP), X-linked IAP (XIAP), surviving, E3 ligases, etc. (Bornstein *et al.*, 2012). These proteins were originally found in baculovirus. They contain a baculovirus inhibitor of apoptosis repeats (BIR) domain, a CARD (Palacios-Rodriguez *et al.*, 2011), and a really interesting new gene domain (RING) (Chaudhary *et al.*, 2016). These proteins suppress apoptosis by interacting with and inhibiting the enzymatic activity of both initiator and effector caspases (Latour and Aguilar, 2015). cellular FLIP shares substantial structural similarities with initiator caspase-8 and -10 and inhibits apoptosis by competing with initiator caspases-8 and -10 for binding to the death-inducing signalling complex (DISC) (Vaculova *et al.*, 2010).

Other mechanisms for the negative regulation of caspase-8 involve the induction of survival signalling pathways that later inhibit caspase-8 activation (Pobezinskaya and Liu, 2012). The cIAP1/2s also contains a baculovirus IAP repeat (BIRD), a CARD and RING E3 ligases and they help to recruit tumour necrosis factor (TNF) receptor-associated factor (TRAF1/2) and inhibit TNF-α apoptotic signalling (Obexer and Ausserlechner, 2014). Although cIAP1/2 are inefficient caspase-8 inhibitors, they execute their inhibitory potential not through direct enzyme inhibition (Pedersen *et al.*, 2014), but, by inducing pro-survival signals (while antagonising the cell death inducing potential of TNF) such as the NF-κB pathway activation downstream of TNFR1 ligation (Ebert *et al.*, 2015, Pedersen *et al.*, 2014). The XIAPs are also regulators of TNFR1 signalling (Moulin *et al.*, 2012), but mainly it directly inhibits the activation of pro-caspase-9 (Latour and Aguilar, 2015). The BIR domains of XIAPs bind the active site of caspases'-3 and -7 active sites and inhibit proteolytic function (Li *et al.*, 2017).

The caspase inhibitory activity of IAPs can be regulated by several mitochondrial molecules such as second mitochondria-derived activator of caspases (SMAC)/direct

inhibitor of (IAP)-binding protein with low pI(Diablo), high-temperature requirement (HtrA)-serine peptidase 2 (HTRA2)/Omi, etc. that are released together with Cytochrome c (Marino *et al.*, 2014). After the release from the inter-membrane space of mitochondria in response to apoptotic signals, they bind via their cleaved N-termini to several IAPs (Garrison *et al.*, 2011, Hui *et al.*, 2011). The SMAC/Diablo in the cytosol binds to cIAP1/2, but mainly to XIAP and neutralises their anti-apoptotic effect by facilitating their degradation by proteasomes (Hamacher-Brady and Brady, 2015, Sensintaffar *et al.*, 2010). However, unlike Cytochrome c, losing SMAC/Diablo, HTRA2/Omi or both proteins do not result in the inability to activate caspases or undergo apoptosis given that other proteins have also been shown to inhibit XIAP (Koff *et al.*, 2015).

1.2.2 Extrinsic pathway

The extrinsic pathway of apoptosis is induced by an instructive process mediated by cell surface death receptors (DR) that transmit apoptotic signals when bound by their cognate death ligands (Ashkenazi, 2015). These DRs, such as DR-3, 4 and 5, First apoptosis signal (Fas) receptor (FasR), TNF-α, are characterised by cysteine-rich extracellular domains required for ligand binding (Lee *et al.*, 2014) and belongs to the TNF-receptor superfamily (TNFRSF). Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligands (Fas-L) are the most studied death ligands that are known to induce apoptosis through binding to their cognate death receptors (Kumazaki *et al.*, 2014, Merkis *et al.*, 2010).

In response to a death inducing stimuli, Fas-L binds to the FasR leading to the trimerisation of its death domain. This leads to the recruitment and binding of the death domain of the adaptor protein, Fas association death domain (FADD), which in turn recruits pro-caspases-8 or -10 forming the DISC (Verbrugge *et al.*, 2010) (figure 2a). The Fas/FasL system has been considered an important apoptosis signal transduction pathway (Hao and Mak, 2010). However, TRAIL, also known as Apo-2 ligand (Apo-2L), have the capability to induce apoptosis in a wide variety of tumour cells while sparing vital normal cells by cross-linking TRAIL-Receptor (TRAIL-R) 1/2, also known as DR4/5 (Fulda, 2015). There are other trans-membrane receptors which TRAIL can bind: TRAIL-R3 and TRAIL-R4 but, they lack the functional cytoplasmic death domain that is required for apoptosis induction resulting in only TRAIL-R1/DR4 and TRAIL-R2/DR5 triggering apoptosis (Lafont *et al.*, 2017).

The trans-membrane receptors including TRAIL-R1/DR4 and TRAIL-R2/DR5 bear high structural similarity and both can trigger apoptosis upon TRAIL-induced cross-linking, furthermore, functional differences between them have been reported (Khan *et al.*, 2014). The TRAIL-R2/DR5 has higher affinity for TRAIL than TRAIL-R1/DR4 however, this higher affinity was found to not necessarily result in enhanced DISC activation as TRAIL-R2/DR5 can be engaged by the soluble ligand which only triggers a comparably weak DISC formation. Supporting the notion that TRAIL-R2/DR5 may require further cross-linking of soluble TRAIL (s-TRAIL), whereas stimulation of TRAIL-R1/DR4 by s-TRAIL appears to be able to trigger apoptosis independently of further crosslinking (Ding *et al.*, 2012).

It is known that the expression of TRAIL-R1/DR4 and/or TRAIL-R2/DR5, together with their adaptor protein TRADD is essential for induction of TRAIL-induced apoptosis (Hartwig *et al.*, 2017). Upon the binding of TRAIL to the TRAIL-death receptors, receptor trimerization occurs, which leads to clustering of their death domains and formation of the DISC comprising of caspases-8 and -10. While caspase-8 is essential, caspase -10 is not required for apoptosis induction by TRAIL. DISC formation results in the activation of caspase-8 through auto-cleavage (Pobezinskaya and Liu, 2012, Wu *et al.*, 2016).

Strong caspase-8/-10 activation induces apoptosis directly by cleaving downstream effector caspases such as pro-caspases-3, -6 and/or -7 (Jin and El-Deiry, 2006). On the other hand, weak caspase-8/-10 activation initiates a mitochondrial amplication loop by cleaving the B-cell lymphoma-2 (Bcl-2) Homology (BH)-3-only protein, BH3 interacting-domain death agonist (Bid), to form truncated Bid (t-Bid). Truncated Bid then translocate to the outer mitochondrial membrane (OMM), leading to the shift in the balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family leading to the induction of the mitochondrial apoptosis pathway (Kantari and Walczak, 2011) Figure 2b.

1.2.3 Intrinsic pathway

The intrinsic or mitochondrial pathway of apoptosis, is activated from within the cell by growth factor withdrawal or DNA damage (Huang and Freter, 2015). The damage triggers the mitochondrial outer membrane permeabilisation (MOMP) and release of mitochondrial proteins (Estaquier *et al.*, 2012). These include Cytochrome c,

apoptosis inducing factor (AIF), endonuclease G (endoG) and SMAC/Diablo (Portt *et al.*, 2011). Once in the cytosol, Cytochrome c forms a complex (which is known as the apoptosome), with apoptosis activating factor-1 (Apaf-1), ATP and pro-caspase-9 (Bratton and Salvesen, 2010, Parrish *et al.*, 2013). This results in the auto-activation of caspase-9, which in turn activates effector caspases-3, -6 or -7 (figure 2b). The SMAC/Diablo protein binds to the IAP preventing them from exerting their anti-apoptotic properties through the inhibition of caspases (Edison *et al.*, 2012, Orrenius *et al.*, 2015). The release of mitochondrial proteins into the cytosol is regulated by the Bcl-2 family of proteins (Chipuk *et al.*, 2010) (figure 2d).

Members of the Bcl-2 protein family are characterised by the presence of one or more BH-only domains. These proteins are classified into anti-apoptotic family members such as Bcl-2, Bcl-2-related protein long form of Bcl-x (Bcl-xl), Bcl-2-like 2 protein (Bcl-w), Myeloid cell leukaemia sequence 1 (Mcl1), etc. The pro-apoptotic effector proteins such as Bcl-2 antagonist killer 1 (Bak), Bcl-2-associated x protein (Bax), BH3-interacting domain (Bid), and Bcl-2-related ovarian killer protein (Bok) which adopt similar globular structure consisting of a helical bundle surrounding a central hydrophobic core helix (α5) (Sharifi *et al.*, 2014). The functions of the various domains that categorise Bcl-2 family of proteins in several groups have been elucidated. The anti-apoptotic proteins contain all four Bcl-2 homology domains (BH1-4) (Morrill and He, 2017). The proteins in this class prevent apoptosis by binding and sequestering their pro-apoptotic counterparts. The second class of pro-apoptotic proteins contain BH1-3 domains. The two proteins Bax and Bak when activated, oligomerise and directly cause MOMP, a critical event during apoptosis (Grobe *et al.*, 2016).

The pro-apoptotic Bcl-2 members are further divided into effector and the BH3-only proteins which are then subdivided into activators and depressors (Llambi *et al.*, 2016) (figure 2c). The activator BH3-only proteins including Bim, tBid are capable of directly interacting with the effector proteins and promoting their oligomerisation leading to MOMP and Cytochrome c release into the cytosol (Schellenberg *et al.*, 2013). On the other hand, depressor BH3-only proteins do not activate Bax/Bak directly, instead they neutralise anti-apoptotic proteins. If a direct activator-Bid is bound by an anti-apoptotic-Bcl-xl, then a subsequent depressor BH3-only protein-

Bad releases the direct activator to promote MOMP (Chipuk and Green, 2008). Thereafter, released Bid will then interact directly with Bax, followed by Bax oligomerisation (Garcia-Saez, 2012). Other depressor proteins such as Bcl-2-interacting killer (Bik) and p53-upregulated modulator of apoptosis (Puma) also function predominantly by binding to the anti-apoptotic proteins such as Bcl-2 and Bcl-xl (Cosentino and Garcia-Saez, 2017, Le-Pen *et al.*, 2016).

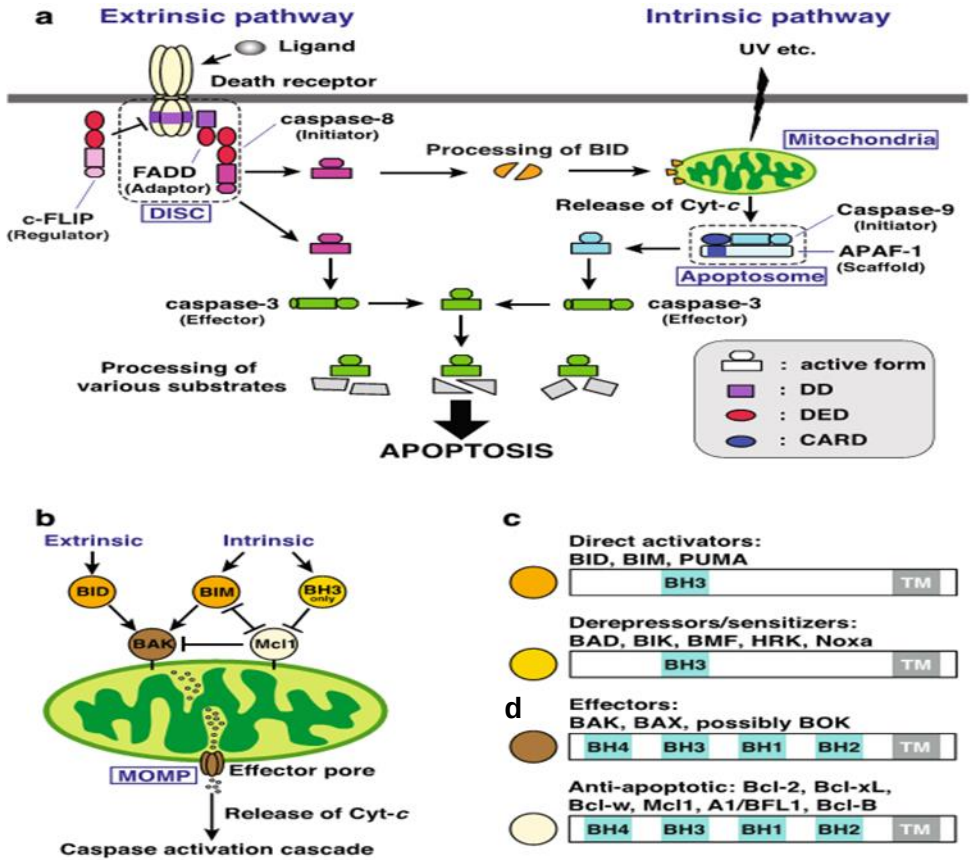

Figure 2: Extrinsic and intrinsic apoptosis pathways **(a)**. The extrinsic pathway is initiated via stimulation of DRs such as Fas or TNFR1 on the cell surface. Stimulation of DRs results in the formation of the DISC through the recruitment and binding of FADD or TRADD and pro-caspase-8. The formation of the DISC leads to the activation of caspase-8 which cleaves and activates caspase-3 and/or Bid to tBid. The tBid translocate to the mitochondria, triggering MOMP and the release of cytochrome c resulting in the activation of caspase-9 through the apoptosome. Caspase-9 then cleaves and activate pro-caspase-3 which then cleaves various cellular components **(b)**. Also, formation of MOMP can be initiated by intracellular death-signals resulting in modulation of the pro- and anti-apoptosis members of the Bcl-2 family of proteins **(c and d)** (Moya *et al.*, 2016).

The Bcl-2 family of proteins can also be regulated by *p53* gene *via* a homo- and/or hetero-dimerisation between each other. The induction of cell death by *p53* gene occurs via regulation of gene expression and by direct action at the mitochondria (Kim *et al.*, 2017). Tumour suppressor protein p53, is a nuclear phosphoprotein that is encoded by the *TP53* gene (Sheikh *et al.*, 2017). *p53* has 3 main domains; a DNA binding domain for binding to other genes, an N-terminal trans-activational domain that forms binding sites for positive or negative regulators and lastly, a C-terminal oligomerisation domain which then undergoes posttranslational modifications and alternative splicing (Mollereau and Ma, 2014, Pflaum *et al.*, 2014).

Under normal conditions, p53 protein is maintained at low levels by the E3 ubiquitin ligase Mouse Double Minute 2 (MDM2). This ligase negatively regulates both p53 stability and activity. The N-terminal domain of MDM2 can directly bind to the N-terminal domain of p53 protein promoting p53's translocation from the nucleus to the cytoplasm. Thus, suppressing its capacity to interact with transcriptional machinery and blocking transcription of target promoters (Nguyen *et al.*, 2017). Following stress, p53 protein becomes stabilised and then become activated through extensive posttranslational modification including: phosphorylation, acetylation, methylation, ubiquitination, neddylation, sumoylation, poly ADP-ribosylation, nitration and addition of *N* -acetylglucosamine (Yakovlev *et al.*, 2010).

These modifications involved affect the stability and activity of p53 protein, either positively or negatively (Terakawa *et al.*, 2012), for example, phosphorylation enhance intrinsic activity of p53 masking it from ubiquitination due to the changes in its electrical properties and conformational dynamics. Translocation of p53 protein from the cell’s nucleus to the mitochondria binds it to the pro-survival members of Bcl-2 family of protein, liberating the multi-domain pro-apoptotic members that then trigger the mitochondrial pathway of apoptosis (Kubli and Gustafsson, 2012).

Puma and Noxa are two BH3-only proteins of the Bcl-2 family that are also involved in promoting apoptosis. Puma, a transcriptional target of p53 directly regulates the transcription-independent, cytosolic and pro-apoptotic function of p53. It has been reported that overexpression of Puma is accompanied by increased Bax expression, Bax conformational change, translocation to the mitochondria, Cytochrome c release and reduction in the mitochondrial membrane potential (Moldoveanu *et al.*, 2014).

Localisation of Puma together with Noxa to the mitochondria it's capable of binding Bcl-xl among the BH3-only proteins, releasing cytosolic p53 protein from an inhibitory complex with Bcl-xl through a dynamic allosteric mechanism. A crucial Trp residue within the BH3 region of Puma engages a His residue in helix a3 of Bcl-xl to induce unfolding of this region of Bcl-xl, thus disrupting a significant portion of the Bcl-xl interface with p53 (Follis *et al.*, 2013). This leads to the freeing up Bax and Bak proteins to activate MOMP and elicit the release of mitochondrial proteins including Cytochrome c (Kubli and Gustafsson, 2012). Another BH3-only protein Bid can be induced by p53 protein following irradiation by gamma radiation and aids in sensitising cells to chemotherapeutic agents (Valente *et al.*, 2013). In addition to regulating apoptosis, p53 has also been reported to play a role in the regulation of the cell division cycle (Sorokina *et al.*, 2016).

1.3 Cell division cycle

Cell division requires a series of separate events collectively known as the cell division cycle. In eukaryotes, the cell division cycle consists of interphase (G1, synthesis (S), G2), and mitotic (mitosis and cytokinesis) phases (Kim *et al.*, 2012). In G1, the cell grows and prepares for S phase, during which DNA replication occurs. Following S phase, G2 phase prepares cells for mitosis (M). Mitosis is a critical cell cycle phase during which duplicated chromosomes are correctly separated into two identical units on the spindle, restoring genome integrity after cell division cycle. Various mechanisms exist to ensure proper mitosis with high accuracy (Fong *et al.*, 2016). During mitotic entry at the G2/M border, cells can abort the process and return to G2 in response to damages or stresses. After committing to mitotic entry, the accuracy of chromosome segregation is further protected by the spindle assembly checkpoint (SAC), which functions to delay anaphase onset until all chromosomes or kinetochores are properly attached to spindle microtubules (Lara-Gonzalez *et al.*, 2012).

The cell division cycle is internally monitored at checkpoints to ensure that the environmental conditions and quality of the replicated genome are acceptable for the progression of the cell division cycle to the next phase. Checkpoint activation can lead to cell division cycle arrest to allow for DNA damage repair, apoptosis, or senescence (Borg and Dixit, 2017). DNA damage checkpoints are positioned before the cell enters the S-phase (G1/S checkpoint) and after DNA replication (G2/M

checkpoint) (Yim *et al.*, 2015). Cell division cycle phase transitions are activated by the phosphorylation of distinct substrates cyclin-dependent kinases (Cdk4/6, 2 and 1) when associated with their cyclin-binding partners. The activity of Cdks involved in cell division cycle regulation is tightly controlled; it is induced by mitogenic signals and their activation can be inhibited at different checkpoints after DNA damage (Chikara *et al.*, 2017, Kaplon *et al.*, 2015). Cyclins (D, E, A and B), which are synthesised and degraded at selected times during the cell division cycle (Bagga and Bouchard, 2014), bind Cdks and become fully active following phosphorylation by a Cdk-activating kinase cycle (Lim and Kaldis, 2013).

In the G1-phase, cells express three kinds of cyclin D (cyclin D1, D2 and D3), combining with and activating Cdk4/6, which is necessary for cells to enter the G1 phase from G0. G0 is a quiescent state which is a home of only cells that are undergoing differentiation (Lim and Kaldis, 2013). Cyclin D differs from other cyclins as it is not expressed periodically but is synthesised in the presence of growth factors (Liao *et al.*, 2015). Cyclin E is also expressed in the G1-phase and combines with Cdk2 for the transition from the G1- to S-phase. The progression of S-phase requires the Cyclin A/Cdk2 complex (Duronio, 2012). These complex phosphorylate/inactivate retinoblastoma (pRb) protein leading to the release of bound transcription factors, such as E2F and promote expression of genes such as *cell division cycle 25* (*cdc25*) necessary to facilitate the G1/S-phase transition (Gerard and Goldbeter, 2014). Cyclin A complexes with Cdk1 to promote the progression and transition of the G2-phase to the M-phase regulated by cyclin B/Cdk1 complex (Bertoli *et al.*, 2013). In the fully active state, Cyclin–Cdk complexes phosphorylate substrates, priming them for ubiquitination and proteasomal destruction.

The overall cellular response to damaged DNA, known as DNA damage response (DDR), is composed of sensor proteins that detect and signal DNA damage to downstream effectors that, in turn, arrest cell division cycle progression and promote repair (Salazar-Roa and Malumbres, 2017). In response to DNA damage, the ataxia telangiectasia mutated (ATM) kinase becomes activated by DNA double strand breaks (DSBs) which then triggers the G1 checkpoint by phosphorylating and activating the Checkpoint Kinase 2 (Chk2) (Denisenko *et al.*, 2016). The Chk2 inhibits Cdc25A, a phosphatase that removes inhibitory phosphorylation of the Cyclin

A/Cdk2 and Cyclin E/Cdk2 complexes, preventing cells from proceeding into S-phase (Parsels *et al.*, 2016). Of note, the G1 checkpoint is critically dependent on p53 protein and ATM induces phosphorylation of p53 leading to p53 stabilisation. Stabilised p53 induces Cdk inhibitor proteins (CKIs), such as p21/CIP1/CDKN1A and p27/Kip1 (El-Deiry, 2016)

Cdks are subjected to negative regulation by Cdk inhibitory proteins (CKIs) as shown on figure 3. Based on their sequence similarity and structure, CKIs are divided into two protein families: the INK4 (inhibitors of Cdk4) and the CIP/Kip families (Cdk Interacting Protein/Kinase Inhibitory Protein) (Otto and Sicinski, 2017). The INK4 family comprises $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. These inhibitors bind to free Cdk4/6 and inhibit their kinase activity by interfering with their associated D-type Cyclins and distorting the ATP binding site. Its ectopic expression arrests the cell division cycle in G1-phase (Bretones *et al.*, 2015). The CIP/Kip family consists of three members: p21/CIP1 (Cdk Interacting Protein 1), p27/Kip1 (Kinase Inhibitory Protein 1) and p57/Kip2 (Kinase Inhibitory Protein 2). These CIP/Kip family members bind to Cyclin A, E, D/Cdk complex and suppress their catalytic activity causing cell division cycle arrest (Karimian *et al.*, 2016). Protein p27 is one of major CKIs and based on structural contact between p27 and Cdk protein, p27 protein mimics as a pseudo ATP to inactivate the Cdk activity by occupying the ATP binding site of Cdk (Otto and Sicinski, 2017). p21/CIP1 was the first member of the CKI proteins to be identified residing in either the nucleus or cytoplasm with Cyclins and Cdks as its role depends on its subcellular distribution (Dixit *et al.*, 2012). p21/CIP1 binds to Cyclin A/Cdk2, E/Cdk2, D1/Cdk4 and D2/Cdk4 complex and as such, inhibits the phosphorylation of pRB protein (Mahale *et al.*, 2015).

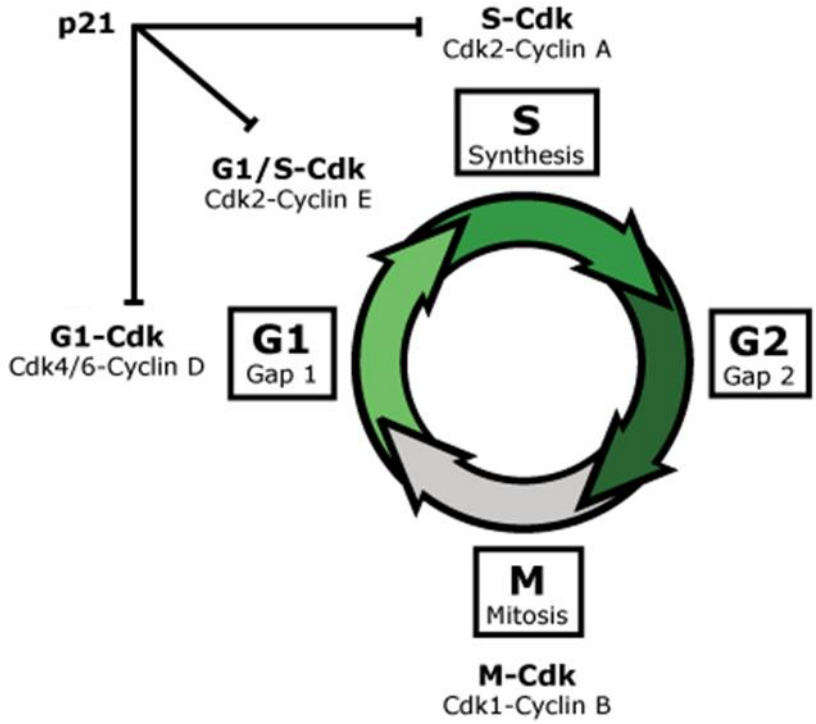

Figure 3: p21^{Cip1} inhibits G1/S-Cdks complex. The association of cyclin E with Cdk2 is active at the G1/S transition and directs entry into S phase. The kinase inhibitor protein group of CKI, p21^{Cip1} and others like p27^{Kip1} and p57^{Kip2} negatively regulate cyclin D/Cdk4/6 and cyclin E/Cdk2 complexes. S phase progression is directed by the cyclin A/Cdk2 complex, and the complex of cyclin A with Cdk1 is important in G2. cyclin B/Cdk1 is necessary for the entry into mitosis (Benson *et al.*, 2014).

Induction of p21/CIP1 by p53 protein upon DNA damage inhibits Cyclin E/Cdk2 thereby inhibiting G1/S transition furthermore. This phenomenon can promote the kinase activity of Cyclin D/Cdk4/6 mitosis (Benson *et al.*, 2014, Visconti *et al.*, 2016). Thus, the progression through G1-phase and overexpression of p21/CIP1 can potentially inhibit Cdk1 and arrest G2/M transition. p21/CIP1 is also known to interact with proliferating cell nuclear antigen (PCNA) that blocks the ability of PCNA to activate pol δ thus inhibiting the DNA replication and the kinase activity of Cyclin A/Cdk1/2 resulting in arrest cell division cycle at intra-S-phase (Karimian *et al.*, 2016, Xu *et al.*, 2014).

DNA damage occurred in S-phase arising from stalled replication forks and nucleotide excision/repair process leads to the intra-S-phase checkpoint activated to prevent further replication. The damage is sensed by the Ataxia Telangiectasia and Rad3-related (ATR) kinase which by activating Chk1, induces Cdc25A proteosomal degradation blocking further progression through S-phase (Chikara *et al.*, 2017). The

ATR and Chk1 kinases also trigger the G2/M checkpoint which prevents cells with damaged DNA from entering mitosis. Mitosis onset requires activity of the master mitotic kinase Cyclin B/Cdk1 (Zhang *et al.*, 2015). Kinase Cdk1 catalytic activity is inhibited during the S- and G2-phases through the phosphorylation on T14 and Y15 induced by the kinases Wee1 and Myt1. These phosphorylations are removed at the G2/M transition by the Cdc25C phosphatase (Visconti *et al.*, 2016). To prevent cells with damaged DNA from entering mitosis, ATR inhibits Cyclin B/Cdk1 activation by stimulating the Cdk1 inhibitory kinase Wee1 and inhibiting Cdc25C *via* Chk1 (Borg and Dixit, 2017). In response to DNA damage ATM and ATR not only stop cell division cycle progression but also initiate DNA repair by phosphorylating several other substrates. If damage cannot be repaired, the cell destiny might be death or permanent growth arrest (senescence) (Shaltiel *et al.*, 2015).

1.4 AIM:

To study the probable anticancer effects and induced mechanism of action of *M. balsamina* crude leaf acetone extract in lung A549 cancer cell lines.

1.4.1 OBJECTIVES:

The objectives of the study were to:

i. Determine the effect of the crude leaf acetone extract on the viability and proliferation of lung A549 cancer cells and Primary Dermal Fibroblast (KMST-6) normal cells using Muse™ count & viability assay kit and Muse™ Ki67 proliferation assay kit, respectively.

ii. Determine the cellular and nuclear morphological features associated with apoptosis using acridine orange/ethidium bromide-dual staining.

iii. Quantitatively determine percentage of cells undergoing apoptosis using Muse™ annexin-V & dead cell kit

iv. Determine the effect of the extract on cell division cycle using Muse™ cell cycle assay kit.

v. Evaluate the effect of the extract on the mRNA (*p21, p53, cdc2* and *cyclin B*) and protein expression profiles of cell division cycle and apoptosis-related proteins using RT-PCR and Proteome profiler.

CHAPTER 2

2. METHODS AND MATERIALS

2.1 Reagents

Acetone (Rochelle chemicals, Johannesburg, SA). Filter papers (Munktell, Ahlstrom, Germany). Dimethylsulfoxide (DMSO), ethidium bromide and propidium Iodide [Sigma-Aldrich, MO, USA]. Muse™ count and viability kit, Muse™ Ki67 proliferation kit, Muse™ cell cycle kit and Muse™ annexin-V & dead cell kit (Merck Darmstadt, Germany). Dulbecco's Minimum Essential Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) (HyClone laboratories, South Logan, USA). Foetal Bovine Serum (FBS) and Trypsin from (Gibco, Life technologies, United State). Phosphate-buffered saline (PBS) [Lonza, Verviers, Belgium]. All tissue culture plates and other plastic ware were from (Nest Biotechnology Co., Ltd, USA). High Pure RNA Isolation kit (Roche Applied Science, Mannheim, Germany). Improm-II™ Reverse Transcriptase kit and GoTaq® G2 Hot Start Green Master Mix (Promega Madison, WI, USA). Acridine orange (Carl Roth GmbH + Co. KG, Germany). Pierce Bicinchoninic Acid (BCA) protein assay Kit (Thermo Fisher Scientific, USA). Polyvinylidene Difluoride (PVDF) membrane [Life technologies, USA]. Mouse primary monoclonal antibodies against Bax and p21, Human Apoptosis Array kit (R&D Systems, Minneapolis, MN, USA). Bcl-2, p53 and Horseradish peroxidase-conjugated (HRP) secondary antibody [BIOCOM biotech, USA]. The C-DiGit ® Blot Scanner (LI-COR Biosciences, Lincoln, Nebraska USA).

2.2 Plant collection and extraction

The plant leaves of *M. balsamina* were collected from Letsitele, Don Village, Limpopo Province (23°C 57' 51" S; 30°C 22' 39" E). Voucher specimen number (UNIN121046) was deposited at the Larry Leach Herbarium (UNIN) at the University of Limpopo. Plant leaves were dried at room temperature and ground to fine powder using a commercial blender. Powdered leaves (20 g) were macerated in 200 ml of absolute acetone with continuous shaking at room temperature for a period of 3 days using a rotary shaker (Labotec, SA). After 3 days, the extract was filtered using Whatman 3HW 90MM filter paper. Acetone was evaporated using an industrial fan at room temperature. The dried extract was dissolved in DMSO to give a stock solution

of 100 mg/ml which was aliquoted in 2 ml micro-centrifuge tubes and stored at -20˚C until use.

2.3 Cell line and culture condition

Lung A549 cancer cell lines and Human Primary Dermal Fibroblast cells (KMST-6) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). A549 and KMST-6 cells were cultured in RPMI-1640 and DMEM, respectively, supplemented with 10% FBS. All the cells were cultured at 37°C in a humidified atmosphere (5% CO_2, 95% air). The cells were sub-cultivated at 80-90% confluence after every 2 days and seeded at a ratio of 1:4 of cells to media. Trypsin (0.05%) was used to detach cells from flasks, either for passaging or seeding during experiments.

2.4 Cell viability assay

To determine the effect of the extract on cell viability, the Muse™ count & viability assay kit was used following the manufacturer's protocol. The assay is based on the quantitative determination of viable cells using dual DNA intercalating fluorescent dyes; i.e. one which stains the DNA of cells with compromised membranes and one that is membrane permeable which stains DNA of live cells. Briefly, A549 and KMST-6 cells were seeded at 7.5×10^4 cells/well in a 24-well plate and allowed to attach for 24 h in a tissue culture incubator. Cells were then treated with 0.3% (v/v) DMSO (negative control), 0-300 µg/ml of the extract and 5 µg/ml of actinomycin D (positive control) for 24 and 48 h. Following treatment, cells were harvested by trypsinisation and stained with the Muse™ count & viability reagent for 5 min at room temperature in the dark. Following staining, about 1000 events for each sample were analysed using the Muse™ cell analyser (EMD Millipore, Darmstadt, Germany). The experiments were performed in duplicate and repeated at least three times.

2.5 Cell proliferation assay

The effect of the extract on cell proliferation was determined using Muse™ Ki67 Proliferation kit according to the manufacturer's instructions. The assay measures the amount of nuclear antigen Ki67 expressed in all phases of the cell division cycle excluding the G0-phase. Briefly, 7.5×10^4 cells/well of A549 were seeded in a 24-well plate and allowed to attach for 24 h in a tissue culture incubator. Cells were then treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 24 and 48 h. Following treatment, cells were permeabilised for 15 min and incubated

with the Muse Ki67-PE reagent for 30 min according to the manufacturer's instructions. Following the addition of the assay buffer, about 2000 events in each sample were analysed using the Muse™ cell analyser (EMD Millipore, Darmstadt, Germany). The experiments were performed in duplicate and repeated at least three times.

2.6 Determination of cellular and nuclear morphological features associated with apoptosis

2.6.1 Acridine orange/Ethidium bromide (AO/EB) dual staining

To determine the effect of the extract on the nuclear and cellular morphology of A549 cancer cells, cells were stained with Acridine orange/ Ethidium bromide dual staining. Acridine orange stains live cells green with an increase in the intensity of AO stain on cells indicating condensation of the nucleus/chromatin. Ethidium bromide which fluoresces red after staining dead cells or cells that have lost their membrane integrity was used as a counter-stain. A549 cells were seeded at a density of 1×10^5 cells/well in 24-well plates and allowed to attach for 24 h in a tissue culture incubator. Cells were then treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 24 and 48 h. Cells were thereafter stained with Acridine orange (10 µg/ml) for 10 min. After incubation, cells were washed once with 1X PBS and further stained with Ethidium bromide (10 µg/ml) for 10 min. After removing the excess dye, cells were observed under an inverted phase contrast microscope (Model: Eclipse-T*i*; Nikon Co., Tokyo, Japan) and photographed at 10X magnification. The experiments were performed in duplicate and repeated at least three times.

2.6.2 Quantitative analysis using Muse™ annexin V & dead cell staining

Distribution of early and late apoptotic cells after treatment with the extract was determined using the Muse™ annexin V & dead cell kit following the manufacturer`s protocol. The kit utilises annexin V to detect phosphatidylserine on the external membrane of apoptotic cells and a dead cell marker 7-AAD is used as an indicator of cell membrane structural integrity. Briefly, 7.5×10^4 cells/well of A549 were seeded in a 24-well plate and allowed to attach for 24 h in a tissue culture incubator. Cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 6 and 24 h. Cells were then trypsinised and centrifuged at 300 × g for 5 min and then

resuspended in RPMI medium with 10% FBS to a final concentration of 1×10^7 cells/ml. Muse™ annexin V & dead cell reagent (100 µl) was added to each tube, cells stained for 20 min at room temperature in the dark and analysed using the Muse™ cell analyser. The experiments were performed in duplicate and repeated at least three times.

2.7 Analysis of cell division cycle

The effect of the extract on cell division cycle progression was assessed using the Muse™ cell cycle assay kit according to the manufacturer's instructions. This assay kit uses a premixed reagent comprising of the nuclear DNA intercalating stain, propidium iodide and RNAse A. Propidium iodide discriminates cells at different stages of the cell division cycle, based on differential DNA content in the presence of RNAse to increase the specificity of DNA staining. Propidium iodide-based staining of DNA content is then utilized to discriminate and measure the percentage of cells in each cell division cycle phase (G0/G1, S, and G2/M). Briefly, A549 cells (7.5×10^4 cells/well) were seeded in a 24-well plate and incubated for 24 h to allow attachment. Cells were then treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 48 h. Cells were then harvested, washed with PBS and fixed with ice-cold 70% ethanol at -20°C for 3 h. Fixed cells were pelleted and stained with cell cycle analysis reagent for 30 min at 37˚C in dark and about 5000 events were analysed on a Muse™ cell analyser. The experiments were performed in duplicate and repeated at least three times.

2.8 Analysis of mRNA expression

Qualitative measure of apoptosis and cell division cycle regulatory genes expressed at mRNA level was achieved by Reverse Transcription Polymerase Chain Reaction (RT-PCR). In this technique, extracted RNA is first transcribed to complementary DNA (cDNA) using a reverse transcriptase enzyme. Transcribed cDNA will then be used as a template for its exponential amplification during PCR reaction. A549 cells at density of 1.5×10^6 cells/ml were seeded in 25 cm^2 culture flasks and allowed to attach for 24 h in an incubator. Cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 24 and 48 h. After the incubation period, floating and adherent cells were harvested and centrifuged at 300 ×g for 5 min. Total RNA was extracted using High pure RNA isolation kit following the manufacture's protocol. Extracted total RNA was reverse transcribed to cDNA using the Improm-

II™ reverse transcriptase kit according to the manufacturer's instructions. Corbett Rotor-gene 6000 series (Qiagen, USA) was used during the reverse transcription. PCR was performed using the GoTaq® G2 hot start green master mix according to the manufacturer's instructions. Amplification by PCR was run with an initial denaturation step for 2 min at 95°C, Denaturation at 95˚C for 30 s, followed by annealing for 30 s at (67˚C for *β-Actin*, 57°C for *cdc-2*; 58˚C for *p53*, *p21* as well as *cyclin B*) and extension step for 1 min at 72 °C for 30 cycles. The PCR product was resolved on 2% agarose gel at 90 V, visualised and photographed using a UV transilluminator (BIO-RAD, USA) and band densities were analysed using GelQuant.NET software provided by biochemlabsolutions.com. The experiments were performed in duplicate and repeated at least three times.

2.9 Analysis of protein expression

To further validate the involvement of apoptosis and its pathways on A549 cells, a proteome profiler antibody array kit was used. The array simultaneously detects the relative expression levels of 35 apoptosis-related proteins thereby measuring the changes in apoptosis-related protein expression compared to the controls. The experiment was carried out in accordance with manufacturer's protocol. A549 cells at density of 2×10^7 cells/ml were seeded in 75 cm^2 culture flasks and allowed to attach for 24 h in an incubator. Cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 48 h. First, approximately 1×10^7 cells/ml of A549 cells were solubilised in lysis buffer 17 (non-denaturing buffered solution with preservatives) and the lysate was rocked gently at 4°C for 30 min. Thereafter, samples were centrifuged at 14000 × g for 5 min and the supernatant was transferred onto a clean test tube. Protein concentrations of the resulting lysates were measured using a BCA protein assay kit according to the manufacturer instructions. The array procedure was performed using the Human apoptosis array kit as per manufacturer's instructions. Briefly, each of antibody-coated array membranes were placed into the provided dish with array buffer 1 and blocked for 1 h at room temperature. The array buffer was aspirated, 400 µg of prepared cell lysates were added onto each well of the dish to incubate at 4°C with gentle shaking overnight. The membranes were washed with 1X wash buffer for 10 min on a rocking platform shaker for a total of three washes and then incubated with 1.5 ml of diluted Detection Antibody Cocktail for 1 hour on a rocking platform shaker. The membranes

were washed and incubated with horse radish peroxidase conjugated streptavidin for 30 min on a rocking platform shaker. After a final wash, membrane intensity was acquired using LumiGLO reserve^{TM} western blotting chemiluminescence substrate and pixel densities were analysed using GelQuant.NET software provided by biochemlabsolutions.com.

2.10 Statistical analysis

Data was analysed using Graphpad Prism, version 6.0; the results were expressed as mean ± standard deviation (SD), from duplicate of 3 independent experiments. Statistical evaluation of the results was determined using the one way analysis of variance (ANOVA), employing the Dunnett's comparison Test. Results were considered significant when *$p \leq 0.05$, ** $p \leq 0.01$.

CHAPTER 3

3. RESULTS

3.1 The effects of *Momordica balsamina* leaf acetone extract on the viability of A549 and KMST-6 cells

The effect of *M. balsamina* acetone extract on the viability of A549 and KMST-6 cells was determined by the Muse™ count & viability assay. Treatment with the extract decreased the percentage viability of A549 cells (figure 3.1A). The decrease was shown to be in a time- and concentration-dependent manner. A significant decrease in cell viability was seen at concentrations above 200 µg/ml and 100 µg/ml at 24 and 48 h of treatment, respectively. On the other hand, extract-treated KMST-6 cells showed a decrease in viability at concentrations above 100 µg/ml at 24 and 48 h (figure 3.1B). Actinomycin D significantly decreased the viability of A549 cells at 48 h and KMST-6 cells at 24 and 48 h.

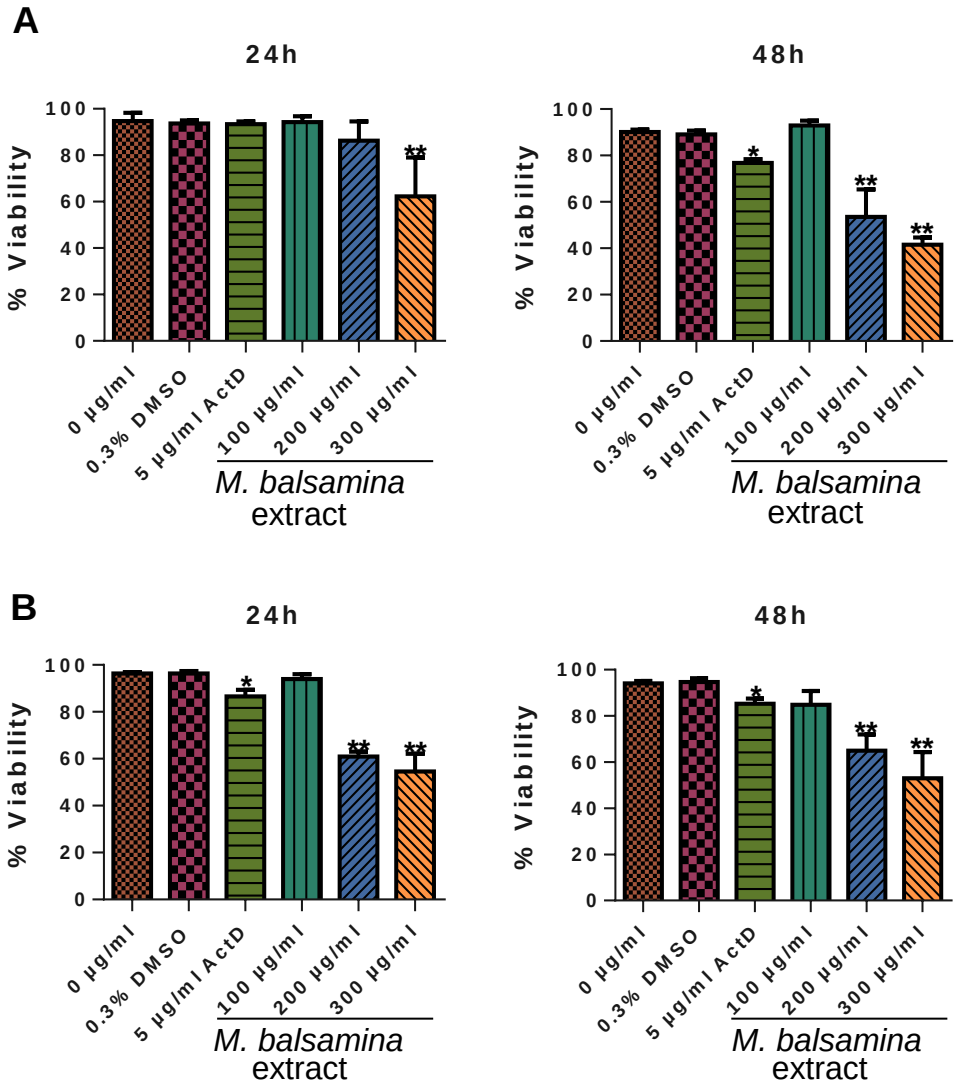

Figure 3.1: Effect of *Momordica balsamina* acetone extract on the viability of A549 (A) and KMST-6 (B) cells. Cells were incubated with 0-300 µg/ml of the extract, 5 µg/ml of actinomycin D and 0.3% (v/v) of DMSO for 24 and 48 h. The effect on cell viability was determined using the Muse™ count & viability assay kit. The data shows the mean ± S.D of three independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicates significant differences to the control.

3.2 The effects of *Momordica balsamina* leaf acetone extract on the proliferation of A549 cells

The effect of *M. balsamina* acetone extract in A549 cell proliferation was determined using the Muse™ Ki67 proliferation kit. The extract significantly inhibited the proliferation of A549 cells in a time- and concentration-dependent manner (figure 3.2). Actinomycin D also significantly inhibited proliferation of A549 cells in a time-dependant manner.

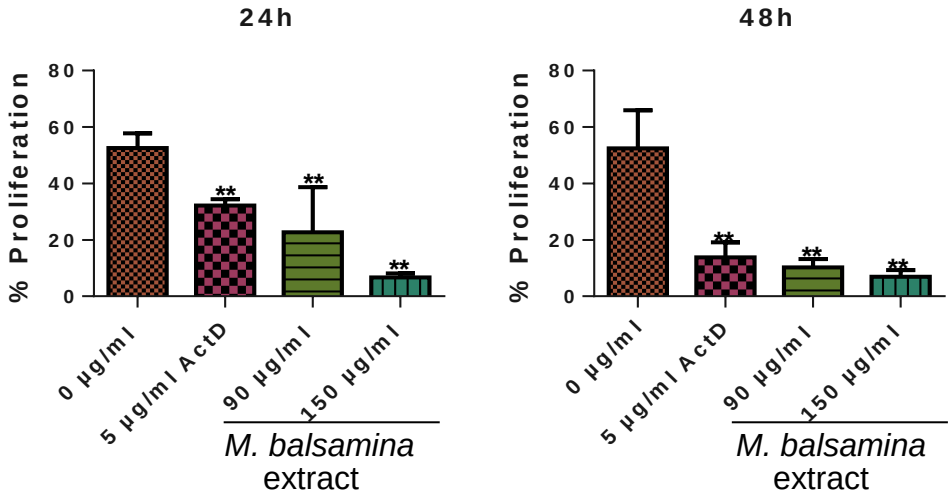

Figure 3.2: Anti-proliferative effect of *Momordica balsamina* leaf acetone extract in A549 cells. A549 cells were treated with 0, 90 and 150 µg/ml of the extract as well as 5 µg/ml actinomycin D for 24 and 48 h. Percentage cell proliferation was assessed by the Muse™ Ki67 proliferation kit and analysed by Muse™ cell analyser. The data shows the mean ± S.D of three independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicates significant differences to the control.

3.3 The effects of *Momordica balsamina* leaf acetone extract on the cellular and nuclear morphology of A549 cells

Features associated with apoptosis were examined by fluorescence microscopy using Acridine orange/Ethidium bromide (AO/EB) dual staining. Extract-treated cells showed a time- and concentration-dependent increase in the number of cells with condensed chromatin as seen by an increase in the intensity of acridine orange (bright green), as compared to untreated cells (figure 3.3). Furthermore, A549 cells treated with 150 µg/ml of the extract showed features, such as loss of membrane integrity and presence of apoptotic bodies, associated with late stages of apoptosis at 48 h. A549 cells that were treated with actinomycin D also revealed chromatin condensation, loss of cell membrane function and presence of apoptotic bodies.

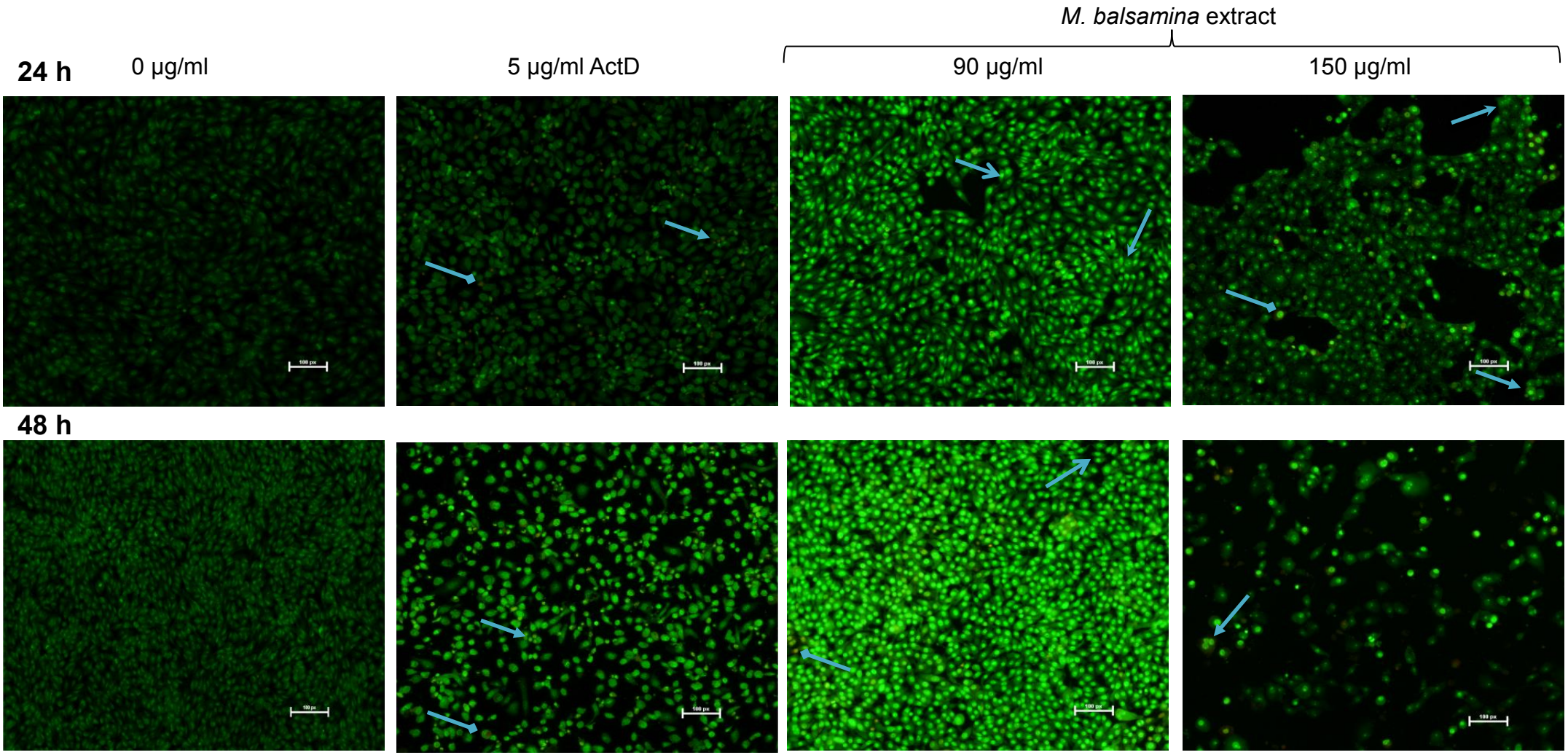

Figure 3.3: Cellular and nuclear morphological changes induced by *Momordica balsamina* leaf acetone extract in A549 cells. Cells were treated with 0, 90 and 150 µg/ml extract and 5 µg/ml actinomycin D for 24 and 48 h. Cells were stained with AO/EB staining then observed under fluorescence microscope (10x). Arrows symbolise the following: Chromatin condensation ( ), Nuclear condensation ( ), Late apoptotic cells ( ) and Apoptotic bodies ( ). The data represent three independent experiments.

3.4 The effects of *Momordica balsamina* leaf acetone extract on the induction of apoptosis in A549 cells.

Quantitative measure of cells undergoing apoptosis was achieved by the Muse™ annexin V & dead cell assay kit. The results on figure 3.4.A represent one set of three independent experiments. Treatment with the extract resulted in a significant time- and concentration-depended increase in the percentage of cells in early [annexin-V (+ve)] and late [annexin-V/7-AAD (+ve)] stages of apoptosis (figure 3.4B). Furthermore, a significant percentage of A549 cells treated with the extract were 7-AAD positive which is indicative of cells undergoing necrosis (figure 3.4B). A549 cells treated with 5 µg/ml actinomycin D showed a time-dependent increase in the percentage of cells in the early stage of apoptosis at 6 and 24 h.

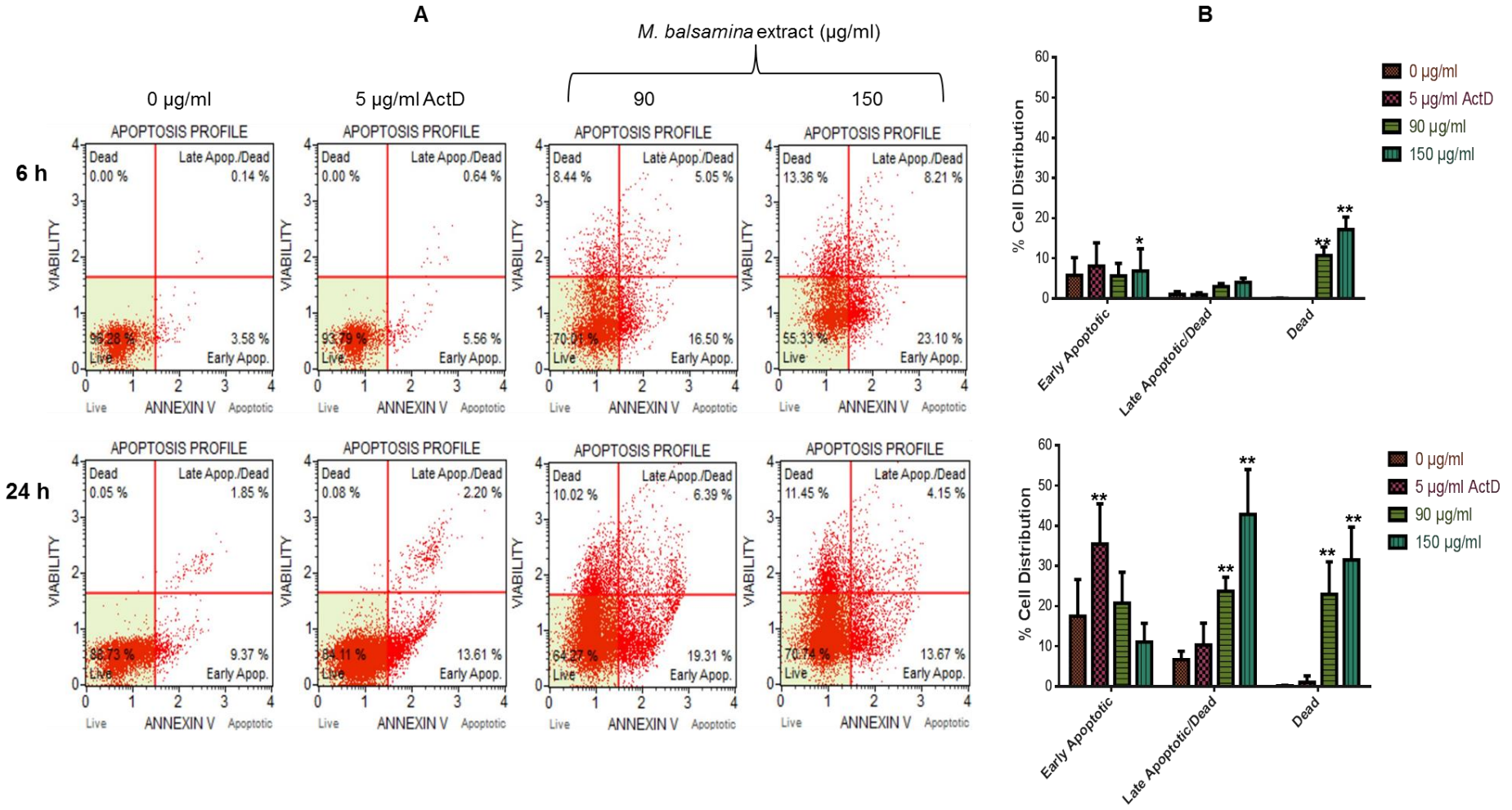

Figure 3.4: The quantitative measure of A549 cells in apoptosis induction. A549 cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 6 and 24 h, then analysed using the Muse™ cell analyser. Representative plots of cell undergoing apoptosis from one of three independent experiments **(A)**. Quantification of apoptotic cells from three independent experiments **(B)**. The data shows the mean ± S.D of three independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicates significant differences to the control.

3.5 The effects of *Momordica balsamina* leaf acetone extract on the distribution of A549 cells during cell division cycle

To examine the mechanism of the extract mediated cell proliferation inhibition, the cell division cycle distribution was evaluated. The results on figure 3.5A represent one set of three independent experiments at 48 h. There was a concentration-dependent cell division cycle arrest of A549 cells treated with the extract at the G0/G1-phase. The extract-treated cells also revealed a decrease in the number of cells at S-phase (figure 3.5B). A549 cells treated with 5 µg/ml actinomycin D showed a non-significant increase at the G0/G1-phase.

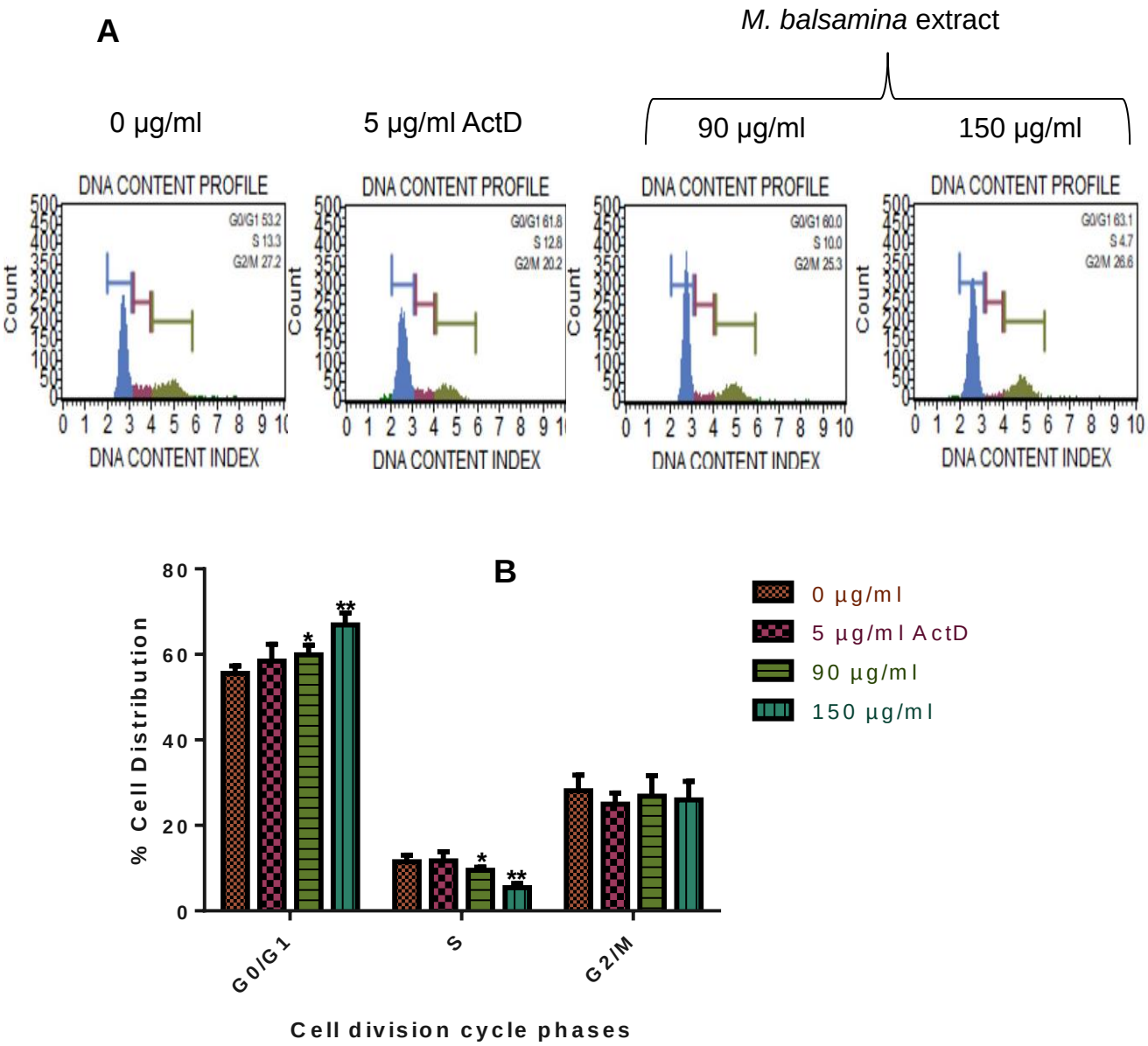

Figure 3.5: *Momordica balsamina leaf acetone* extract induce cell cycle arrest in A549 cells. Cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 48 h. Cells were harvested, fixed with 70% ethanol followed by incubation with the cell cycle reagent. Cell division cycle progression was then analysed using the Muse™ cell analyser. Representative plots of cell population during cell cycle from one of three independent experiments **(A)** and a quantitative measure of cells in G0/G1, S and G2/M phases **(B)**. The data shows the mean ± S.D of three independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicates significant differences to the control.

3.6 The effects of *Momordica balsamina* leaf acetone extract in modulating the mRNA expression level of cell division cycle-regulatory genes

The mRNA expression levels of cell division cycle regulatory genes (*cyclin B, cell division cycle-2, p53* and *p21*) were analysed using RT-PCR. The results demonstrated an increase in the mRNA expression of *p53* at 24 and 48 h in A549 cells treated with 5 µg/ml actinomycin D, 90 and 150 µg/ml of extract (figure 3.6A). This increase was more pronounced at 24 h as compared to at 48 h. An increase in the mRNA expression of *p21* in A549 cells treated with 5 µg/ml actinomycin D and 150 µg/ml of extract was also observed. However, a decrease in *p21* mRNA expression level was observed in A549 cells treated with 90 µg/ml at 24 h. Furthermore, the extract and actinomycin D-treated A549 cells showed a decrease in *p21* mRNA expression at 48 h (figure 3.6.B). There was no relative change in the mRNA expression of *cdc-2* at 24 h, however, an increase was observed at 48 h in cells treated with 90 µg/ml extract (figure 3.6C). There was an increase in *cyclin B* mRNA expression in cells treated with 90 and 150 µg/ml extract at 24 h. At 48 h increase in the mRNA expression of *cyclin B* was only observed in cells treated with 90 µg/ml while treatment with 150 µg/ml of the extract led to a decrease in the mRNA expression of *cyclin B*. Actinomycin D-treated cells showed an increase in the mRNA expression of *cyclin B* at 24 h followed by a decrease at 48 h (figure 3.6D).

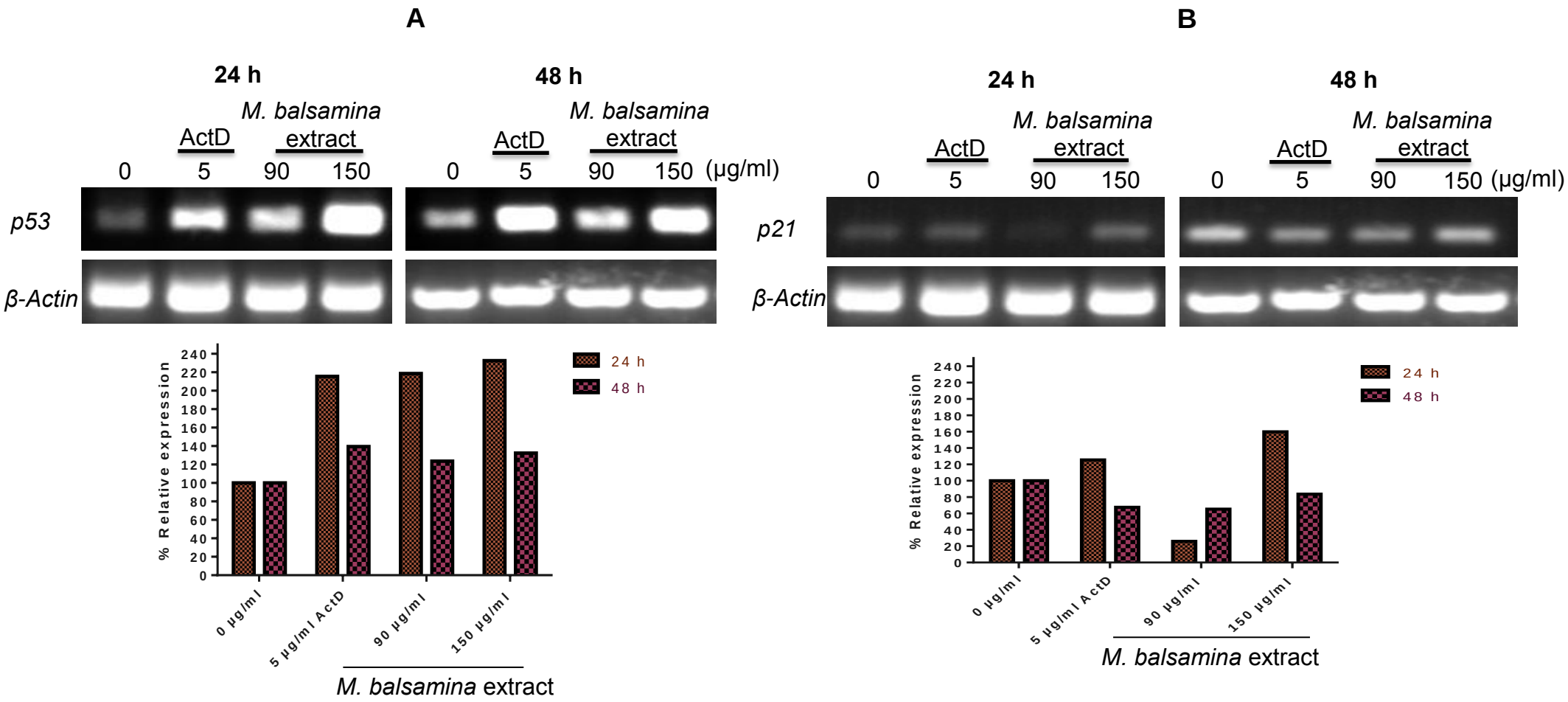

Figure 3.6A: Analysis on the expression of cell division cycle associated genes *p53* (A) and *p21* (B). A549 cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 24 and 48 h. Total RNA was harvested and cDNA constructed for the detection of expressed genes by RT-PCR. Densitometric analysis was performed using GelQuant.NET software provided by biochemlabsolutions.com and values obtained were plotted as histograms.

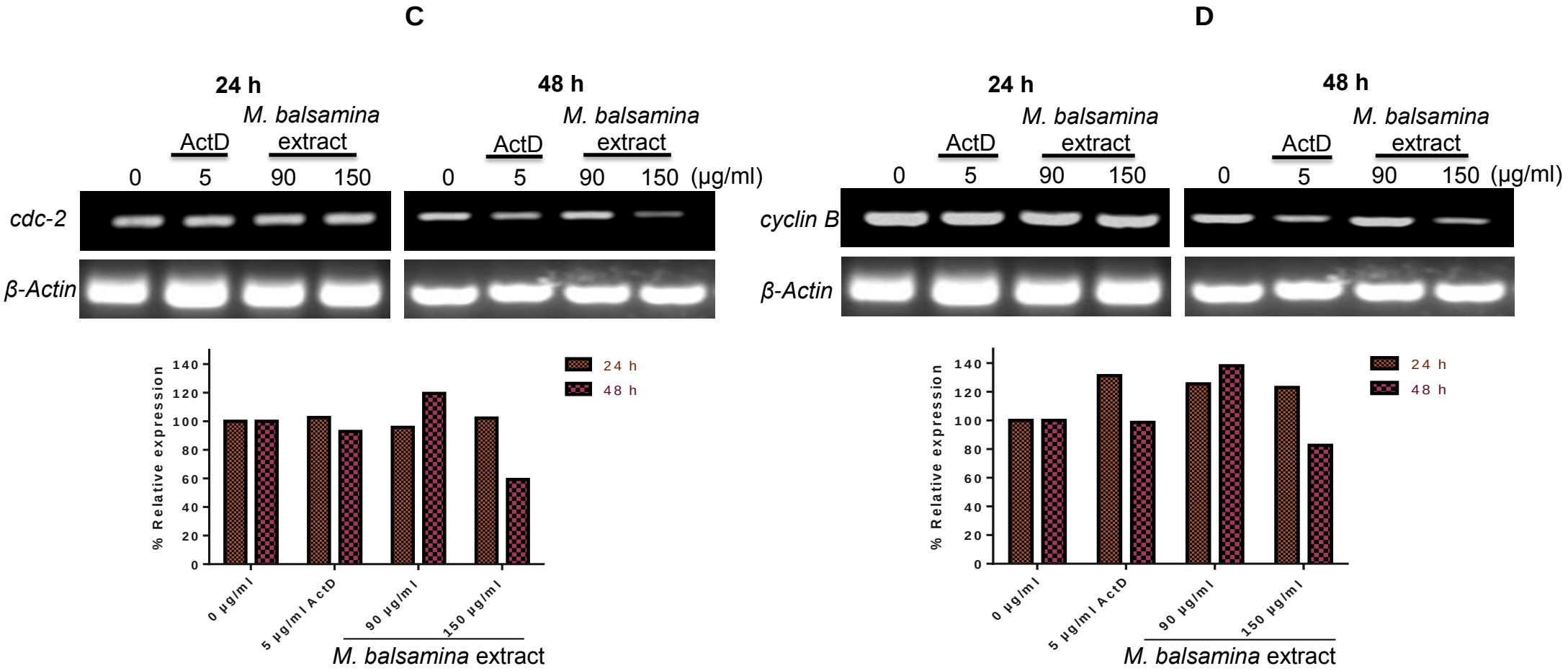

Figure 3.6B: Analysis on the expression of cell division cycle associated genes *cdc-2* (C) and *cyclin B* (D). A549 cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 24 and 48 h. Total RNA was harvested and cDNA constructed for the detection of expressed genes by RT-PCR. Densitometric analysis was then performed using GelQuant.NET software provided by biochemlabsolutions.com and values obtained were plotted as histograms.

3.7 The effects of *Momordica balsamina* leaf acetone extract on the relative-expression levels of apoptosis and cell division cycle regulatory proteins.

3.7.1 Effects of *Momordica balsamina* on the expression levels of the Bcl-2 family and mitochondrial proteins

Expression levels of apoptosis regulatory proteins involved in the mitochondrial pathway was measured using the proteome profiler antibody array. Treatment with the extract resulted in the downregulation in the expression levels of pro-apoptotic protein, Bax. A significant downregulation in Bax protein expression was observed in cells treated with 90 µg/ml of the extract. The expression level of Bad protein was significantly downregulated in cells treated with 90 and 150 µg/ml of the extract. However, actinomycin D-treated cells showed a significant upregulation in the expression level of Bax protein and a downregulation in the expression levels of Bad protein. A non-significant downregulatory effect on the expression levels of anti-apoptotic protein Bcl-2 was observed in extract- and actinomycin-treated cells. A significant downregulation in Bcl-xl protein expression was seen in extract- and actinomycin D-treated cells (figure 3.7.1A). There was a release of mitochondrial proteins Cytochrome c and HTRA2/Omi, in A549 cells treated with the extract and actinomycin D. However no notable change in the amounts of SMAC/Diablo protein were observed in cells treated with the extract and actinomycin D (figure 3.7.1B).

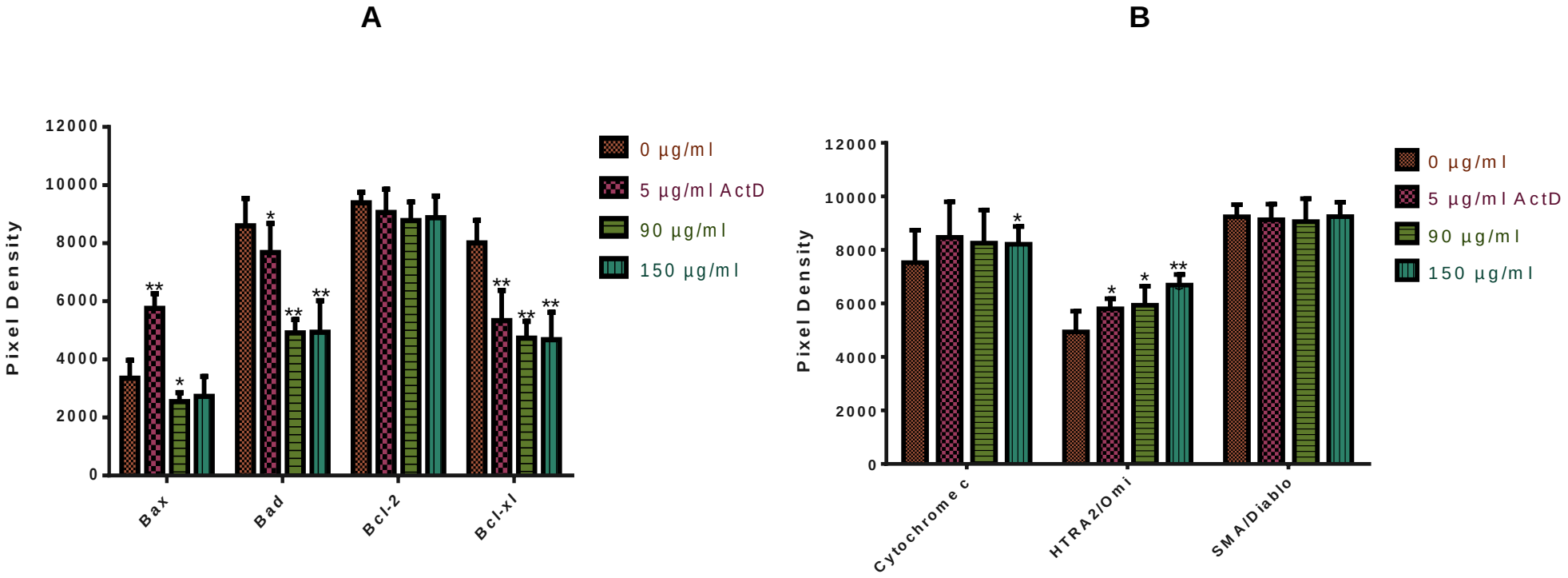

Figure 3.7.1: Human apoptosis array analysis on Bcl-2 and mitochondrial proteins. A549 cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 48 h. Cells were solubilised in lysis buffer. The samples were detected following the manufacturer's instructions in the Human apoptosis antibody array kit. Membrane intensity were acquired using the C-DiGit ® Blot Scanner (LI-COR Biosciences, USA) and relative pixel densities were measured using GelQuant.NET software provided by biochemlabsolutions.com. Values obtained were plotted as bar graphs and data points represent the mean ± S.D of two independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicates significant differences to the control.

3.7.2 Effects of *Momordica balsamina* on the expression levels of membrane receptors and death domains in the extrinsic pathway

The expression levels of the membrane receptors and death domains involved in the extrinsic apoptosis pathway were measured using proteome profiler antibody array kit. TRAIL R1/DR4 showed a non-significant downregulation in A549 cells treated with actinomycin D and 150 µg/ml of the extract. However, following treatment with 90 µg/ml of the extract, a significant upregulation in the expression level of TRAIL R1/DR4 as compared to untreated cells was observed (figure 3.7.2). A significant downregulation was observed in the expression levels of TRAIL R2/DR5 in A549 cells treated with actinomycin D; however, there was a trend towards downregulation in A549 cells treated with 150 µg/ml of the extract. Fas/TNFRSF6/CD95 showed a significant downregulation in A549 cells treated with actinomycin D but a non-significant downregulation in cells treated with the extract. A death domain FADD had a non-significant downregulation in A549 treated cells (figure 3.7.2).

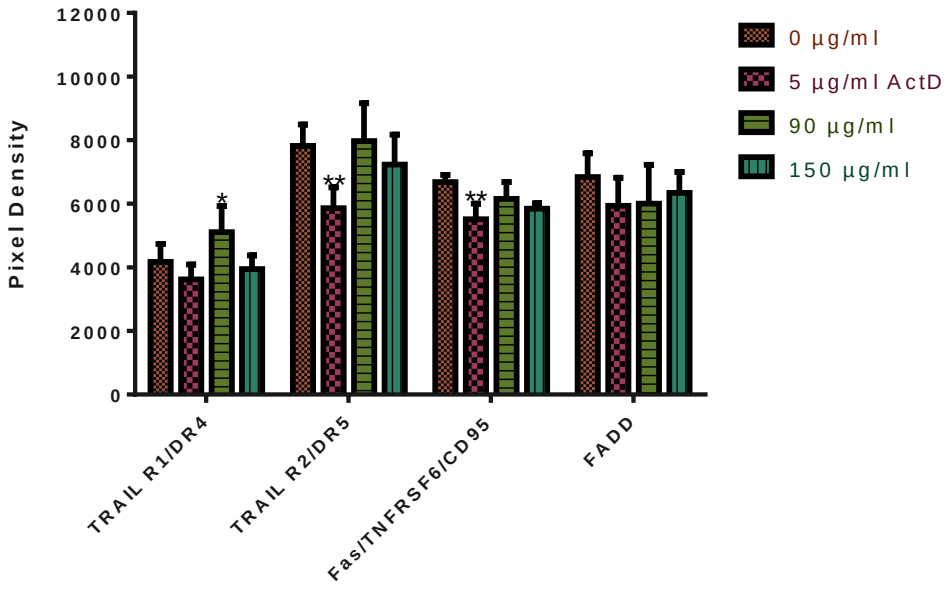

Figure 3.7.2: Human apoptosis array analysis on proteins involved in extrinsic pathway. A549 cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 48 h. Cells were solubilised in lysis buffer. The samples were detected following the manufacturer's instructions in the Human apoptosis antibody array kit. Membrane intensity was acquired using the C-DiGit ® Blot Scanner (LI-COR Biosciences, USA) and relative pixel densities were measured using GelQuant.NET software provided by biochemlabsolutions.com. Values obtained were plotted as bar graphs and data points represent the mean ± S.D of two independent experiments performed in duplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicates significant differences to the control.

3.7.3 Effects of *Momordica balsamina* on the expression levels of Caspases and Inhibitors of apoptosis proteins

A significant downregulation in pro-caspase-3 expression accompanied by a significant concentration-dependent upregulation in cleaved-caspase-3 expression was observed in A549 cells treated with the extract. There was no change in the expression levels of pro-caspase-3 in A549 cells treated with actinomycin D was observed, however, there was a significant upregulation in the expression levels of cleaved-caspase-3 (figure 3.7.3). The expression level of cIAP1 was significantly upregulated in A549 cells treated with 90 µg/ml extract. There was no relative change in the expression levels of cIAP2 and XIAP inhibitors of apoptosis in extract- and actinomycin D-treated cells, however, an upregulation in the expression levels of XIAP in cells treated with150 µg/ml of the extract was observed (figure 3.7.3).

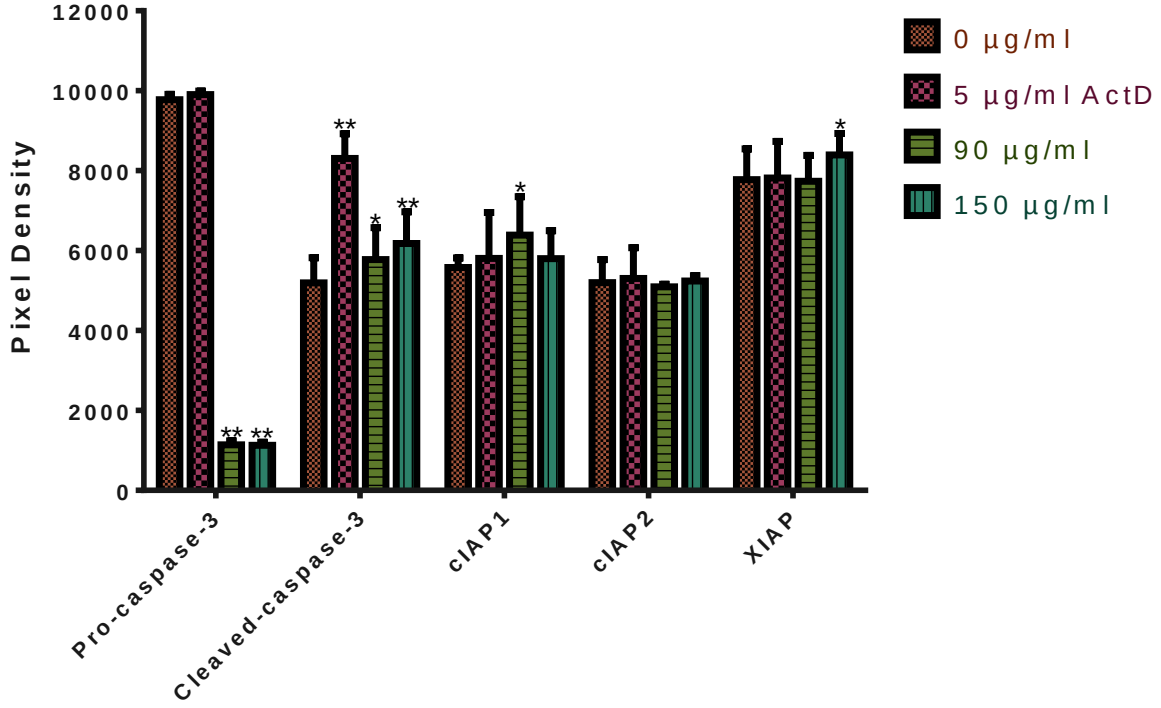

Figure 3.7.3: Effect of *Momordica balsamina* on the expression levels of Caspase and Inhibitors of apoptosis proteins. A549 cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 48 h. Cells were then solubilised in lysis buffer. The samples were detected following the manufacturer's instructions in the Human apoptosis antibody array kit. Membrane intensity was acquired using the C-DiGit ® Blot Scanner (LI-COR Biosciences, USA) and relative pixel densities were measured using GelQuant.NET software provided by biochemlabsolutions.com. Values obtained were plotted as bar graphs and data points represent the mean ± S.D of two independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicates significant differences to the control.

3.7.4 Effects of *Momordica balsamina* on the expression levels of proteins involved in cell division cycle

The expression levels of proteins involved in cell division cycle (p53, p21/CIP1/CDKN1A and p27/Kip1) was determined using a proteome profiler antibody array. A549 cells treated with 150 µg/ml of the extract revealed a significant phosphorylation of p53 at serine15. However, non-significant phosphorylation of p53 at serine15 was observed in A549 cells treated with 90 µg/ml extract and 5 µg/ml actinomycin D. There was no phosphorylation of p53 at serine46 observed in extract- and actinomycin D-treated cells. Additionally, a non-significant phosphorylation of p53 at serine635 was observed in A549 cells treated with 90 µg/ml extract and 5 µg/ml actinomycin D. Moreover, treatment with 150 µg/ml of the extract had no phosphorylation of p53 at serine392. There was a non-significant downregulation on the expression levels of p21/CIP1/CDKN1A protein in the extract- and actinomycin D-treated cells. However, a significant concentration-dependent upregulation in the expression levels of p27/Kip1 protein in extract-treated A549 cells was observed. Actinomycin D-treated cells showed a non-significant upregulation in the expression levels of p27/Kip1 protein.

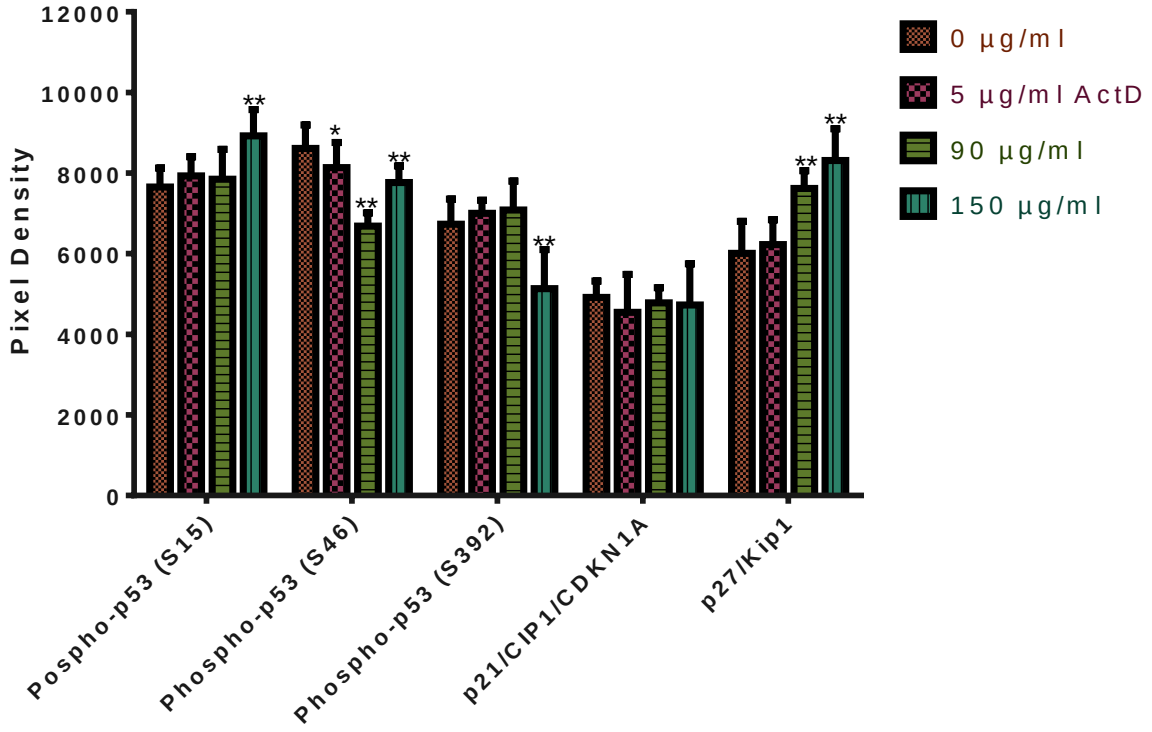

Figure 3.7.4: Human apoptosis antibody analysis of cell division cycle related proteins. A549 cells were treated with 0, 90 and 150 µg/ml of the extract and 5 µg/ml of actinomycin D for 48 h. Cells were then solubilised in lysis buffer. The samples were detected following the manufacturer's instructions in the Human apoptosis antibody array kit. Membrane intensity was acquired using the C-DiGit ® Blot Scanner (LI-COR Biosciences, USA) and relative pixel densities were measured using GelQuant.NET software provided by biochemlabsolutions.com. Values obtained were plotted as bar graphs and data points represent the mean ± S.D of two independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicates significant differences to the control.

CHAPTER 4

4. Discussion and Conclusion

The development of effective anticancer agents with the ability to induce apoptosis without affecting normal proliferating cells is being sourced from natural plants (Fallahian *et al.*, 2017). Currently about 60% of the drugs that are in clinical trials such as curcumin (Amalraj *et al.*, 2017), or already used as anticancer therapy such as paclitaxel (Pandi *et al.*, 2013) are plant-derived medicines (Carrasco *et al.*, 2014, Seca and Pinto, 2018). However, mortality rate in cancer is continually increasing, possibly as many as 1.5 million death are expected to occur in Africa by 2020 (Stefan *et al.*, 2013) together with an estimated number of 11.5 million new cancer cases by 2030 worldwide (Kuete *et al*., 2016). Recently in our lab, the anticancer activity of *M. balsamina* leaf acetone extract was investigated on breast MCF-7 cancer cells and the findings revealed anti-metastatic and pro-apoptosis effects of *M. balsamina* crude leaf acetone extract (Boshielo, 2016). Given the differences in the sensitivity of different cancer cell lines towards treatment with medicinal plants; the current study aimed to study the probable anticancer efficacy and induced mechanism of action of *M. balsamina* crude leaf acetone extract in lung A549 cancer cells.

The crude leaf acetone extract of *M. balsamina* was tested for bioactivity and it exhibited the ability to decrease the viability and inhibit the proliferation of A549 cells with an IC_{50} of 150 µg/ml at 48 h (figure 3.1A). Compared to the previous study by Boshielo, (2016) that indicated inhibition in proliferation of MCF-7 cells at lower concentrations following 24 h incubation, the proliferation of A549 cells, in this study, was inhibited at a relatively higher concentration following 48 h of treatment (figure 3.2). This confirms the differences in the sensitivity of different cancer cell lines towards the medicinal plant being utilised. In addition, the report by the NCI (United States National Cancer Institute of Plant Screening Program) showed that a plant extract following incubation between 48 and 72 h is generally considered to have active cytotoxic effect if it has an IC_{50} value equal to or lesser than 20 µg/ml (Banerjee *et al*., 2016, Rahman *et al*., 2013). However, data in this study showed that the extract had an IC_{50} of 150 µg/ml which is above that recommended by the

NCI. Even though the IC_{50} value was higher than 20 µg/ml, treatment with the extract was non-cytotoxic to the normal KMST-6 cells (figure 3.1B). The strong growth inhibitory activity of the acetone fraction prompted more studies to determine its mechanism(s) of action.

Several modes of cell death exist and certain stimulus may result in activation of one or more of these death processes within a cell. The decision taken by a cell to undergo different mechanisms is regulated by various factors, including the energy/ATP levels (autophagy), the extent of damage or stress (apoptosis) and the presence of inhibitors of specific pathway (caspase inhibitors) (Necrosis/Necroptosis) (Long and Ryan, 2012). Apoptosis is considered as one of the most relevant forms of programmed cell death. This is because it eliminates damaged or dying cells with minimal damage to surrounding cells and tissues (Redza-Dutordoir and Averill-Bates, 2016). Cell shrinkage, loss of cell membrane function, formation of membrane blebs, chromatin condensation, phosphatidylserine externalisation, activation of caspases and apoptotic bodies are morphological and biochemical features characteristic of cells undergoing apoptosis (Nagappan *et al.*, 2016).

Our findings revealed the presence of features characteristic of cells undergoing apoptosis in the cells treated with the extract, these included; chromatin condensation, nuclear condensation and apoptotic bodies (figure 3.3). Furthermore, externalisation of phosphatidylserine was observed. Interestingly, a population of cells that were stained only with 7-AAD was also observed. This suggested the occurrence of necrotic mode of cell death in addition to apoptosis. Suzuki *et al*., (2015) reported that if more than one mode of cell death occurs at the same time, one mechanism dominates the other. This was the case in extract-treated A549 cells wherein a high percentage of cells were undergoing apoptosis as compared to those undergoing necrotic mode of cell death (figure 3.4.B). Thus the data suggests apoptosis as the mode of cell death that can be associated with the observed decrease in cell viability and inhibition of cell proliferation.

There are two well characterised pathways that are involved in the induction of apoptosis depending upon the stimulus: the extrinsic and intrinsic pathways (Beesoo *et al.*, 2014). The intrinsic pathway is activated by non-receptor stress stimuli such as hypoxia, damaged DNA, etc. The activated intrinsic pathway is mostly regulated by

the pro- and anti-apoptosis members of Bcl-2 family of proteins which control the mitochondrial membrane permeability through homo- and hetero-dimerisation (Shrivastava *et al.*, 2015). The overall balance between pro- and anti-apoptosis proteins ultimately governs the fate of each cell (Zhou *et al*., 2018). An increase in the ratio of anti-apoptosis proteins over pro-apoptosis proteins favours the survival of cells. On the other hand an increase in the ratio of pro-apoptosis over anti-apoptosis proteins leads to the permeability of the mitochondrial membrane leading to Cytochrome c release and other mitochondrial proteins in to the cytosol. The release of Cytochrome c prompts activation of pro-caspase-9, through the apoptosome complex which then activates the effector pro-caspase-3 leading to cell apoptosis (Maji *et al.*, 2018). An additional class of pro-apoptotic protein known as the 'sensitiser' BH3-only protein, Bad, promote apoptosis by binding competitively to the anti-apoptotic proteins such as Bcl-xl, thus relieving the suppression of anti-apoptosis signals (Hennessy, 2016). Although there was no apparent shift in the Bax to Bcl-2 ratio, presence of Cytochrome c and other mitochondrial proteins was detected in the cytosol (figure 3.7.1B).

Akl *et al.*, (2014) and Lindsay *et al.*, (2011) reported that Bad is phosphorylated by survival signals such as the PI3K/Akt pathway which promotes binding to 14–3–3 scaffold proteins thus, sequestering it in the cytoplasm. Loss of survival signal leads to Bad dephosphorylation and dissociation from 14–3–3 scaffold proteins, where it can translocate to the mitochondria and interact with Bcl-xl and thus enhancing membrane permiabilisation. Hence, Cytochrome c responsible for the formation of the apoptosome complex and HTRA2/Omi proteins were released from the mitochondria (figure 3.7.1B). Formation of the complex converts a potential cytoplasmic protease pro-caspase-9 to its active form caspase-9 which then cleaves pro-capase-3 to activated caspase-3 (Jiang *et al.*, 2016b). In this study the presence of cleaved-caspase-3 (figure 3.7.3) was detected which suggest the formation of the apoptosome complex and the initiation of a caspase cascade including the activation of caspase-9. Furthermore, the presence of nuclear morphological features associated with apoptosis suggests the activation of this caspase cascade.

Death receptors are the most important modulators of the extrinsic apoptosis pathway (Elrod and Sun, 2014). In this study no notable change in the expression levels of death receptors and their death domains involved in the extrinsic pathway

was observed following treatment with the extract (figure 3.7.2), which suggested that the extract modulates apoptosis in A549 cells *via* the intrinsic apoptosis pathway.

The first IAP antagonists' pro-apoptotic molecule SMAC/Diablo eradicates the inhibitory role of inhibitor of apoptosis proteins, such as XIAP, allowing for full activation of caspase-3 and -9 (Kantari and Walczak, 2011). The second IAP antagonists' pro-apoptotic molecule HTRA2/Omi competitively bind to the BIR domains of IAPs *via* the IAP-binding motif, so that the BIR-bound caspases are released and reactivated (Kavitha *et al.*, 2017). The activation of caspases in A549 cells treated with the extract suggests that the observed release of the mitochondrial protein molecule HTRA2/Omi (figure 3.7.1B) suppressed the activity/expression of XIAP, cIAP1 and cIAP2 proteins (figure 3.7.3).

As stated previously, the mitochondrial apoptosis pathway is activated in response to diverse stress stimuli, including DNA damage or nutrient deprivation. These stress stimuli activate several proteins such as p53 (Aubrey *et al*., 2018). Additionally, most empirically-based chemotherapeutic agents kill tumour cells by inducing irreparable genomic damage, leading to a p53-dependent apoptosis (Thomas *et al.*, 2015). The levels of p53 protein rise substantially because several signalling pathways that are activated in response to the aforementioned stressors converge upon the inhibition of MDM2, whereas some lead to modifications (e.g. acetylation, phosphorylation) in the p53 protein itself (Freed-Pastor and Prives, 2012, Hernandez-Valencia *et al.*, 2018). Phosphorylation of p53 at various residues generally result in p53 stabilisation and activation (Berger, 2010). Reed and Quelle, (2015) reported that, phosphorylation of p53 at serine15, 20, 33, 37 and threonine18 causes selective modulation of gene targets (positive modulation of *p21*; negative modulation of *noxa*).

In this study, phosphorylation of p53 at serine15 was observed accompanied by a downregulation in p21 expression (figure 3.7.4). Even though no upregulation in p21 protein expression was observed, phosphorylation and activation of p53 was accompanied by the upregulation of the protein expression of another regulator of Cdks protein, p27 (figure 3.7.4). Protein p21 and p27 inhibits progression at G1/S-phase checkpoint during cell division cycle (Visconti *et al.*, 2016), hence the G1/S-

phase cell division cycle arrest that was observed in figure 3.5B. The cell division cycle arrest observed might also be due to that, the highest levels of p27 protein expression during Cdks regulation is at the G1-phase and then quickly declined at the S-phase (Du *et al.*, 2015, Kang *et al.*, 2016). Furthermore, p27 is known to bind to a broader spectrum of Cyclin/Cdk complexes already formed including; Cyclin A-E/Cdk2, Cyclin D/Cdk4-6 and Cyclin A-B/Cdk1 heterodimers inhibiting their activities (Yuan *et al.*, 2015).

In this study, low expression levels of *cdc2* and *cyclin B* genes (figure 3.6C and D) led to a more significant cell division cycle arrest of cells at G1/G0-phase (figure 3.5B). According to Nguyen *et al*., (2016), *cdc2* gene drives cells into mitosis and phosphorylation of *cdc2* gene subsequently binds *cdc2* to *cyclin B* forming the maturation-promoting factor complex that mediate their export to mitosis (Spencer *et al.*, 2013) and thus their low expression levels prevents this export. Moreover, Tan *et al*., (2017) reported that levels of *cyclin B* promote entry into mitosis after its accumulation in the cytoplasm during S-phase, thus in this study, the significant decrease in the number of extract-treated cells at S-phase was also due to the decrease levels of *cyclin B* gene.

In conclusion, the results of this study clearly demonstrated the anticancer activity of *M. balsamina* crude leaf acetone extract triggering apoptosis in lung A549 cancer cells. The presence of several secondary metabolites might be the principal cause of the anticancer activity of the crude leaf acetone extract (Madala *et al*., 2016). Features of a necrotic mode of cell death were revealed, however, necrosis mode of cell death was not effective over apoptosis. Hence the Cytochrome c release together with the subsequent activation of caspase-3 further indicates that apoptosis was induced *via* the mitochondrial (intrinsic) apoptosis pathway. The non-absolute change observed on the receptors and the domains involved in the extrinsic pathway together with the inhibited activity of IAPs following the extract treatment further confirms the mitochondrial apoptosis pathway induction. Anti-proliferative activity of the extract resulted in a G0/G1-phase cell division cycle arrest induced furthermore by interactions between *p53*, *p21*, *cdc2*, *cyclin B* genes and their related proteins including p27 protein. This suggested involvement of a p53-mediated mechanism of apoptosis. The results of this study have provided a novel mechanistic insight into this type of traditional herb as a cancer therapeutic and therefore have the potential

to contribute to overall understanding and the future development of *Momordica balsamina* leaf acetone extract as a novel cancer therapeutic.

CHAPTER 5

5. REFERENCES

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