# The Effects of Crude Methanolic Extract of *Commelina benghalensis* Linn on the Expression of Apoptotic and Cell Division Cycle Genes in Jurkat T and Wil-2 NS Cancer Cell Lines.

by

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# DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree of **Philosophiae Doctor (Ph.D.)** in **Biochemistry** has not been previously submitted by me for a degree at this or any University; that it is my work in design and in execution, and that all material contained therein have been duly acknowledged.

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# DEDICATION

This work is dedicated to my mother **Sibongile Gladys Mbazima**, my late father **Dennis Mthangu Mbazima** and my late grandmother **Anna Topini Themba**.

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# LIST OF CONFERENCE PRESENTATIONS AND PUBLICATIONS

- Mbazima, V.G and Mampuru, L.J. Growth inhibitory and apoptosis inducing effects of crude methanolic extracts of *Commelina* species through modulation of apoptosis regulatory genes in Wil-2 NS and Jurkat T cancer cell lines. SASBMB XIX$^{th}$ conference, Stellenbosch University, South Africa, January 16-20, 2005.

- Mbazima, V.G and Mampuru, L.J. Pro-apoptotic effects of *Commelina* species on Jurkat T cancer cell line, Indigenous plant use forum (IPUF) conference, University of Botswana, Botswana, July 3-6, 2006.

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| | |
|---|---|
| Ac-DEVD-pNA | N-acetyl-asp-glu-val-asp-*para*-nitroaniline |
| AIF | Apoptosis inducing factor |
| ALL | Acute lymphoblastic leukemia |
| ANOVA | Analysis of variance |
| Apaf-1 | Apoptosis protease activating factor-1 |
| ATCC | American type culture collection |
| ATP | Adenosine triphosphate |
| Bad | Bcl-2 antagonist of cell death |
| Bak | Bcl-2 antagonist killer 1 |
| Bax | Bcl-2 associated X protein |
| BCA | Bicinichonic acid |
| Bcl-2 | B-cell lymphoma-2 |
| Bcl-$x_L$ | B-cell lymphoma-extra large |
| BH | Bcl-2 homology |
| bHLH-LZ | Basic helix-loop-helix-leucine-zipper |
| Bid | Bcl-2 interacting domain death agonist |
| Bik | Bcl-2 interacting killer |
| Bim | Bcl-2 interacting mediator of cell death |
| BIR | Baculovirus IAP repeat |
| BSA | Bovine serum albumin |
| c-AIP | Cellular-inhibitor of apoptosis |
| $^oC$ | Degrees centigrade (Celsius) |
| CARD | Caspase recruitment domain |
| Cdc2 | Cell division cycle-2 |

| | |
|---|---|
| Cdk | Cyclin-dependent kinase |
| Cdki | Cyclin-dependent kinase inhibitor |
| cDNA | Complementary DNA |
| cFLIP | Cellular FLICE-like inhibitory protein |
| CMECB | Crude methanolic extract of *Commelina benghalensis* L |
| $CO_2$ | Carbon dioxide |
| $C_t$ | Cycle threshold |
| dATP | Deoxyadenosine triphosphate |
| DED | Death effector domain |
| DISC | Death-inducing signaling complex |
| DMEM | Dulbecco s modified eagle s medium |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleoside triphosphates |
| DNase | Deoxyribonuclease |
| DR3 | Death receptor-3 |
| DR4 | Death receptor-4 |
| E | Efficiency |
| EDTA | Ethylenediaminetetraacetic acid |
| EGTA | Ethylene glycol-*bis* (beta-amino-ethyl-ether) - N, N, N', N'-tetraacetic acid |
| Fas | Fas-1 induced apoptosis signal |
| FasL | Fas-1 induced apoptosis signal ligand |
| FADD | Fas-associated death domain |
| FBS | Fetal bovine serum |

| | |
|---|---|
| GAPDH | Glyceraldehyde-phosphate dehydrogenase |
| $G_1$ | Gap 1 |
| $G_2$ | Gap 2 |
| h | Hour |
| HCl | Hydrochloric acid |
| HRP | Horseradish peroxidase |
| IAP | Inhibitor of apoptosis proteins |
| ICAD | Inhibitor of caspase activated DNase |
| IgG | Immunoglobulin G |
| INK4 | Inhibitors of cdk4 |
| KCl | Potassium chloride |
| KIPs | Kinase inhibitor proteins |
| LEHD-pNA | Leu-Glu-His-Asp-*para*itroanilide |
| mA | milliampere |
| Max | Myc associated protein-X |
| MB | Myc Box |
| Mcl-1 | Myeloid cell leukemia-1 |
| MDM2 | Mouse double minute-2 |
| MgCl | Magnesium chloride |
| ml | Millilitres |
| mM | Millimolar |
| M phase | Mitosis |
| mRNA | Messenger RNA |
| MTT | 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide |
| MuLV | Murine leukaemia virus |

| | |
|---|---|
| NaCl | Sodium chloride |
| $NaVO_3$ | Sodium orthovanadate |
| NAIP | Neuronal apoptosis inhibitory protein |
| NP-40 | Nonidet P-40 |
| OMM | Outer mitochondrial membrane |
| PARP | Poly (ADP-ribose) polymerase |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| pRb | Retinoblastoma protein |
| PSN | Penicillin, streptomycin, neomycin |
| Puma | p53-upregulated modulator of apoptosis |
| PVDF | Polyvinylidene fluoride |
| qrt-PCR | Quantitative real-time polymerase chain reaction |
| *rb* | Retinoblastoma gene |
| REST | Relative expression software tool |
| RING | Really interesting new gene |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute |
| RT-PCR | Reverse transcription polymerase chain reaction |
| rRNA | Ribosomal RNA |
| S.D | Standard deviation |
| SDS | Sodium dodecylsulphate |
| SDS-PAGE | Sodium dodecylsulphate-polyacrylamide gel electrophoresis |

| | |
|---|---|
| SMAC/DIABLO | Second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI |
| S phase | DNA synthesis phase |
| tBid | Truncated Bcl-2 inhibitor of death |
| TBS | Tris-buffered saline |
| TEMED | N, N, N', N'–Tetramethylenethylenediamine |
| TNF | Tumour necrosis factor |
| TNFR1 | Tumour necrosis factor receptor 1 |
| TRAIL | Tumour necrosis factor alpha- related apoptosis inducing ligand |
| Tris | Trihydroxymethylaminomethane |
| µg | Microgram |
| µl | Microliter |
| UV | Ultraviolet |
| V | Volts |
| XIAP | X-linked inhibitor of apoptosis protein |

## Abstract

*Commelina benghalensis* Linn is used in traditional medicine in several Asian and African countries for the treatment of various ailments such as stomach irritations, burns, sore throat and feet, diarrhoea and as an anti-inflammatory agent. Recently, our laboratory showed that the crude methanolic extract of *Commelina benghalensis* L (CMECB) exhibits growth inhibitory and pro-apoptotic effects in Jurkat T and Wil-2 NS cancer cell lines. In this study, the precise molecular mechanism(s) associated with CMECB-induced growth inhibitory and apoptosis inducing effects in Jurkat T and Wil-2 NS cell lines were investigated. This was achieved by investigating the effects of the extract on the cell division cycle distribution profile as well as its effects on various cell division cycle and apoptosis regulatory genes. Ground stems of *C. benghalensis* L were extracted with absolute methanol to obtain a crude extract. To assess the effect of CMECB on cancer cell growth, experimental cell cultures were exposed to various concentrations (0 to 600 µg/ml) of CMECB for up to 72 hours. The results demonstrated a significant reduction in cell viability and inhibition of proliferation of experimental cell cultures as determined by the trypan blue dye exclusion assay and the Coulter counter method, respectively. Analysis of nuclear morphological changes in cells stained with Hoechst 33258 confirmed apoptosis as the mode of cell death that is associated with the growth inhibitory effects of CMECB in both the Jurkat T and Wil-2 NS cell lines. This assertion was based on the observed presence of nuclear morphological changes such as chromatin condensation and fragmentation and apoptotic bodies in cells exposed to CMECB. In order to get an insight on the pro-apoptotic mechanisms of CMECB, Western blot

and quantitative real-time PCR (qrt-PCR) were used to investigate the expression profiles of various apoptosis and cell division cycle regulatory genes. Qrt-PCR results showed a lack of a clear up- and/or down-regulatory effects of CMECB on the mRNA expression levels of *bax* and *bcl-2* in both Jurkat T and Wil-2 NS cells.

Western blot analyses demonstrated that CMECB induced apoptosis by facilitating Bax protein translocation from the cytosol to the mitochondria in both Jurkat T and Wil-2 NS cells. In addition, CMECB down-regulated Bcl-2 protein expression which, as a result, led to the shift in the Bax/Bcl-2 protein ratio at certain time points and concentration in both Jurkat T and Wil-2 NS cells. The modulation of the Bcl-2 family members led to mitochondrial cytochrome c release into the cytosol and activation of caspases-9 and -3; this was also confirmed by caspase activity assays and eventual degradation of PARP. Furthermore, CMECB induced Jurkat T and Wil-2 NS cell division cycle arrest at the $G_2$/M phase as determined by flow cytometric analysis. Western blot analyses of $G_2$/M phase regulatory proteins demonstrated that the CMECB-induced cell division cycle arrest was associated with the down-regulation of cyclin B1 and Cdc2 protein expression levels. Western blot analyses results further revealed that the arrest of Wil-2 NS cells at the $G_2$/M phase was independent of p21 protein activity. However, Jurkat T cell division cycle arrest was found to be mediated, in part, by p21. Quantitative real-time PCR results did not show a clear trend in terms of the down- or up-regulatory effects of the extracts on the $G_2$/M phase regulatory genes. The CMECB-induced apoptosis and $G_2$/M arrest was found to occur in a p53-independent

manner due to the lack and down-regulation of p53 protein levels in both Jurkat T and Wil-2 NS cells, respectively. In conclusion, CMECB induces its anticancer activity by inducing $G_2$/M phase arrest and mitochondrial-mediated apoptosis independent of p53 protein activity. Although the study did not perform *in vivo* experiments to ascertain the efficacy of extracts of CMECB against specific tumour types in animal models, the present findings somehow validate the traditional use of *C. benghalensis* L as an anticancer agent. A more definitive study needs to be done to ascertain this assertion.

# Chapter 1

## 1. Introduction

Cancer still remains the leading cause of death in many countries. Because carcinogenesis is a complex process, finding effective therapies often depends on new discoveries about the underlying cellular mechanisms. A number of studies are examining pharmaceutical agents that might interfere with cellular mechanisms in cancerous cells so as to block the growth and progression of tumours (Lin *et al*., 2007). Extensive research has identified various molecular targets that can potentially be used not only for the prevention of cancer but also for its treatment. However, the lack of success with targeted monotherapy resulting from bypass mechanism has forced researchers to employ either combination therapy or agents that interfere with multiple cell signaling pathways (Aggarwal and Shishodia, 2006).

Recently, studies have shown evidence that support the anticancer activity of plant extracts. Ashwagandha leaf extract is reported to have *in vivo* tumour suppressive activity against highly malignant human fibrosarcoma (HT1080) cells that were subcutaneously injected in nude mice to form tumours (Widodo *et al.,* 2007). In another study, Triphala, a herbal formulation consisting of three equal parts of medicinal dried plant fruits *Emblica officinalis, Terminalia belerica* and *Terminalia chebula,* has been reported to induce pancreatic tumour growth inhibition and apoptosis in nude mice (Shi *et al.,* 2008). In addition, a wide array of phytochemicals has shown quite promising anticancer results in this direction, with little or no cytotoxicity to normal cells. These agents are present in edible plants as well as medicinal plants and

have been reported to interfere with multiple signaling pathways. The ability of these agents to interfere with multiple cell signaling pathways stems from their possession of substantial antioxidant, antimutagenic and anticarcinogenic activities (Issa *et al*., 2006; Singh, R.P., *et al*., 2002). These phytochemicals include resveratrol (found in red grapes, peanuts and berries), allicin (garlic), lycopene (tomato), catechins (green tea), beta carotene (carrots), limonene (citrus fruits), ursolic acid (apples, pears and prunes), and dietary fibre (Aggarwal and Shishodia, 2006). The effects of phytochemicals on most of the cell signaling cascades are ultimately linked to the modulation of cell division cycle regulatory molecules leading to growth inhibition and/or apoptotic death of cancer cells (Hsu *et al*., 2006; Srivastava and Gupta, 2006). Indeed, the induction of apoptosis by certain anticancer chemotherapeutic agents is regarded as the preferred way to manage cancer (Brown and Wouters, 1999).

Accordingly, in recent years, non-nutrient phytochemicals, ethno-pharmacologically used herbs and plant extracts are being extensively explored for their potential protective effects against cancer. This is because phytochemicals are relatively non-toxic, inexpensive and available in an ingestive form (Gali-Muhtasib *et al*., 2006). As a result, there is an increasing trend worldwide to integrate traditional medicine with primary health care. This renewed interest in traditional pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for the plants usage but also with the discovery of novel compounds of pharmaceutical value (Fennel *et al*., 2004).

While fruits and vegetables, herbs and other plant extracts are recommended for prevention of cancer and other diseases, their active ingredients (at the molecular level) and their mechanisms of action are less well understood. In an effort to contributing towards understanding the mechanism of action by which phytochemicals protect against cancer, the molecular pathway of the crude methanolic extract of *Commelina benghalensis* L induced apoptosis and its effect on cell division cycle progression will be investigated in this study.

## 1.1. *Commelina benghalensis* Linn

*Commelina benghalensis* Linn [family: Commelinaceae] commonly known as the Benghal dayflower or tropical spiderwort, is an annual or perennial herb with fleshy creeping stems that root readily at the nodes. It is native to the tropical and subtropical Asia and Africa. Although native to Asia and Africa, *C. benghalensis* L is also found in other countries such as the United States of America and Australia where it is classified as one of the troublesome weeds that invades agricultural sites (Webster *et al*., 2005).

In its native areas, *C. benghalensis* L is widely used as a traditional medicine for the treatment of various ailments in the form of a concoction or paste, eaten as a vegetable and also used as fodder for livestock. In India, different parts of *C. benghalensis* L are used in traditional medicine for the treatment of sore feet, sore throat, burns, eye irritation, thrush in infants, stomach irritations (van der Burg, 2004), scabies, wound healing (Anitha *et al*., 2008), fever, snake bites (Sikdar and Dutta, 2008), nervous disorders, swelling and as a laxative (Immanuel and Elizabeth, 2009). In addition, *C. benghalensis* L is

said to be used for softening skin, soothing of internal inflamed or irritated surfaces (Singh, A.K., *et al*., 2002), rabies (Sandhya *et al*., 2006), diarrhoea, leprosy, fever, as well as a stimulant (Parekh and Chanda, 2008). In Nepal, India, the leaves of the plant are said to be used as a paste for the treatment of burns, while the root juice is used to treat indigestion (Manandhar and Manandhar, 2002). In the Chhattisgarh province of India, *C. benghalensis* L (locally called common Rice weed or *Kaua-Kaini*) is used by senior traditional healers in the treatment of cancer (http://botanical.com/site/column_poudhia/articles/_11840.html).

In Pakistan, *C. benghalensis* L is commonly used for treating bedsores, breast sores, pimples (Qureshi *et al*., 2009), skin inflammation and as a laxative (Jabeen *et al*., 2009). In China, *C. benghalensis* L is medicinally used as a diuretic, febrifuge and an anti-inflammatory agent (Hong and DeFillipps, 2000). The stem sap squeezed from *C. benghalensis* L is topically used, in South African traditional medicine, for the treatment of skin lumps or skin outgrowths (K. Mabela, personal communication). Other reported traditional medicinal uses of *C. benghalensis* L include treatment of malaria and soothing of urethral pains (Iwalewa *et al*., 2007).

Alkaloids, caffeine and volatile oils are reported as some of the phytochemical constituents of *C. benghalensis* L (Parekh and Chanda, 2008). Thus, the various bioactivities of the plants may be, in part, attributed to the presence of these chemical groups. Furthermore, some studies have also reported

alkaloids (Wong *et al.*, 2009) and caffeine's (Bode and Dong, 2007) ability to exert anticancer activity by activating cancer cell apoptosis (discussed below)

## 1.2. Apoptosis

Apoptosis or programmed cell death is a well regulated physiological form of cellular suicide. It plays an essential role in maintaining cell numbers, eliminating cells that are redundant, damaged or infected. It is also vital for normal development, maintenance of tissue homeostasis and for an effective immune system (Adams, 2003). Apoptosis is a delicately regulated and balanced process in physiological context. Failure of this regulation results in pathological conditions, such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Jin and El-Deiry, 2005).

Apoptosis was first described in 1972 by Kerr and colleagues. It was described or defined based on morphological changes that had been repeatedly observed in various tissue and cell types when they die. Characteristic apoptotic morphological features include: nuclear condensation, nuclear fragmentation, membrane blebbing, cellular fragmentation into membrane-bound bodies, reduction in cellular size, phagocytosis of the dying cell and lack of an ensuing inflammatory response (Kerr *et al*., 1972; Wyllie *et al*., 1980). Furthermore, these apoptosis-associated distinct morphological changes are choreographed by the activation of a series of proteases called caspases (Fuentes-Prior and Salvesen, 2004).

## 1.3. Caspases: Initiators and executioners of apoptosis

Caspases (cysteine-dependent aspartate-specific proteases) are a family of cysteine proteases that cleave target proteins at specific aspartate residues. These proteases are synthesised as inactive zymogens called procaspases consisting of N-terminal pro-domains of variable length and amino acid sequence, a large (p20) and a small subunit (p10). The domains are separated by an inter-domain region consisting of one or more aspartate residues that are cleaved during the activation of procaspases to active enzymes (Wang and Lenardo, 2000; Denault and Salvesen, 2002; Fan *et al*., 2005). There are approximately 14 known mammalian caspases that have been identified. Based on their structural differences and their role in cellular processes, caspases can be classified into two major groups: inflammatory caspases and apoptotic caspases. The apoptotic caspases can be further subdivided into initiator and executioner caspases (Fig. 1.1) (Boatright and Salvesen, 2003; Degrerev *et al*., 2003).

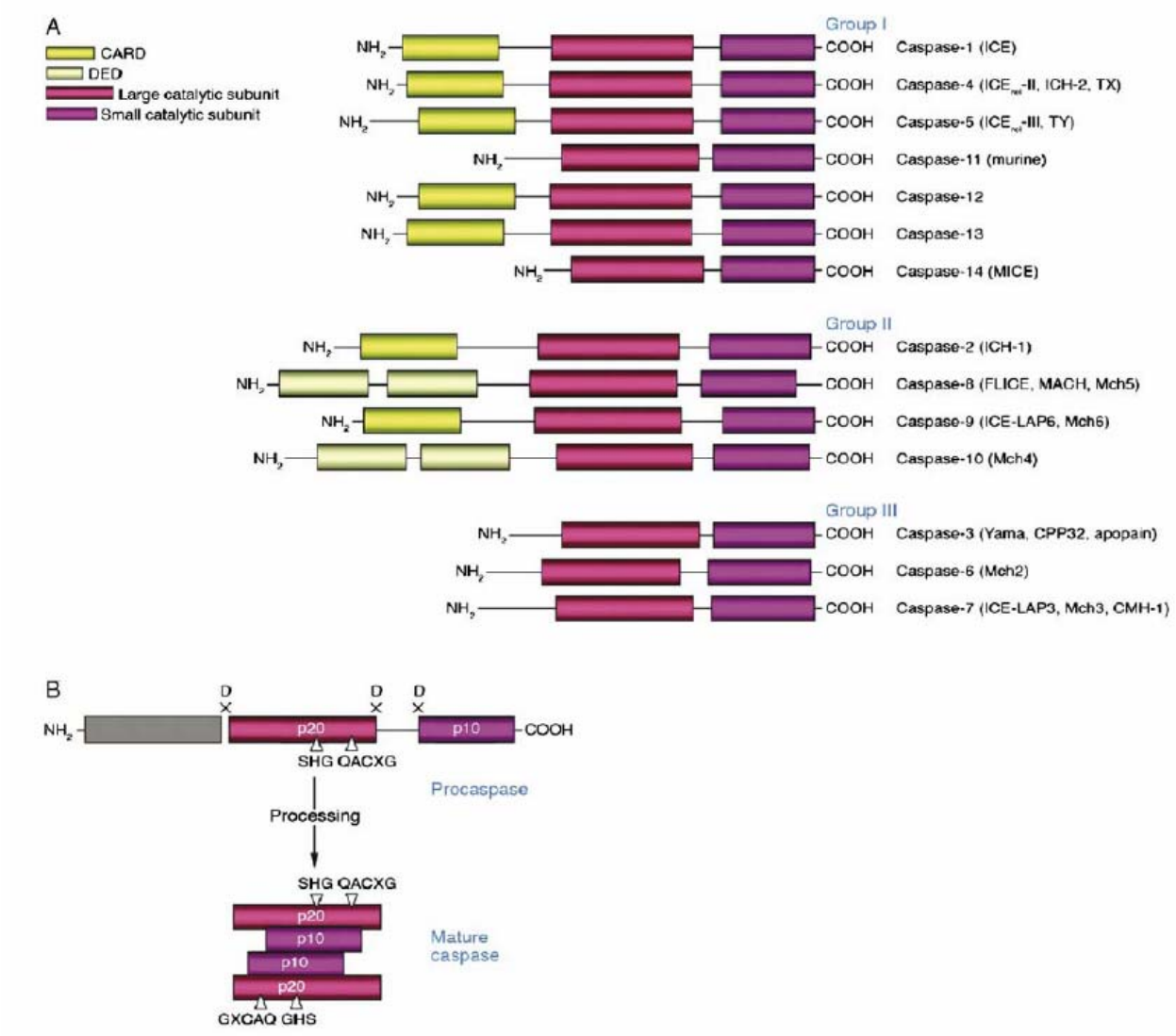



**Figure 1.1 Caspase structure and activation.** (A) The two major classes of caspases: inflammatory caspases (group I) and apoptotic caspases which are further subdivided into initiators (group II) and executioners (group III) of apoptosis. (B) The cleavage of aspartate residues within the linker region found between the N-terminal pro-domain, the large subunit (p20) and the small subunit (p10) of procaspases to generate an active caspase enzyme (Rupinder *et al*., 2007).

### 1.3.1 Initiator caspases

The group of apoptotic initiator caspases consist of caspases-2, -8, -9 and -10. In their procaspase form, initiator caspases possess a long N-terminal pro-domain consisting of motifs called the death effector domain (DED) in caspases-8 and -10 or a caspase recruitment domain (CARD) in caspases-2 and -9. The DED and CARD enable procaspases to interact with adaptor proteins forming part of multi-protein complexes that promote the recruitment and dimerisation of initiator procaspases. As a result of dimerisation, the N-terminal pro-domain is proteolytically removed leading to the formation of an active enzyme consisting of two p20 and two p10 subunits (Boatright *et al*., 2003; Chang *et al.,* 2003; Donepudi *et al.,* 2003, Lavrik *et al.,* 2005). Once activated, initiator caspases proteolytically cleave and activate downstream executioner caspases (Stennicke and Salvesen, 2000).

### 1.3.2. Executioner caspases

The group of executioner caspases comprises of caspases-3, -6 and -7 and are structurally characterised by the presence of a short N-terminal pro-domain (Boatright and Salvesen, 2003; Bossy-Wetzel and Green, 1999). They are activated when proteolytically processed by upstream initiator caspases at aspartate residues found in the inter-domain region between the p10 and p20 domains and also between the N-terminal and the p20 domain (Creagh and Martin, 2001; Denault and Salvesen, 2002). The active executioner cleaves cytoskeletal proteins, cellular DNA repair proteins and cell division cycle-related proteins ultimately leading to the aforementioned morphologic

manifestations of apoptosis (Ghobrial *et al*., 2005; Jin and El-Deiry, 2005; Rupinder *et al*., 2007).

## 1.4. Apoptosis signaling pathways

There are two major apoptosis signaling pathways leading to the activation of a caspase cascade: the extrinsic and intrinsic pathways. The extrinsic or receptor-mediated pathway is mediated by death receptors from the tumour-necrosis factor (TNF) receptor superfamily. The intrinsic or mitochondrial pathway is mediated by the mitochondria and regulated by the B-cell leukaemia-2 (Bcl-2) family of proteins (Fig. 1.2) (Ghobrial *et al*., 2005; Hsu *et al*., 2004). These apoptosis signaling pathways are discussed below.

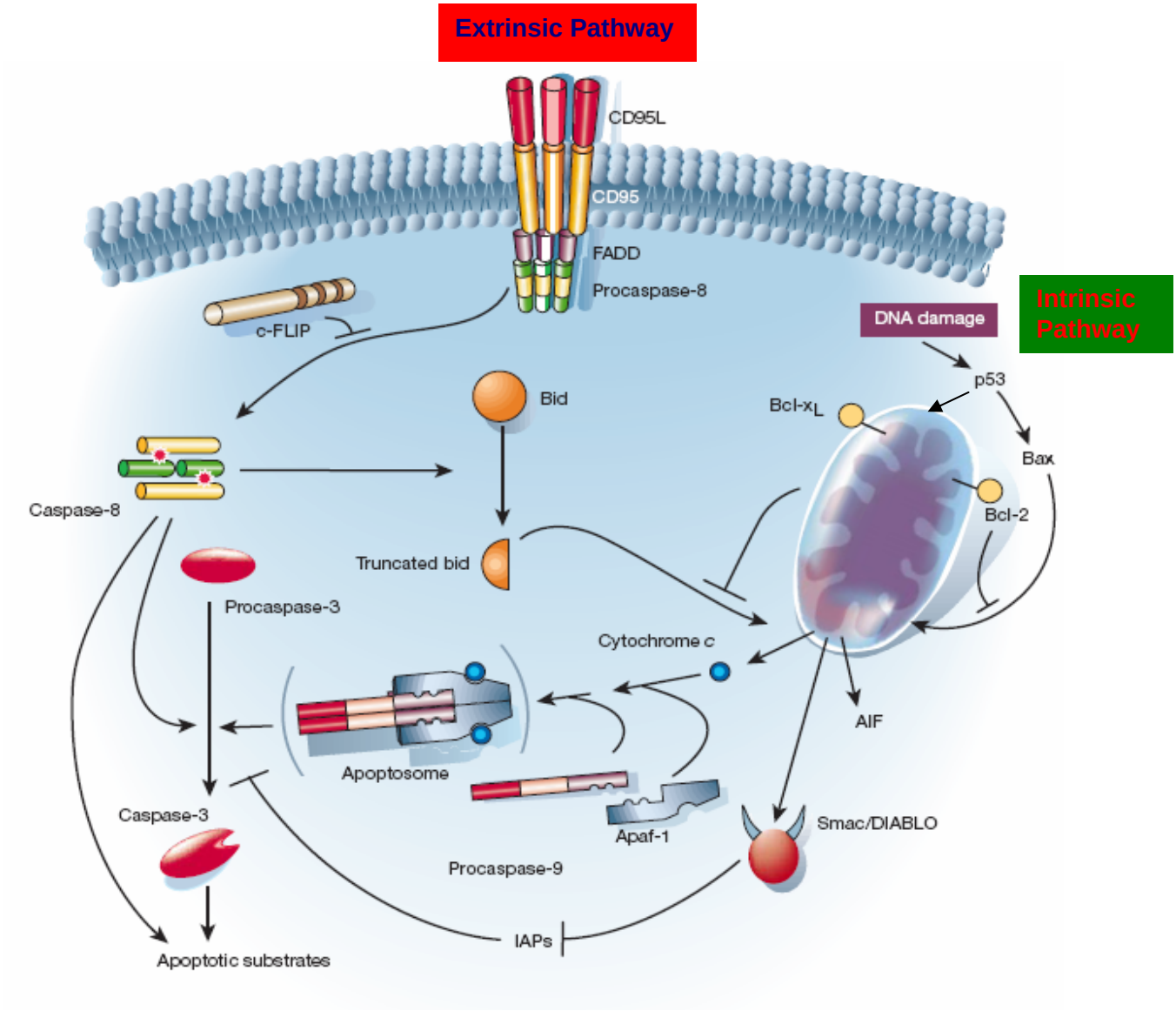



**Figure 1.2 The two main apoptosis signaling pathways.** The extrinsic pathway is mediated by death receptor proteins resulting in the activation of caspases-8 and/or -10. The intrinsic pathway is triggered when cytochrome c is released from mitochondria leading to the activation of the initiator caspase-9. The activation of the above mentioned initiator caspases lead to the activation of executioner caspases and induction of apoptosis (Hengartner, 2000).

### 1.4.1 Extrinsic apoptotic pathway

The extrinsic or receptor-mediated apoptotic pathway begins outside of the cell, when conditions in the extracellular environment dictate that a cell must die. This pathway is mediated by type I transmembrane proteins belonging to the tumour-necrosis factor (TNF) growth factor superfamily of cell surface receptors such as Fas/CD95, DR3, DR4 and tumour necrosis factor receptor type 1 (TNFR1). These receptors are characterised by a conserved extracellullar cysteine rich domain (Ghobrial *et al*., 2005; Zörnig *et al*., 2001), which makes it possible for them to recognise and bind their cognate ligands with specificity (Ashkenazi, 2002). On the cytoplasmic side of the cell, death receptors comprise of death domains which recruit and bind to adaptor molecules required for the initiation of a caspase cascade (Strasser *et al.*, 2000; Zimmermann *et al.,* 2001).

When the cell receives extracellular death signals, ligands [e.g., Fas-ligand (FasL) and TNF] which are type II membrane proteins, initiate apoptosis by binding to type I cell surface receptors, e.g., Fas/CD95. Following binding of a ligand, death-domains of death receptors oligomerise forming trimers leading to the recruitment and binding of the adaptor protein Fas-associated death domain (FADD) *via* death domain-death domain interactions. In addition to the death domain, FADD also contains a death effector domain (DED) in its structure. This DED is involved in the recruitment and binding of initiator caspases-8 and/or -10 to the death domain/FADD complex leading to the formation of a death-induced signaling complex (DISC). Within this complex, procaspases come into close proximity resulting in their dimerisation and

auto-processing giving rise to active caspases-8 and/or -10. (Boatright *et al*., 2003; Chang *et al.,* 2003; Donepudi *et al.,* 2003; Lavrik *et al.,* 2005; Strasser *et al.,* 2000).

Activated caspases-8 and/or-10, in turn, cleave and activate downstream executioner caspases such as caspase-3 and caspase-7 (Qin *et al*., 2001). The activated executioner caspases then attack defined cellular molecules, thus leading to typical biochemical and morphological features of apoptosis (Fig. 1.2) (Ghobrial *et al*., 2005; Holdenrieder and Stieber, 2004; Jin and El-Deiry, 2005; Rupinder *et al*., 2007). Other death receptors are also thought to activate caspase-8 through the same induced proximity mechanism although slightly detailed and complicated sets of adaptors are used by some receptors (Ashkenazi and Dixit, 1999).

In some cell types, the signals of the activated initiator caspases are too weak to trigger cell death. Therefore, the activated initiator caspase-8 cleaves Bcl-2 inhibitory domain (Bid) to truncated Bid (tBid) in order to amplify the death signal and induce cell death. The cleavage of Bid, a pro-apoptotic member of the Bcl-2 family of proteins which controls release of cytochrome c from mitochondria, to tBid in turn activates other pro-apoptotic proteins such as Bcl-2 associated protein X (Bax) and/or Bcl-2 antagonist killer 1 (Bak). The activated Bak/Bax translocate to the outer mitochondrial membrane where they promote the release of cytochrome c into the cytoplasm. As a result of cytochrome c release, the mitochondrial or intrinsic pathway (Fig. 1.2) is

activated (Bremmer *et al*., 2006; Broaddus *et al.,* 2005; Li *et al.,* 2002) in a manner described below.

### 1.4.2 Intrinsic or mitochondria-mediated apoptosis

Intrinsic apoptosis can be initiated by a number of stress stimuli such as UV radiation, heat shock, DNA damage, some tumour suppressor proteins and most chemotherapeutic agents. Upon sensing these stress signals, the outer mitochondrial membrane is permeabilised resulting in the release of intermembrane space contents such as cytochrome c and other pro-apoptotic molecules into the cytoplasm (Kroemer, 2003; Marsden and Strasser, 2003). In the cytoplasm, cytochrome c binds to the C-terminal of the adaptor molecule apoptosis protease-activating factor 1 (Apaf-1) in the presence of ATP/dATP. This induces a conformational change in Apaf-1 leading to the exposure of its caspase associated recruitment domain (CARD) which is located on the N-terminal domain. Following the conformational change, Apaf-1 oligomerises forming a wheel-like structure that recruits and binds procaspase-9 *via* CARD-CARD interactions (Acehan *et al.,* 2002; Green and Kroemer, 2005). This leads to the formation of a DISC-like complex called the apoptosome wherein procaspase-9 is activated to caspase-9 *via* induced proximity dimerisation (Chang *et al.,* 2003; Pop *et al.,* 2006). The activated caspase-9 in turn proteolytically cleaves and activates effector caspases such as caspase-3 (Rich *et al*., 2000; Shi, 2002a). Caspase-3 has been shown to be the primary executioner caspase, necessary for cleavage of poly(ADP-Ribose) polymerase (PARP) and the inhibitor of caspase-activated deoxyribonuclease (ICAD), thus leading to DNA fragmentation and other

morphological changes associated with apoptosis (Hsu *et al.,* 2004).

## 1.5. Regulation of apoptosis

Activation of a caspase cascade during the advent of apoptosis is regulated and executed by different interplay of many genes responsive to various stimuli (Huang *et al*., 2003). Dysregulation of apoptosis resulting in increased or decreased activity is associated with a variety of chemical disorders including cancer, autoimmunity, neurodegenerative diseases and infertility (Rathmell and Thompson, 2002). Some of the proteins involved in the regulation of apoptosis by regulating the activation of caspases are the Bcl-2 family of proteins, the inhibitor of apoptosis (IAP) family of proteins and the cellular Flice inhibitory proteins (cFLIPs).

### 1.5.1. Bcl-2 family of proteins

The cytochrome c-mediated activation of caspases in both mitochondrial-mediated intrinsic and extrinsic apoptotic pathway is regulated by the Bcl-2 family of proteins. This family comprises of approximately 30 members which are characterised by the presence of one or more of the Bcl-2 homology (BH1-BH4) domains (Fig. 1.3). The BH domains make it possible for Bcl-2 family members to interact and form homo- or heterodimers which leads to their inhibition or activation (Reed *et al.,* 2004). Family members are classified as pro- or anti-apoptotic based on their ability to either promote or inhibit cytochrome c release by modulating the permeability of the outer mitochondrial membrane (Bouchier-Hayes *et al*., 2005; Gabriel *et al.,* 2003). Anti-apoptotic members, such as Bcl-2 and B-cell lymphoma extra-large (Bcl-

$x_L$), consist of all four BH domains. The pro-apoptotic members consist of multi-domain pro-apoptotic proteins such as Bax and Bak, consisting of three BH domains (BH1 to BH3) and a subgroup which is composed only of the BH3 domain called the BH3-only pro-apoptotic proteins; examples of this group include Bid, Bim and Bad (Fig. 1.3) (Danial and Korsmeyer, 2004; Strasser, 2005).

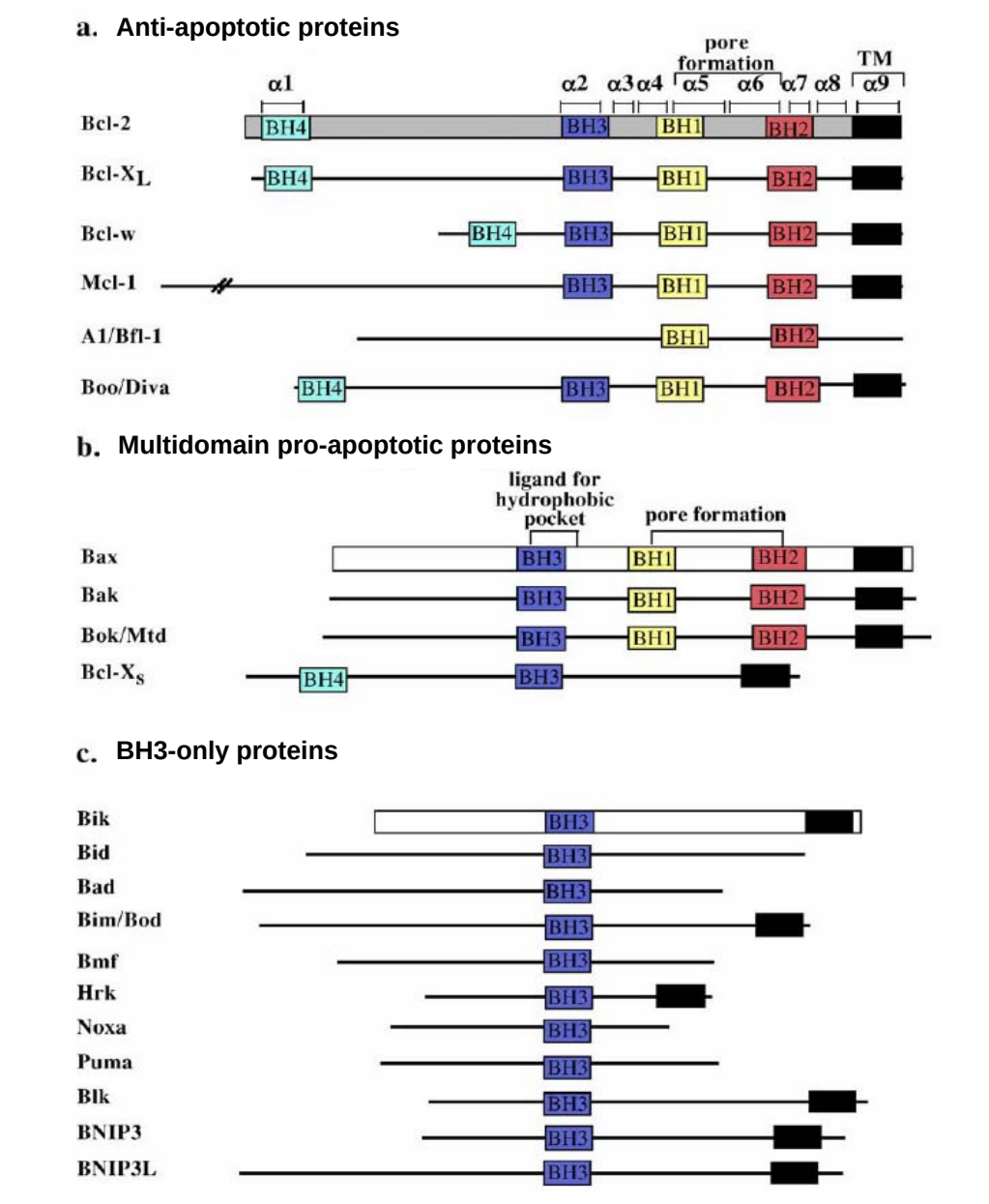



**Figure 1.3. Members of the Bcl-2 family of proteins.** Bcl-2 family members are classified as pro- or anti-apoptotic based on their cellular function. (a) The anti-apoptotic members are characterised by the presence of four conserved Bcl-2 homology (BH1-BH4) domains. Pro-apoptotic members are subdivided into (b) multi-domain and (c) BH3-only pro-apoptotic proteins (Er *et al*., 2006).

### 1.5.2. BH3-only and multi-domain pro-apoptotic proteins

BH3-only proteins are found in most parts of the cell and they serve as sensors of stress signals (Cory and Adams, 2002). Upon sensing stress signals, BH3-only proteins are activated in several ways. These include transcriptional upregulation [p53-upregulated modulator of apoptosis (Puma)], subcellular relocalisation [Bcl-2 interacting mediator of cell death (Bim)], dephosphorylation [Bcl-2 antagonist of cell death (Bad)] and proteolysis (Bid) (Kirkin *et al.,* 2004; Puthalakath and Strasser, 2002). Once active, these proteins can either activate or sensitise cells to undergo apoptosis by interacting with multi-domain pro-apoptotic or anti-apoptotic proteins (Borner, 2003; Letai, 2005).

The sensitiser BH3-only proteins include Bad and Bcl-2 interacting killer (Bik) and they exert their pro-apoptotic activity by binding to anti-apoptotic proteins such as Bcl-2, and thus neutralising them. This leads to the release of the second group of BH3-only proteins called activators, such as Puma, Bid/tBid and Bak. These activator BH3-only and multi-domain pro-apoptotic proteins normally exist as inactive proteins or bound to anti-apoptotic proteins in non-apoptotic cells. Following their release from anti-apoptotic proteins, activator BH3-only proteins then bind and activate multi-domain pro-apoptotic proteins such as Bax and/or Bak (Fig. 1.4) (Galoneck and Hardwick, 2006; Kim *et al.,* 2006).

Bax is a multi-domain pro-apoptotic member of the Bcl-2 family that resides in the cytosol or loosely bound to the outer mitochondrial membrane as a monomeric protein (Cory *et al.,* 2003). Upon receipt of apoptotic signals, activator BH3-only proteins bind and induce conformational change in Bax and/or Bak proteins. As a result, Bax and/or Bak oligomerise forming homodimers and migrate to the outer mitochondrial membrane. The Bax and/or Bak oligomers insert themselves on the outer mitochondrial membrane to form transmembrane channels. Bak and/or Bak oligomers are also suggested to increase the permeability of the outer mitochondrial membrane by interacting with the voltage-dependent ion channel. The disturbance of the outer mitochondrial membrane leads to the release of contents of the intermembrane space, such as cytochrome c and apoptosis-inducing factors (AIFs) into the cytosol and subsequent activation of a caspase cascade (Fig. 1.4) (Danial and Korsmeyer, 2004; Eskes *et al.,* 2000; Letai, 2005).

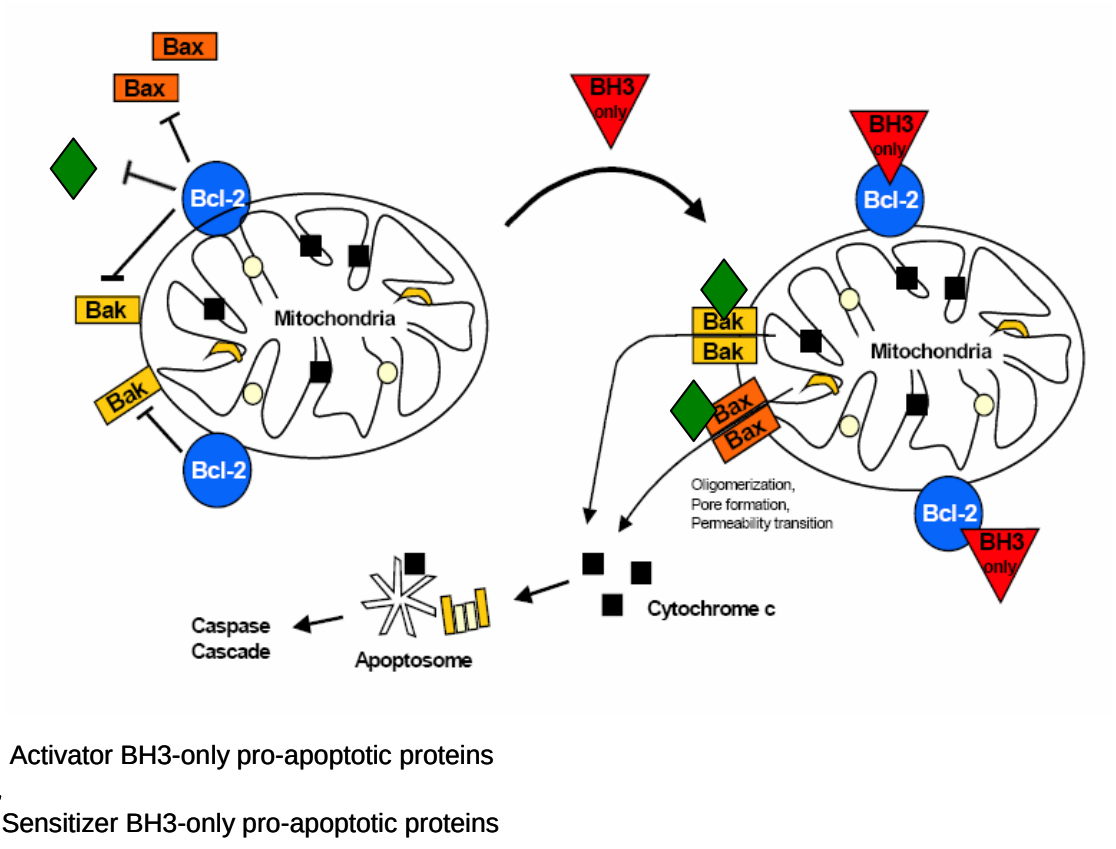



**Figure 1.4. Activation of multi-domain pro-apoptotic proteins**. Upon receipt of apoptotic signals, activated BH3-only pro-apoptotic proteins bind to anti-apoptotic proteins (e.g. Bcl-2, Bcl-xl). This leads to the displacement of activator BH3-only and multi-domain (Bax and/or Bak) pro-apoptotic proteins from the anti-apoptotic proteins. The activator BH3-only proteins then bind and activate multi-domain pro-apoptotic proteins resulting in the formation of pores on the outer mitochondrial membrane. As a consequence of pore formation cytochrome c is released into the cytosol and a caspase cascade is activated *via* the formation of an apoptosome (Gewies, 2003).

### 1.5.3. Bcl-2 and other anti-apoptotic proteins

Bcl-2 protein is an integral, mitochondrial membrane-associated protein with anti-apoptotic effects. This protein and other anti-apoptotic proteins are reported to govern ion transport and protect against breaches in the membrane (Packham and Stevenson, 2005). The Bcl-2 proteins predominantly localise the outer mitochondrial membrane and mediate anti-apoptotic effects by stabilising the mitochondrial membrane. The stabilisation of the mitochondrial membrane leads to the inhibition of pore formation, the release of cytochrome c and the subsequent activation of caspases (Green and Kroemer, 2005). It is suggested that Bcl-2 anti-apoptotic proteins prevent cytochrome c release by sequestering activator BH3-only and forming heterodimers with multi-domain pro-apoptotic proteins. This prevents the BH-3-only pro-apoptotic proteins induced oligomerisation and activation of multi-domain pro-apoptotic proteins such as Bax (Fig. 1.4) (Zörnig *et al*., 2001). Evidence indicates that over-expressed Bcl-2 prevents the activation of caspases in apoptosis and hypoxia-induced necrosis, suggesting that Bcl-2 prevents cell death by acting upstream of the activation of caspases (Puthalakath and Strasser, 2002).

In summary, the sensitivity of cells to apoptotic stimuli depends upon the relative ratios of Bax/Bax homodimers, Bcl-2/Bax heterodimers and Bcl-2/Bcl-2 homodimers. An excess of Bax homodimers promotes cell death, whereas Bax complexed with Bcl-2 favours survival (Danial and Korsmeyer, 2004; Zörnig *et al*., 2001). As mentioned, these proteins regulate apoptosis in part by affecting the mitochondrial compartmentalisation of cytochrome c.

### 1.5.4. Cellular Flice inhibitory proteins (cFLIPs)

Cellular Flice inhibitory proteins are structurally similar to initiator caspases-8 and -10 but lack a functional catalytic domain. To date two variants of Flips have been identified in mammalian cells: $cFLIP_L$, and $cFLIP_S$. $cFLIP_L$ is a long form of cFLIP characterised by presence of two death effector domains located on the N-terminal and a non-functional catalytic domain consisting of large (p20) and small (p12) domains. $cFLIP_S$ is a short form of cFLIP, like the long form, $cFLIP_S$ contains death effector domains on its N-terminal but lack the non-catalytic domain on the C-terminal (Fig. 1.5) (Krueger *et al.,* 2001; Panka *et al.,* 2001).

Both forms of FLIPs are reported to inhibit caspase activation in the receptor-mediated apoptotic pathway at the DISC by binding to FADD *via* their death effector domains and thus preventing the recruitment and the activation of initiator caspases-8. Furthermore, FLIPs are reported to inhibit caspase activation by directly binding to initiator procaspases-8 through DED-DED interactions. This prevents the association of initiator procaspases with the adaptor molecule FADD as well as the formation of a functional DISC which is required for the activation of initiator procaspases (de Thonel and Eriksson, 2005).

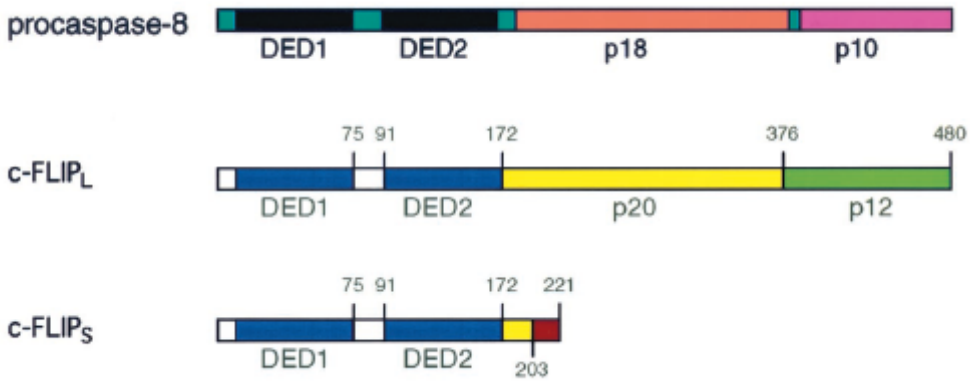



**Figure 1.5. The Structural similarities between procaspase-8 and the two variants of Flice inhibitory proteins** (Krueger *et al*., 2001).

### 1.5.5. Inhibitor of apoptosis proteins

The inhibitor of apoptosis proteins (IAP) family is also regarded as key regulators of apoptosis due to their ability to bind and inhibit active initiator and effector caspases activity (Schimmer, 2004). To date, six members of the IAP protein family have been identified in mammalian cells namely cellular-IAP1 (c-IAP1), cellular-IAP2 (c-IAP1, X-linked IAP (XIAP), neuronal apoptosis inhibitory protein (NIAP), survivin and livin. These proteins are characterised by the presence of between one and three baculovirus IAP repeats (BIR) domain(s) at the N-terminal (Shi, 2002b). c-IAP1, c-IAP2 and XIAP consist of three BIR domains and a C-terminal really interesting new gene (RING) domain that has an E3 ubiquitin ligase activity (Fig. 1.6) (Dean *et al.,* 2007; Yang *et al.,* 2000). In addition to the BIR and RING domains, c-IAP1 and c-IAP2 also contain a CARD which is suggested to play a regulatory role rather than recruiting and binding caspases (Dean *et al*., 2007).

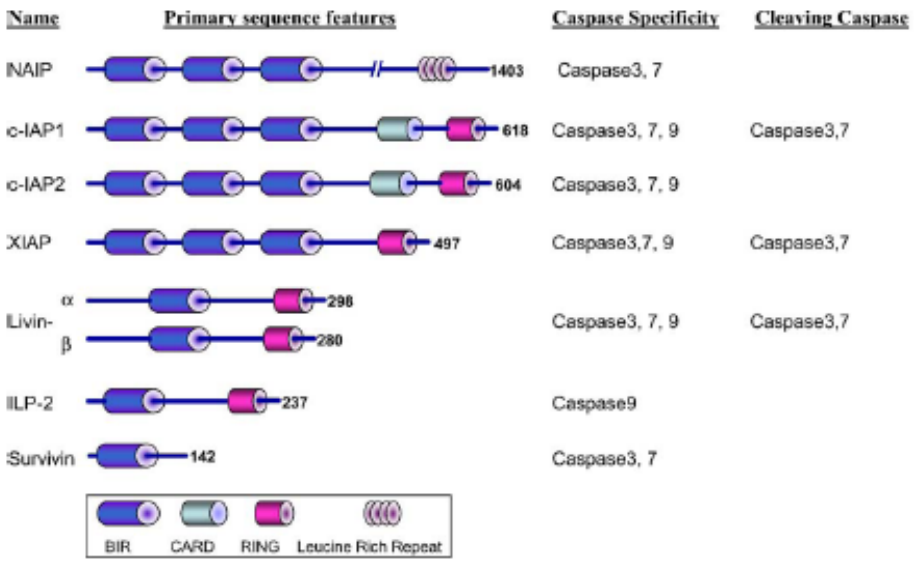



**Figure 1.6. Inhibitor of apoptosis protein (IAP) family members.** The IAP family members are characterised by the presence of one or more BIR domain(s) at the N-terminal. Some members also contain a C-terminal RING or Leucine rich domain (Boaz *et al*., 2004).

The IAP members exert their anti-apoptotic activity by directly binding and inhibiting the active initiator caspase-9 and effector caspases-3 and -7 (Salvesen and Duckett, 2002). There are two ways in which these proteins are suggested to inhibit caspase activity. One involves the BIR domains at the N-terminal and the other involves C-terminal RING domain. XIAP, cIAP1 and cIAP2 BIR3 is reported to specifically inhibit active caspase-9 by directly binding to it. The amino acid sequence linking BIR1 and BIR2 domains is reported to be essential for the inhibiting effector caspases-3 and -7. It is reported that binding of IAP such as XIAP, cIAP1 and cIAP2 to either caspase-3 or -7 results in the linker region lying on the caspase active site. This in turn prevents the effector caspases from binding and cleaving their substrates (Du *et al.,* 2000; Ekert *et al.,* 2001). Although poorly understood, Zimmermann *et al.* (2001) reported that cIAP1 and cIAP2 may also inhibit caspase-9 maturation *via* CARD-CARD interaction found near the C-terminal RING domain.

In addition to their ability to directly bind and inhibit various caspases, IAP members also possess ubiquitilation activity which targets IAPs and proteins bound to them for degradation by the proteosome. This activity is seen as both anti-apoptotic and pro-apoptotic. It is pro-apoptotic in that it facilitates self ubiquitination of IAP and thereby targeting them for degradation, anti-apoptotic in a sense that it can also ubiquinate and target caspases bound to IAP for degradation (Schimmer, 2004; Yang *et al.,* 2000). For the cells to maintain the balance between cell death and proliferation, apoptosis is intimately linked to the cell division cycle (Yu *et al*., 2005).

## 1.6. The cell division cycle

The cell division cycle governs cell proliferation, growth and cell division. Eukaryotic cell division cycle is divided into four distinct phases: gap 1 ($G_1$), DNA synthesis (S phase), gap 2 ($G_2$) and mitosis (M phase). In the S phase DNA is synthesised and during the M phase cells divide into two daughter cells. These events are separated by $G_1$ and $G_2$ phases during which proteins that are required for S and M phase control and progression are synthesised, respectively (Collins and Garrett, 2005; Sherr and Roberts, 1999) (Fig. 1.7). The cell division cycle also consists of two check points ($G_1$/S and $G_2$/M) found at the boundaries between the $G_1$/S and $G_2$/M phases, respectively. These checkpoints are there for the detection of damaged DNA and/or malfunctional organelles or structures such as mitotic spindle which are required during mitosis thus preventing the replication of damaged DNA and proliferation of cells with genetic abnormalities (Bode and Dong, 2007; Park and Lee, 2003; Roberts, 1999). In addition to the above mentioned phases, the cell division cycle also consists of the $G_0$ or quiescent phase wherein cells neither proliferate nor divide. However, the cells grow and perform their functions (Sherr and Roberts, 1999).

The progression of cell division cycle phases and the transition from one phase to the next is facilitated by cyclin-dependent kinases (cdks). The cell division cycle promoting activity of cdks is activated through forming complexes with cyclins. Cdks are also subject to regulation by cdk-activating kinases through phosphorylation and dephosphorylation mechanism(s). There are four known cyclins that are involved in the progression of the cell division

cycle and are expressed at different phases of the cycle. The cyclin D/cdk4/6 and cyclin E/cdk2 complexes are responsible for $G_1$ phase entry and progression and for the transition from $G_1$ to S phase, respectively. Cyclin A/cdk2 is required for S phase progression while $G_2$/M transition and progression are characterised by cyclin B/cdc2 and cyclin A/cdc2 complexes (Fig. 1.6) (Oren, 2003; Sancar *et al*., 2004; Senderowicz, 2003, Swanton, 2004). Cyclin/cdk complexes (cyclin D/cdk4/6 and cyclin E/cdk2) promote cell division cycle entry and progression by phosphorylating the retinoblastoma protein (pRb) which functions as a negative regulator of cell division cycle. The phosphorylation of pRb leads to the activation of E2F transcription factors which in turn induce the transcription of various genes that are required for $G_1$/S transition and DNA synthesis (Nevins, 2001; Stevens and La Thangue, 2003).

The activity of cdks is negatively regulated by a group of proteins called cyclin-dependent kinase inhibitors (cdki) which are classified into two, namely: the inhibitor of cdk4 (INK4) and kinase inhibitor proteins (KIPs). The INK4 class of cdki comprises of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. These proteins negatively regulate the cell division cycle by binding to cdk4/6 thereby inhibiting cyclin D/cdk4/6 complex formation which is required to promote cell division cycle progression. The KIP class (p21, p27 and p57) is also referred to as universal cdk inhibitors because of their ability to bind and inhibit a broad range of cyclin/cdk complexes (Fig. 1.6) (Lin *et al*., 2007; Müller and Helin, 2000; Sherr and Roberts, 1999).

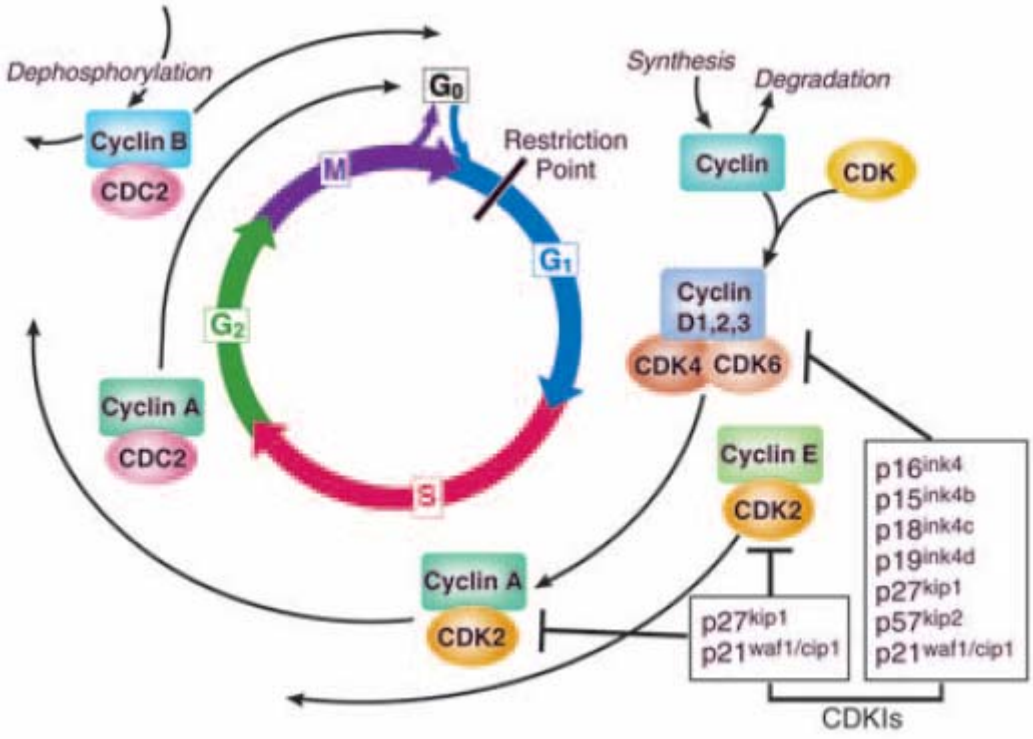



**Figure 1.7. The cell division cycle**. The cell division cycle consists of four non-overlapping phases ($G_1$, S, $G_2$ and M). The transition from one phase to the next is governed by cyclin/cdk complexes which are in turn negatively regulated by a group of proteins called cyclin dependent kinase inhibitors (CDKIs) (Schwartz and Shah, 2005).

## 1.7. Interconnection between the cell division cycle and apoptosis

Increased cellular proliferation and/or decreased cell death in the form of apoptosis are some of the causes of cancer. In addition, cancer can also be a consequence of a failure of cells to differentiate which may result from the breakdown of normal cellular processes that regulate differentiation (Singh, R.P., *et al*., 2002). Multicellular organisms maintain proper control of cell proliferation by keeping a balance between cell proliferation and apoptosis. To maintain this balance, cell division cycle and apoptosis are interlinked through sharing several regulatory proteins (Khosravi-Far and Esposti, 2004; King and Cidlowsky, 1995). This is based on the findings in which several cell division cycle regulatory proteins have been seen playing a regulatory role in the induction and/or repression of apoptosis. Furthermore, this link is observed when cells are exposed to various stresses. Some of the proteins reported to play a regulatory role in both apoptosis and cell division cycle include pRb/E2Fs, c-Myc and p53 (Yu and Zhang, 2005).

### 1.7.1 The c-Myc protein

The c-Myc protein is a member of short-lived nuclear phosphoproteins that transcriptionally regulate various proteins involved in cell proliferation, growth and apoptosis. Other members of the Myc family of proteins include L-Myc and N-Myc (Dang, 1999). The c-Myc protein consists of an N-terminal which comprises of two regions known as Myc Box 1 (MB1) and Myc Box 2 (MB2) which are involved in the activation of transcription. On the C-terminal c-Myc consists of a basic helix-loop-helix-leucine-zipper (bHLH-LZ) which is involved in DNA binding. The bHLH-LZ also facilitates the binding of c-Myc to another

bHLH-LZ protein known as Myc associated protein-X (Max) leading to the formation of a heterodimeric complex. Formation of this complex activates c-Myc's transcriptional regulatory activity and also enhances its ability to bind to specific DNA sequences (Grandori *et al.,* 2000; Oster *et al.,* 2002; Pelengaris and Khan, 2003).

Stimulation of c-Myc protein levels in response to growth factors is reported to promote cell proliferation by inducing S phase entry and progression in cells at the $G_0$ phase of the cell division cycle. The c-Myc protein achieves this through its transcriptional targets cdc25A, cyclin D2, and cdk4 (Dang *et al.,* 2006). C-Myc-mediated cdc25A elevation is reported to activate cyclin E/cdk2 complex formation. Induction of cyclin D2 and cdk4 *via* c-Myc is reported to promote cyclin D/cdk4/6 complex formation. In addition, the accumulation of cyclin D2 is also suggested to repress the inhibitory activity of the cyclinE/cdk2 inhibitor, $p27^{kip}$. All the above mentioned events lead to the release and activation of the family members of transcription factors E2F (E2F2 and E2F3) through the phosphorylation of the retinoblastoma protein (pRb) mediated by cyclin D/cdk4/6 and cyclin E/cdk2 complexes. Activation of E2Fs then facilitates the transcription of various proteins required for $G_1$/S phase progression (Bouchard *et al.,* 2001; Dey *et al.,* 2000; Sherr and Roberts, 1999).

In the absence of growth factors or in response to various stresses, accumulation of c-Myc protein is reported to lead to the induction of apoptosis. The molecular mechanism by which c-Myc promotes apoptotic cell death is

unclear or poorly understood. However, it is suggested that c-Myc executes its pro-apoptotic activity by transcriptionally up-regulating the expression of Mouse double minute 2 (Mdm2) negative regulators, $p19/14^{ARF}$. The accumulation of $p19/14^{ARF}$ proteins blocks Mdm2 activity which results in the accumulation and stabilisation of the p53 protein and apoptosis (Matsumura *et al.,* 2003; Sherr, 2006; Tsantoulis and Gorgoulis, 2005).

### 1.7.2 pRB and E2F complex

The *rb* gene product retinoblastoma protein (pRb) is a phosphoprotein belonging to a group of proteins called pocket proteins which also include proteins such as p107 and p130. These proteins are reported to be involved in the regulation and progression of $G_1$/S phase and in the induction of apoptosis. pRb executes its apoptosis and cell division cycle regulatory activity through interacting with members of the E2F family of transcription factors. The binding of pRb to the transcription factors is in turn regulated by phosphorylation and dephosphorylation mechanisms (Giangrande *et al.,* 2004; Israels and Israels, 2005).

In quiescent cells, pRb is hypophosphorylated (active form) and is normally found associated with E2F proteins. The E2F proteins are a family of transcription factors that promote entry into the S phase in quiescent cells. The binding of pRb to these proteins represses their transcriptional activity and as a result $G_1$/S entry and progression is inhibited. Following growth stimulation signals, pRb is inactivated (hyperphosphorylated) through the activity of cyclin D/cdk4/6 complexes. The hyperphosphorylated pRb is unable

to bind to E2F transcription factors and this leads to their activation and subsequent stimulation of cells at $G_0$ to enter the cell division cycle (Singh, R.P., *et al.,* 2002).

pRb is also suggested to play a regulatory role in apoptosis. Hypophosphorylated pRb is reported to inhibit apoptosis by binding and inhibiting E2F1 which has been reported to induce the transcription of $p14^{ARF}$-p53 mediated apoptosis as described above. In contrast, pRb is also reported to have pro-apoptotic activities. It is reported to promote cell death by binding to p53-Mdm2 complex. As a result the p53 inhibitory activity of Mdm2 is inhibited and a p53-mediated apoptosis is induced (Godefroy *et al.,* 2006). In another mechanism of pRb-mediated apoptosis, hyperphosphorylation of pRb is suggested to increase E2F1 protein levels which in turn lead to the induction of the p53 homolog p73. Activated p73 then induces the transcription of pro-apoptosis genes (Bracken *et al.,* 2004; Phillips *et al.,* 1999).

### 1.7.3 p53

The tumour suppressor protein p53 is a transcription factor that regulates various genes involved in the regulation of cellular response to DNA-damage, cell division cycle control and induction of apoptosis (Liu *et al*., 2007; Xie and Shaikh, 2006). In unstressed cells, p53 is produced as an inactive protein and its levels are generally kept low due to its short half-life (Kasibhatla and Tseng, 2003; Sutcliffe and Brehm, 2004). Mdm2 protein is mainly responsible for keeping p53 protein levels low and blocking its transcriptional activity.

Mdm2 protein promotes the degradation of p53 protein by binding and ubiquitinating p53, through its ubiquitin ligase activity. The ubiquitinated p53 protein is then exported from the nucleus to the cytoplasm and targeted for proteosome degradation (Tweddle *et al*., 2003). In addition, Mdm2 is reported to down-regulate p53's transcriptional activity by binding within p53 transactivation domain located on the N-terminus of the protein and thus preventing its interaction with components of the transcription machinery (Bode and Dong, 2004).

In response to various stimuli such as DNA damage, oxidative stress and exposure to anticancer agents, p53 undergoes posttranslational modifications such as phosphorylation and/or acetylation which are believed to be crucial for the stabilisation, activation and functional activity of the protein (Shu *et al*., 2007). As a result of these modifications, Mdm2 is inhibited from binding to p53 and thus prevent ubiquitination and degradation of the protein (Ryan *et al*., 2001). Phosphorylation of p53 has been reported to not only stabilise p53 by abolishing its interaction with Mdm2, and regulate cellular localisation by inhibiting nuclear export, it also stimulates the transactivation activity of p53 (Sutcliffe and Brehm, 2004). Mdm2 is also reported to undergo phosphorylation when the cell experiences genotoxic or other cellular stress. Phosphorylated Mdm2 has been shown to be less able to promote p53 degradation (DeHaan *et al*., 2001).

Accumulation and activation of p53 in the nucleus stimulates its activity as a transcription factor that can induce growth arrest and/or apoptosis (Lee *et al*.,

2008). Growth arrest mediated by p53 is achieved *via* the transactivation of p53 response gene *p21* which acts by inducing cell division cycle arrest either in the $G_1$/S or $G_2$/M phase (Fuster *et al*., 2007; Shu *et al*., 2007). The p21 protein is a universal inhibitor of cdks; its effect on cdks blocks the formation of the catalytic complexes between cdks and cyclins which are crucial for the control and transition of the cell division cycle from one phase to the next (Kim and Zhao, 2005). The arrest of the cell division cycle allows time for the cell to assess and repair the damage on the DNA, thus preventing the growth of tumour prone cells (Fuster *et al*., 2007). If the damage is extensive, p53 triggers the extrinsic or intrinsic apoptotic cell death (Haupt *et al.,* 2003).

In the extrinsic pathway p53 is reported to transcriptionally activate tumour necrosis factor (TNF) receptor superfamily members such as Fas/CD95, DR4 and DR5/KILLER, a receptor for the death ligand TRAIL (Schuler and Green, 2001; Yu and Zhang, 2005). Activation of these receptor proteins leads to the formation of a death-inducing signaling complex that triggers a caspase cascade leading to apoptosis. p53 is reported to induce the intrinsic apoptotic cell death by transcriptionally activating pro-apoptotic protein Bax (Chipuk and Green, 2006). The activation of Bax protein in turn antagonises the outer mitochondrial membrane-stabilising activity of the anti-apoptotic protein Bcl-2 resulting in pore formation and release of cytochrome c into the cytosol. This leads to the formation of an apoptosome and the activation of a caspase cascade leading to the induction of apoptosis (Debatin, 2004; Khosravi-Far and Esposti, 2004).

In addition several other studies have reported that p53 may also induce apoptosis in a p53-independent transactivation pathway. In this pathway, a tetrameric form of p53 is reported to translocate to the outer mitochondrial membrane where it binds and antagonise the anti-apoptotic activity of the Bcl-2 protein. The p53/Bcl-2 interaction is believed to lead to the formation of pores on the outer mitochondrial membrane and the release of apoptogenic molecules into the cytosol (Liu *et al.,* 2007; Shiah *et al*., 2007).

## 1.8. Motivation of the study

Recently, research on natural diet based agents for anticancer has been focused on activity capable of selective or preferential elimination of cancer cells by inhibiting cell division cycle progression and/or causing apoptosis. *Commelina benghalensis* L is traditionally used for the treatment of skin outgrowth, stomach disorders and as an anti-inflammatory agent. Several studies have reported that various anti-inflammatory agents play an important role in the prevention of cancer. The anticancer activity of these agents is suggested to be attributed to their ability to inhibit inflammation by scavenging free radicals which may cause DNA damage and lead to carcinogenesis. Based on the prevalent usage of *C. benghalensis* L in African and Asian traditional medicine as an anti-inflammatory agent, we hypothesized that the plant may also have anticancer activity. Indeed, we demonstrated from our previous study that treatment of Jurkat T cancer cell line with CMECB resulted in a p53-dependent induction of apoptosis which was associated with the activation of Bax expression and repression of Bcl-2 expression. In addition, the extract induced a p53-dependent Wil-2 NS cells apoptosis which was

associated with the repression of Bcl-2 expression (Mbazima, 2005). Therefore, based on these findings, the present study aims to delineate the precise molecular mechanism(s) of CMECB-induced apoptosis in Jurkat T and Wil-2 NS cancer cell lines. In order to characterise the apoptotic mechanism(s) in these cells, expression levels of key molecules endemic to the intrinsic and extrinsic apoptotic signaling pathways are investigated. Special attention was focused on the possible involvement of cytochrome c, caspases-3 and -9 and PARP in this study model. In addition, the effects of CMECB on p21 protein expression profiles and cell division cycle distribution profiles are evaluated.

## 1.9. Hypotheses:

I. CMECB alters the Bax-to-Bcl-2 proteins expression ratio in Jurkat T and Wil-2 NS cells that leads to mitochondrial release of cytochrome c; this further triggers a caspase cascade that results in the cleavage of PARP and apoptosis.

II. CMECB induces apoptosis in Jurkat T and Wil-2 NS cancer cells and this induction is accompanied by protein p21-mediated cell division cycle arrest.

## 1.10. Aim

The aim of the study was to delineate the molecular mechanisms of CMECB-induced apoptosis and to investigate its effect on the cell division cycle distribution profiles in Jurkat T and Wil-2 NS cancer cell lines.

### 1.10.1. Objectives

The specific objectives of this study were to:

I. Analyse the mRNA and protein expression profiles of *bax, bcl-2* and *p53* after treatment with CMECB using quantitative real-time PCR and Western blot analysis.

II. Investigate the downstream apoptotic events, such as:

- The release of mitochondrial cytochrome c into the cytosol using Western blot analyses.
- The effect of CMECB on caspases-3 and -9 using Western blot analyses and caspase activity assays.

III. Investigate the effect CMECB on the cell division cycle distribution profiles of Jurkat T and Wil-2 NS cells using flow cytometry.

IV. Analyse the mRNA and protein expression profiles of cell division cycle regulatory genes using quantitative real-time PCR and Western blotting, respectively.

V. Establish a possible apoptotic model of CMECB-induced apoptosis in Jurkat T and Wil-2 NS cancer cells.

# Chapter 2

# Materials and Methods

## 2.1 Equipment

- Büchi Rotary evaporator R-205 (Büchi Labortechnik AG, Switzerland).
- $CO_2$ Incubator (NAPCO model, Instrulab cc, RSA)
- Coulter counter model $Z_1$ [Beckman Coulter (Pty), Ltd, RSA]
- Centrifuges (model GS-6R and GS-15R, Beckman Instruments Inc., USA)
- DU730 spectrophotometer (Beckman Coulter, USA)
- EC 120 mini-vertical gel system (E-C apparatus corporation, USA)
- Easy-cast electrophoresis system (Owl Scientific, Inc., USA)
- Hybaid Omnigene thermal cycler (Hybaid Limited, UK)
- Light and Fluorescence Microscope (Zeiss, Germany)
- ChemiDoc XRS image analyser, microtiter plate reader model 550, Western blot transfer system and power-pack (Bio-Rad Laboratories Inc., USA)
- Hypercassette (Amersham, UK)
- Tissue culture flasks (Greiner Bio-one, Germany),
- Rotor-Gene 6000 real-time PCR machine (Corbett Research, Australia)
- Commercial blender (Waring products division, USA)
- Epics Alba flow cytometer (Beckman-Coulter, USA)

## 2.2 Cells, culture media and biochemicals

- Jurkat T and Wil-2 NS cell cultures (American Type Culture Collection, USA)
- RPMI-1640 media, DMEM, foetal bovine serum (FBS), penicillin, streptomycin and neomycin (PSN) mixture (Gibco, New Zealand)
- Acetic acid, bromophenol blue, coomassie blue R200, ponceau S, sodium chloride, ethanol, methanol, $Na_2HPO_4$, $KHPO_4$, EDTA [Saarchem (Pty) Ltd, RSA]
- Monoclonal IgG primary antibodies and goat anti-mouse IgG-HRP conjugated secondary antibodies (Santa-Cruz Biotechnology Inc., USA)
- Caspases-3 and -9 colorimetric assay kits (R&D Systems, Inc., USA)
- High capacity cDNA reverse transcription kit (Applied Biosystems, USA)
- SensiMix dT kit (Quantace, UK)
- Trypan blue, ammonium persulphate, TEMED, sodium orthovanadate, Tris, diethyl pyrocarbonate, EGTA, 2-mercaptoethanol, MTT, sodium dodecyl-sulphate, Hoechst 33258 (Sigma-Aldrich, USA)
- High pure RNA isolation kit, protease inhibitor cocktail (Roche Diagnostics GmbH, Germany)
- PVDF membrane (Pall Corporation, USA)
- Acrylamide and *bis*-acrylamide, nonidet P-40, skimmed powder milk (Fluka Biochemica, Switzerland)
- Tween-20, DMSO**,** KCl [Merck Laboratory Suppliers (Pty) Ltd, RSA]

- BCA protein assay kit, supersignal West Pico chemiluminescent substrate (Thermo Scientific, USA)
- Glycerol [NT Laboratory Supplies (Pty) Ltd, RSA]
- PCR primer pairs (Invitrogen Life Technologies, UK)

## *2.3. Preparation of extract*

The stems of *C. benghalensis* L were harvested from a maize plantation field at Acornhoek, Mpumalanga province in February 2006 and 2007. Stems were stored at 4°C during transportation and were stored at -20°C until extracted. Crude methanol extracts were obtained by extraction of putative bioactive compounds from fresh stems of *C. benghalensis* L. Briefly, the fleshy stems of *C. benghalensis* L were ground into small pieces under liquid nitrogen using a blender (Waring Products Division, USA), followed by extraction with absolute methanol at room temperature for 48 hours. The methanol extracts were dried under vacuum using a rotary evaporator R-205 (Büchi Labortechnik AG, Switzerland) set under low pressure at 40°C. The dried residue was weighed and re-dissolved in dimethyl sulphoxide (DMSO) to give a stock concentration of 200 mg/ml. The quantified extracts were stored at –20°C under a stream of nitrogen in the dark until required.

## *2.4. Cell lines, cell culture maintenance and treatment*

### 2.4.1. Experimental cancer cell lines

**Jurkat T cells** are T lymphocyte cells that were originally derived from peripheral blood of a 14 year old with acute lymphoblastic leukemia (ALL). They have a round shape and grow in suspension as single cells or clumps in RPMI-1640 medium supplemented with 15% foetal bovine serum (FBS) and have a doubling of 25-35 hours (Schneider *et al*., 1977).

**Wil-2 NS cells** are non-immumoglobulin secreting B lymphocytes. They are variants of Wil-2 derived from a spleen of a Caucasian male with hereditary

spherocytic anaemia. They have a lymphoblast shape and grow in suspension as single cells or clumps in RPMI-1640 medium supplemented with 10% FBS. Under these culture conditions the Wil-2 NS cells have a doubling time of 18-24 hours (Levy *et al*., 1971).

### 2.4.2. Cell culture and treatment

Jurkat T and Wil-2 NS experimental cell cultures were obtained from American type culture collection (ATCC, USA). Cells were maintained and grown in tissue culture flasks at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ in RPMI-1640 supplemented with 10% (v/v) heat inactivated FBS and 1% antibiotic (penicillin, streptomycin and neomycin, PSN) mixture. Normal monkey Vero cell line was kindly donated by Prof J Eloff (University of Pretoria) and maintained at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ in DMEM medium supplemented with 5% heat inactivated FBS and 1% PSN.

For the treatment of Jurkat T and Wil-2 NS cultures, the stock solution was diluted with RPMI-1640 medium supplemented with 10% FBS to give final concentrations ranging from 0 to 600 µg/ml. The solutions were filter sterilised through a 0.22 µm pre-sterilised GP express plus steritop™ filter (Millipore Corporation, USA) before testing. The final concentration of DMSO in the CMECB-treated experimental cultures did not exceed 0.1%. Control or untreated cultures were exposed to 0.1% of the drug vehicle DMSO. For experiments, cells were seeded at a concentration of 2 x $10^5$ cells/ml and

treated with 0, 100, 400 (for Jurkat T cells) or 350 (for Wil-2 NS cells) and 600 µg/ml of CMECB, unless stated otherwise.

## 2.5. *Cell proliferation and assessment of cell viability*

To determine the effect of the crude methanolic extracts of CMECB on cell proliferation and viability, Jurkat T and Wil-2 NS cells were treated with different concentrations of the extract and incubated for 24, 48 and 72 hours. To assess the effect of the extract on cell proliferation, cells were sampled and counted at different times of incubation using a Coulter counter model $Z_1$ (Coulter Electronics, RSA). Percentage cell viability was assessed by staining cells with 0.4% trypan blue dye. Numbers of dead and viable cells were counted using a haemocytometer under a light microscope (Zeiss, Axiolab, Germany), and cell viability expressed as percentage per 100 cells counted.

The effect of CMECB on the normal monkey Vero cells was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay. Briefly, normal monkey Vero cells were seeded in a 96-well microtiter plate at 1 $x10^5$ cells/well and exposed to various concentrations of CMECB (0-1000 µg/ml) for 24 hours after allowing them to attach for overnight. After treatment, 50 µl of MTT (5 mg/ml) solution was added to each well and the plate was incubated for an additional 4 hours at 37°C. Following the careful removal of the medium and MTT, 100 µl of DMSO was added to each well to dissolve the formazan crystals formed within the cells. The amount of the dissolved formazan was measured at 570 nm using a microtiter plate reader model 550 (Bio-Rad Laboratories Inc., USA). The percentage survival was calculated

using the following formula: % survival = (Experimental absorbance values/ Control absorbance values) x 100.

## 2.6. Examination of apoptotic nuclear morphology

Nuclear morphological changes in Jurkat T and Wil-2 NS cells exposed to CMECB for 3 hours were examined using fluorescence microscopy. Experimental cell cultures were exposed to different concentrations of CMECB for 3 hours. Following treatment, cells were harvested by centrifugation at 100 *x g* for 10 minutes and washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4. After washing, cells were stained with 50 μg/ml of Hoechst 33258 stain (Sigma-Aldrich, USA) for 10 minutes. Nuclear morphological changes, occurring in the cells as an indicator of the advent of apoptosis, were then viewed under fluorescence microscopy (Zeiss, Axioskop, Germany).

## *2.7. Quantitative real-time PCR analysis*

The effects of CMECB on mRNA expression levels of various apoptosis and cell division cycle regulatory genes were analysed using quantitative real-time polymerase chain reaction (qrt-PCR). Experimental cells were treated with various concentrations of CMECB and then harvested at 1, 6, 12 and 24 hours. The cells were washed twice with ice-cold PBS, pH 7.4; total RNA was then isolated using high pure RNA isolation kit according to the manufacturer′s instructions (Roche Diagnostics GmbH, Germany). The isolated RNA was quantified by reading the absorbance at 260 nm with an OD

of 1 being considered equivalent to 40 µg/ml of RNA. The quality of RNA was evaluated by determining the $A_{260}/A_{280}$ ratio.

Complementary DNA (cDNA) strands were synthesised by reverse transcribing 1 µg of RNA in a reverse transcription reaction mixture containing 1x PCR buffer, 0.5 mM deoxy-nucleoside triphosphates (dNTPs) mix, one unit of RNAse inhibitor, random RT-PCR primers and 2.5 units of MuLV reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA). After 10 minutes of incubation at room temperature to allow primer annealing, the reaction mixture was incubated at 42ºC for 15 minutes, heated to 95ºC for 5 minutes, and chilled at 4ºC for 5 minutes in a Hybaid Omnigene thermal cycler (Hybaid Limited, UK). The resultant cDNA was quantified by reading the absorbance at 260 nm with an OD of 1 being considered equivalent to 33 µg/ml of DNA.

For quantitative real-time PCR, cDNA (equivalent to 100 ng) was amplified in 25 µl reaction mixtures containing 1x SensiMix master mix, 1x Sybr green (Quantace, UK) and 0.8 µM of each reverse and forward primers. The cycling conditions consisted of 10 minutes polymerase activation at 95$^{o}$C and 30 (for*, GAPDH*) or 40 (for *bax, bcl-2, cdc2, cyclin B1, p53 and p21*) cycles of annealing at 58°C (for *bcl-2, cdc2, cyclin B1, GAPDH, p53 and p21*) or 60°C (for *bax*) for 15 seconds, elongation at 72$^{o}$C for 20 seconds and denaturation at 95$^{o}$C for 10 seconds, using the Rotor-Gene 6000 system (Corbett Research, Australia). The primer sequences listed in Table 1 were used for

the amplification of the respective genes and were obtained from the PrimerBank and synthesised by Invitrogen Life Technologies, UK.

Quantification of the molecular concentration of template cDNA was performed with the standard curve method for relative quantification. The real-time PCR efficiencies (E) were calculated by the Rotor-Gene 6000 series software 1.7.87 (Corbett Research, Australia). The baseline and the threshold were automatically set by the Rotor-Gene 6000 series software 1.7.87. The crossing point of the amplification curve with the threshold represented the cycle threshold ($C_t$). In addition, the real-time reaction of the products was checked by melting point analysis after each reaction. The expression levels of the genes of interest were determined relative to *GAPDH* using the relative expression software tool (REST-RG) (Pfaffl ***et al.,*** 2002).

Table 1. Primer sequences used in quantitative real-time PCR analysis

| | |
|---|---|
| Bax | Forward primer: 5′-GGG TGG TTG GGT GAG ACT C-3′ |
| | Reverse primer: 5′-AGA CAC GTA AGG AAA ACG CAT TA-3′ |
| Bcl-2 | Forward primer: 5′-CTG TCT GGA ATA GAA GGC ACT CT-3′ |
| | Reverse primer: 5′-ACC CTC GTC TTT TAG AAA CAG GA-3′ |
| p53 | Forward primer: 5′-ATT GCC AGA GGT TTT ACC GAG-3′ |
| | Reverse primer: 5′-CGA AAC TCC CAC GGA TAG AAT CT-3′ |
| cyclinB1 | Forward primer: 5′-AGA AGA CGC CTC TTT CCC AGT-3′ |
| | Reverse primer: 5′-GGG TAA AGT TGA CCT GGT TCA G-3′ |
| Cdc-2 | Forward primer: 5′-ACC AGA TCG ACT TGA TCG TGC-3′ |
| | Reverse primer: 5′-CGC CCT TTT CTT AGG GTC GTA-3′ |
| GAPDH | Forward Primer: 5′-ACC CAC TCC TCC ACC TTT G-3′ |
| | Reverse Primer: 5′-CTC TTG TGC TCT TGC TGG G-3′ |
| p21 | Forward primer: 5′-TGC AAC ATT TTC GGC AGC TAA-3′ |
| | Reverse primer: 5′-TCC TCA AAT TCG TCA AAG GGT TC-3′ |

## *2.8. Extraction of total cellular and mitochondrial proteins*

Experimental cultures exposed to different concentrations of CMECB were harvested by centrifugation at *100 x g* and washed twice with ice-cold PBS, pH 7.4. Total cellular protein extracts were prepared by resuspending cells in a lysis buffer [2 mM Tris-HCl, pH 8.0, 1% Nonident P-40, 13.7 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate ($NaVO_3$) and protease inhibitor cocktail (Roche Diagnostics GmbH, Germany)] for 20 minutes on ice. Lysates were centrifuged at 14 000 *x g* at 4°C for 15 minutes and the supernatants stored at -20°C until needed for Western blot analysis.

For extraction of mitochondrial proteins, experimental cultures that were seeded at 5 x $10^5$ cells/ml were exposed to different doses of CMECB for up to 24 hours. After treatment, cells were harvested as described above and washed with ice-cold PBS, pH 7.4. Cell were then lysed in isotonic sucrose buffer (250 mM sucrose, 10 mM HEPES-KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, pH 8.0, 10 µM digitonin and protease inhibitor cocktail) for 5 minutes at 37°C. After centrifuging at 15 000 *x g* for 10 minutes at 4°C, the resultant pellets were lysed with ice-cold 0.5% Triton X-100 in isotonic sucrose buffer for 10 minutes on ice. Lysates were centrifuged at 15 000 *x g* for 10 minutes and the supernatants containing mitochondrial proteins were stored at -20°C for Western blot analysis. Protein concentration of total cellular and mitochondrial protein extracts was determined by using the BCA assay kit (Thermo Scientific, USA).

## 2.9. Western blot analysis

Aliquots containing equal amount (30–40 µg) of total cellular or mitochondrial proteins were boiled in 2x sodium dodecyl sulphate (SDS) sample loading buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8)] before being resolved on a 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Proteins on the gels were electro-blotted onto PVDF membrane (Pall Corporation, Pensacola, FL, USA) using a blotting buffer (20% methanol, 25 mM Tris, 192 mM glycine, pH 8.3) at 200 mA for 90 minutes at 4°C. Following electro-blotting, the membranes were blocked with 0.05% TBS-Tween (25 mM Tris, 125 mM NaCl, pH 7.4) containing 3% skimmed milk for 1 hour at room temperature. After blocking, the membranes were washed three times for 10 minutes each with wash buffer (0.05% TBS-Tween without milk), and then incubated with specific primary goat monoclonal anti-mouse antibodies: Bcl-2 (1: 500), caspases-3 and -9 (1: 500), cytochrome c (1: 000), cyclin B1 (1: 500), Cdc2 (1: 500), p21 (1: 500), PARP (1: 500), Bax (1: 250), and p53 (1:500) at 4°C overnight. After washing three times with washing buffer for 10 minutes each, membranes were further incubated for 1 hour in the presence of a peroxidase-conjugated goat IgG secondary antibody (1: 10 000) diluted with blocking buffer. The membranes were washed again as described above and immunoreactive proteins were then detected using the supersignal west pico chemiluminescent substrate (Thermo Scientific, USA) and visualised using the ChemiDoc XRS image analyser (Bio-Rad Laboratories Inc., USA). To ensure that equal amounts of protein were loaded, GAPDH was used as an internal control.

## 2.10. *Caspase activity assays*

The effect of CMECB on the activities of caspase-3 and caspase-9 was assessed using caspase-3 and caspase-9 colorimetric assay kits (R&D Systems, Inc., USA) following the manufacturer's instructions. Briefly, Jurkat T and Wil-2 NS cells (3 x $10^5$ cells/ml) were exposed to various concentrations of CMECB for 0.5, 1, 3, 6 and 12 hours. After treatment, the control and treated cells were collected by centrifugation at 100 *x g*, washed twice with ice-cold PBS, pH 7.4, and lysed by incubating in a lysis buffer for 10 minutes on ice. Following centrifugation at 10 000 *x g* for 1 minute at 4°C, aliquots of the supernatants were used to determine protein concentration using BCA assay kit (Thermo Scientific, USA). Caspases-3 and -9 activities were assayed by incubating equal amount of protein lysates in reaction mixtures containing caspase-3 (Ac-DEVD-pNA) or caspase-9 (LEHD-pNA) substrates and caspase-3 or caspase-9 assay reaction buffers for 2 hours at 37°C. The release of *para*-nitroaniline (pNA) was measured at 405 nm using a microtiter plate reader model 550 (Bio-Rad Laboratories Inc., USA).

## *2.11. Cell division cycle distribution analysis*

Cell division cycle synchronisation of Jurkat T and Wil-2 NS cells was achieved by culturing cells (8 x $10^5$ cells/ml) in RPMI-1640 medium supplemented with 0.04% FBS for 24 hours. This resulted in the arrest of cells at the $G_0/G_1$ phase of the cell division cycle. Synchronised cells (1 x $10^5$ cells/ml) were treated with designated concentrations of CMECB for 24, 48 and 72 hours in RPMI-1640 medium supplemented with 10% FBS. After treatment, cells were harvested by centrifugation at 100 *x g* for 10 minutes,

washed twice with ice-cold PBS, pH 7.4, and fixed in 70% ethanol at -20°C. Fixed cells were washed twice with 1% BSA in PBS and re-suspended in 0.5 ml of DNA staining buffer [0.05 mg/ml propidium iodide, 40 mM, 1 mg/ml RNAse (DNAse free), 0.1% NP-40 and 40 mM sodium citrate, pH 7.6] in the dark for 30 minutes at 37°C. Following incubation, cell division cycle distribution was assessed using Epics Alba flow cytometer (Beckman Coulter, USA). Data from 10 000 cells per sample were collected and analysed by using the cell fit analysis program (Beckman Coulter, USA).

## *2.12. Data presentation and statistical analysis*

All values are expressed as mean ± standard deviation (SD). The data was analysed by one-way ANOVA followed by Dunnett's comparison test using GraphPad InStat Software. Differences between the means of control and CMECB-treated cells were considered significant at $p \leq 0.05$.

# Chapter 3

## Results

### 3.1. CMECB inhibits cancer cell growth

The first objective was to investigate whether CMECB treatment inhibits the growth of Jurkat T and Wil-2 NS cancer cells. As shown in Figs, 3.1A and 3.2A, CMECB inhibited the proliferation of Jurkat T and Wil-2 NS cells in a dose-dependent manner as determined electronically by the Coulter counter method. The inhibition of proliferation suggested cell death in the experimental cultures. To confirm this, the viability assay was conducted using the trypan blue dye exclusion assay. The results shown in Figs, 3.1B and 3.2B revealed that CMECB not only inhibited the proliferation of test cell cultures but also was able to decrease the percentage viability of the cell cultures in a dose- and time-dependent manner. Significant inhibition of cell proliferation and reduction in cell viability was observed in cells exposed to 400 and 600 µg/ml of CMECB. As a control experiment, treatment with CMECB concentrations of up to 1000 µg/ml did not have an inhibitory effect on the viability of normal Vero monkey cells (Fig. 3.3). These results demonstrate that the CMECB has a cell growth inhibitory activity that is specific against the experimental cell cultures and not against the normal cells.

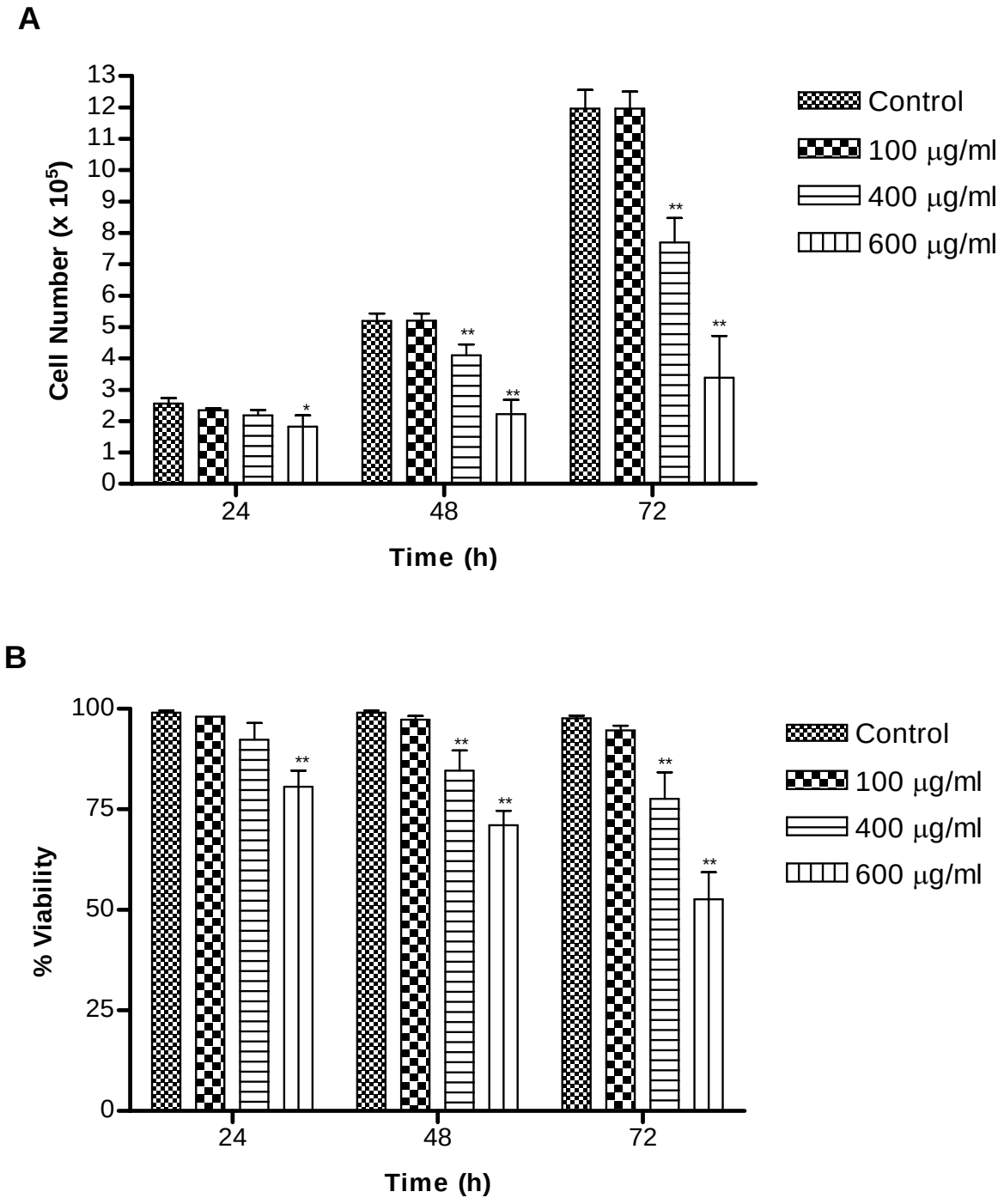



**Figure 3.1.** The effect of CMECB on the proliferation (A) and viability (B) of Jurkat T cells. Cells were cultured with or without different concentrations of CMECB for 24, 48 and 72 hours, and the cell number and viability were determined using the Coulter counter and the trypan blue dye exclusion assay, respectively. Each data point represents the mean± S.D. of three independent experiments. *p< 0.05, **p< 0.01.

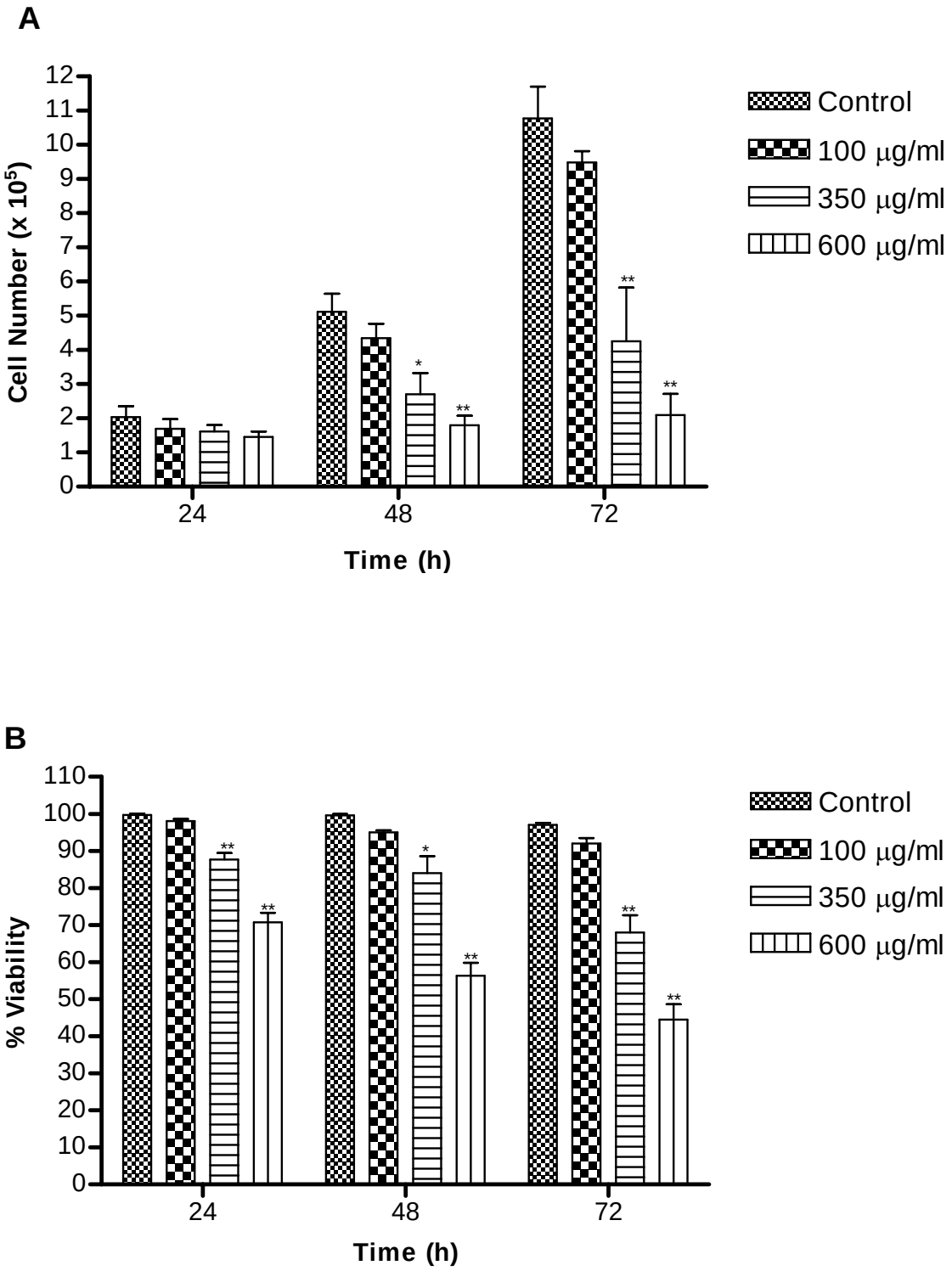



**Figure 3.2.** The effect of CMECB on the proliferation (A) and viability (B) of Wil-2 NS cells. Cells were cultured with or without different concentrations of CMECB for 24, 48 and 72 hours, and the cell number and viability were determined using the Coulter counter and the trypan blue dye exclusion assay, respectively. Each data point represents the mean± S.D. of three independent experiments. *p< 0.05, **p< 0.01.

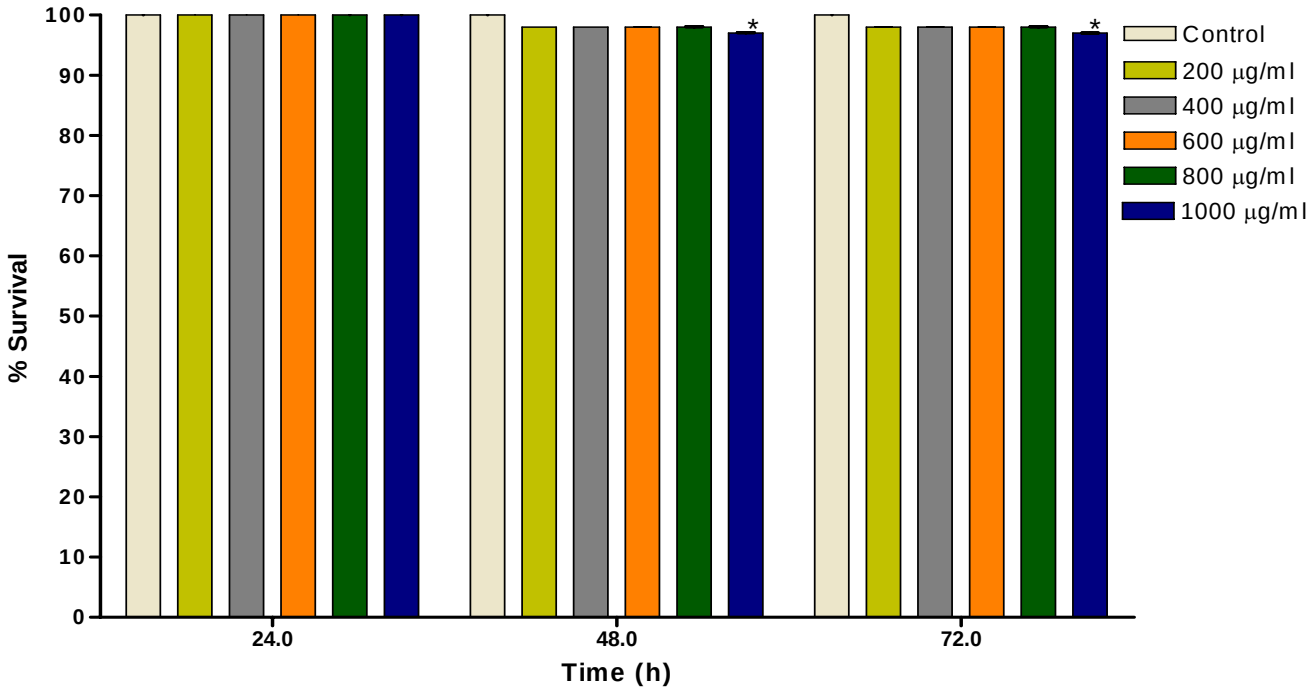



**Figure 3.3.** The effect of CMECB on the viability of normal Vero monkey cells. Cells were cultured with or without different concentrations of CMECB for up to 72 hours and the cell viability was assessed by the MTT assay. Each data point represents the mean± S.D. of three independent experiments performed in duplicates. *p< 0.05.

## 3.2. CMECB induces nuclear morphological changes indicative of apoptosis

To investigate whether the antiproliferative activity and the decrease in cell viability of Jurkat T and Wil-2 NS cells were a result of the induction of apoptosis, nuclear morphology of both cells lines, treated with or without CMECB for 3 hours, was examined. CMECB-treated Jurkat T and Wil-2 NS cells showed typical nuclear morphological changes indicative of the induction of apoptosis. These features include condensation and margination of the chromatin as evidenced by the light blue staining of the chromatin and the formation of apoptotic bodies (Figs. 3.4 and 3.5). On the other hand, the control cells showed intact and uniform blue staining of the chromatin which was less bright when compared to apoptotic cells, while few showed features that are characteristic of apoptosis (Figs. 3.4 and 3.5); this was not a surprising observation since apoptosis is a normal physiological process. These results give an indication that CMECB-induced anticancer activity in Jurkat T and Wil-2 NS cells is associated with the induction of apoptotic cell death.

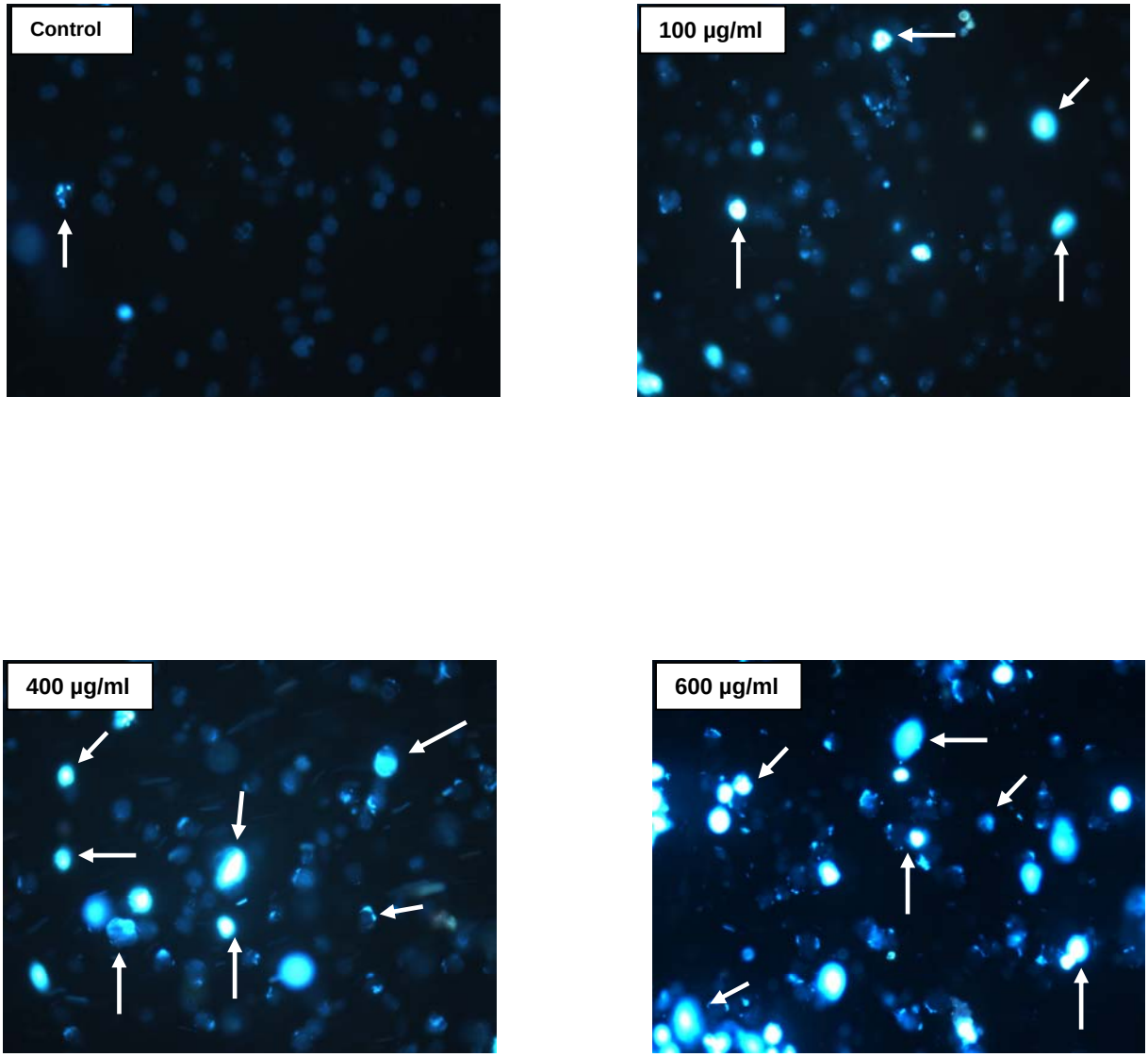



**Figure 3.4.** CMECB-induced nuclear morphological changes in Jurkat T cells. Cells were exposed to various concentrations of CMECB for 3 hours, stained with Hoechst 33258 and viewed under a fluorescence microscopy. Arrows are highlighting the presence of apoptotic features such as chromatin condensation and margination as well as the formation of apoptotic bodies. Magnification: 40X.

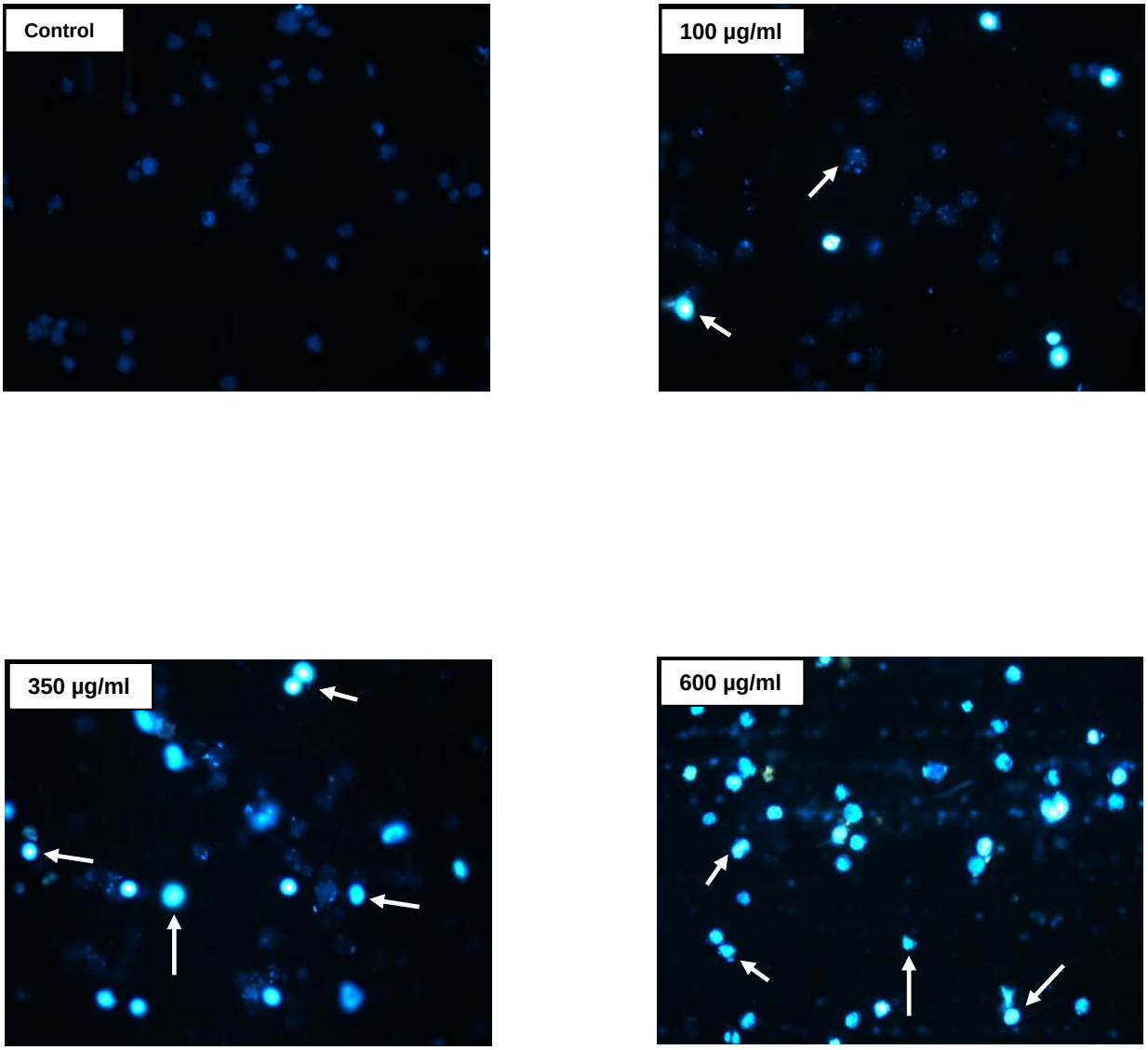



**Figure 3.5.** CMECB-induced nuclear morphological changes in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB for 3 hours, stained with Hoechst 33258 and viewed under a fluorescence microscopy. Arrows are highlighting the presence of apoptotic features such as chromatin condensation and margination as well as the formation of apoptotic bodies. Magnification: 40X.

## 3.3. CMECB induces Bax translocation and Bcl-2 down-regulation in Jurkat T and Wil-2 NS cells

Members of the Bcl-2 family of proteins are known to play a crucial role in the promotion or inhibition of apoptosis. To investigate the involvement of the members of the Bcl-2 family of proteins in CMECB-induced apoptosis in Jurkat T and Wil-2 NS cells, Bax and Bcl-2 protein expression profiles were assessed using Western blotting. As shown in Fig. 3.6, the exposure of Jurkat T cells to various concentrations of CMECB resulted in the down-regulation of Bcl-2 protein expression as compared to the control cells at 3 and 6 hours. A dose-dependent decrease of Bcl-2 protein expression was observed in Jurkat T cells exposed to CMECB for 0.5, 1, 12 and 24 hours. Exposure of Jurkat T cells to 400 and 600 µg/ml of CMECB resulted in the maximal down-regulation of Bcl-2 protein levels ranging between 83-97% and 76-99% after 12 and 24 hours, respectively (Fig. 3.6). Western blot analysis of Bcl-2 protein expression in Wil-2 NS cells treated with different concentrations of CMECB revealed a dose-dependent decrease in Bcl-2 protein expression at all experimental time intervals evaluated (Fig. 3.10). The down-regulation of Bcl-2 protein expression was detected as early as 0.5 hour after treatment and lasted up to 24 hours, with the maximal down-regulatory effects of 91 and 98% being observed after 12 hours in the presence of 400 and 600 µg/ml of CMECB, respectively (Fig. 3.10).

Treatment of Jurkat T and Wil-2 NS cell lines with various concentrations of CMECB had no notable or down-regulatory effects on the total cellular protein levels of the pro-apoptotic protein, Bax, as compared to the vehicle-treated cells (Figs. 3.7 and 3.11). This was attributed to the translocation of Bax

protein from the cytosol to the outer mitochondrial membrane as evidenced by the dose-dependent increase of Bax protein levels in the mitochondrial protein fraction (Figs. 3.8 and 3.12). As a result, an increase in Bax to Bcl-2 ratio, following the exposure of both experimental cultures to various concentration of CMECB for 0.5, 1, 12 and 24 hours, was observed (Figs. 3.9 and 3.13). These results suggest that CMECB-induced apoptosis in Jurkat T and Wil-2 NS cells is mainly mediated through the down-regulation of the anti-apoptotic protein, Bcl-2, which is triggered by the translocation and accumulation of Bax in the mitochondria.

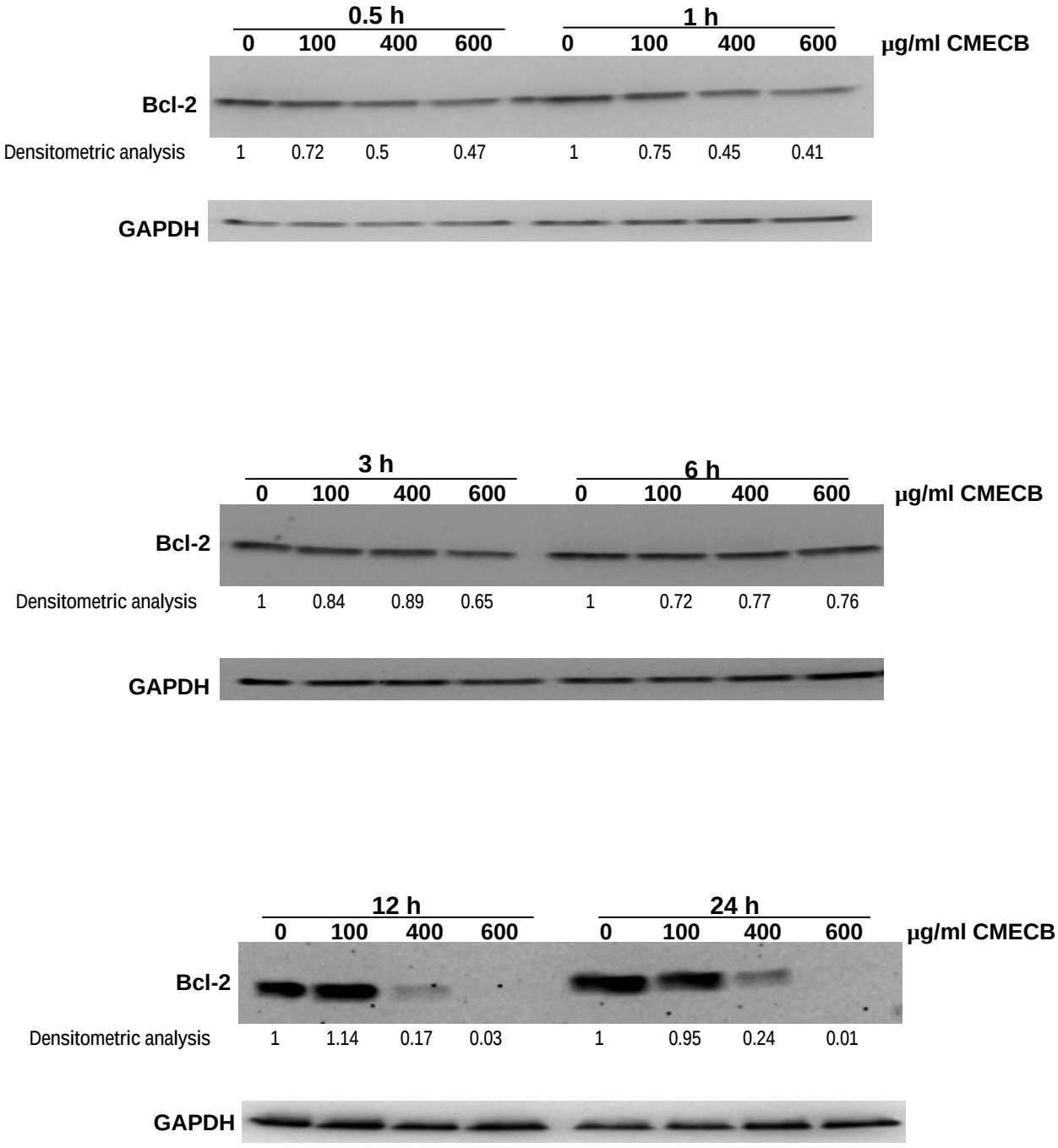



**Figure 3.6.** The effect of CMECB on Bcl-2 protein expression in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 0.5-24 hours and protein expression was analysed by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

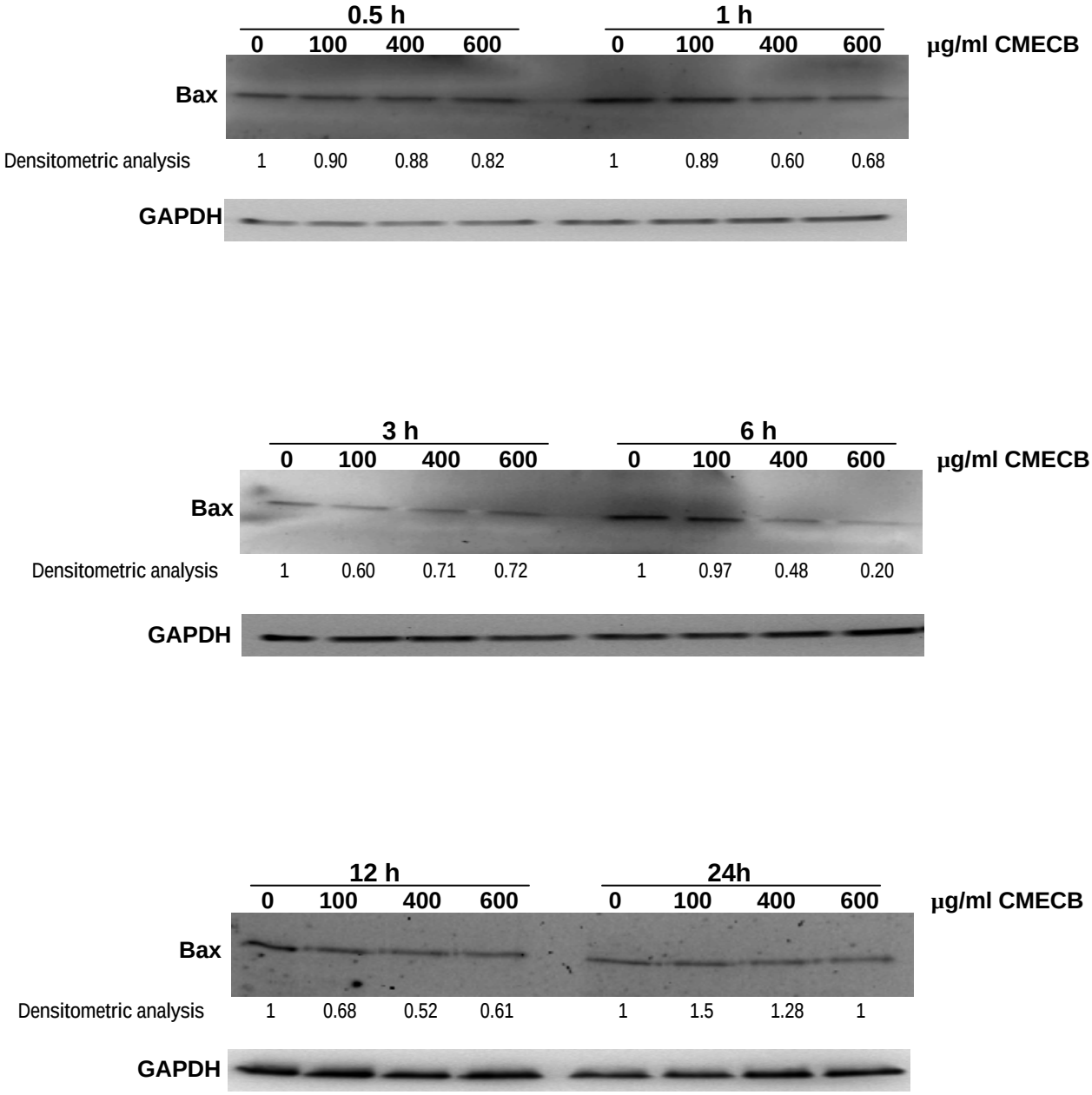



**Figure 3.7.** The effect of CMECB on Bax protein expression in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 0.5-24 hours and protein expression was analysed by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

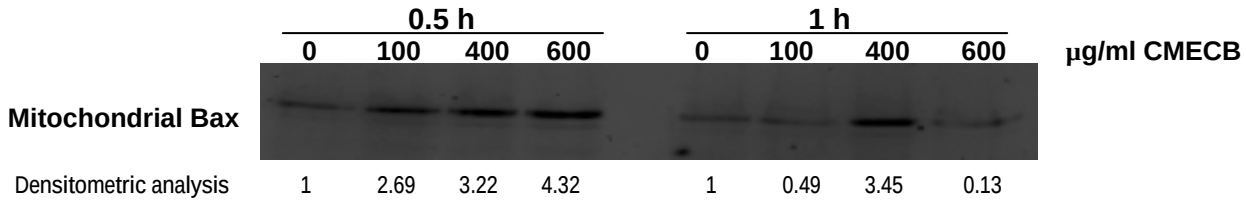



**Figure 3.8.** Effects of CMECB on the translocation of Bax protein to the mitochondria in Jurkat T cells as determined by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

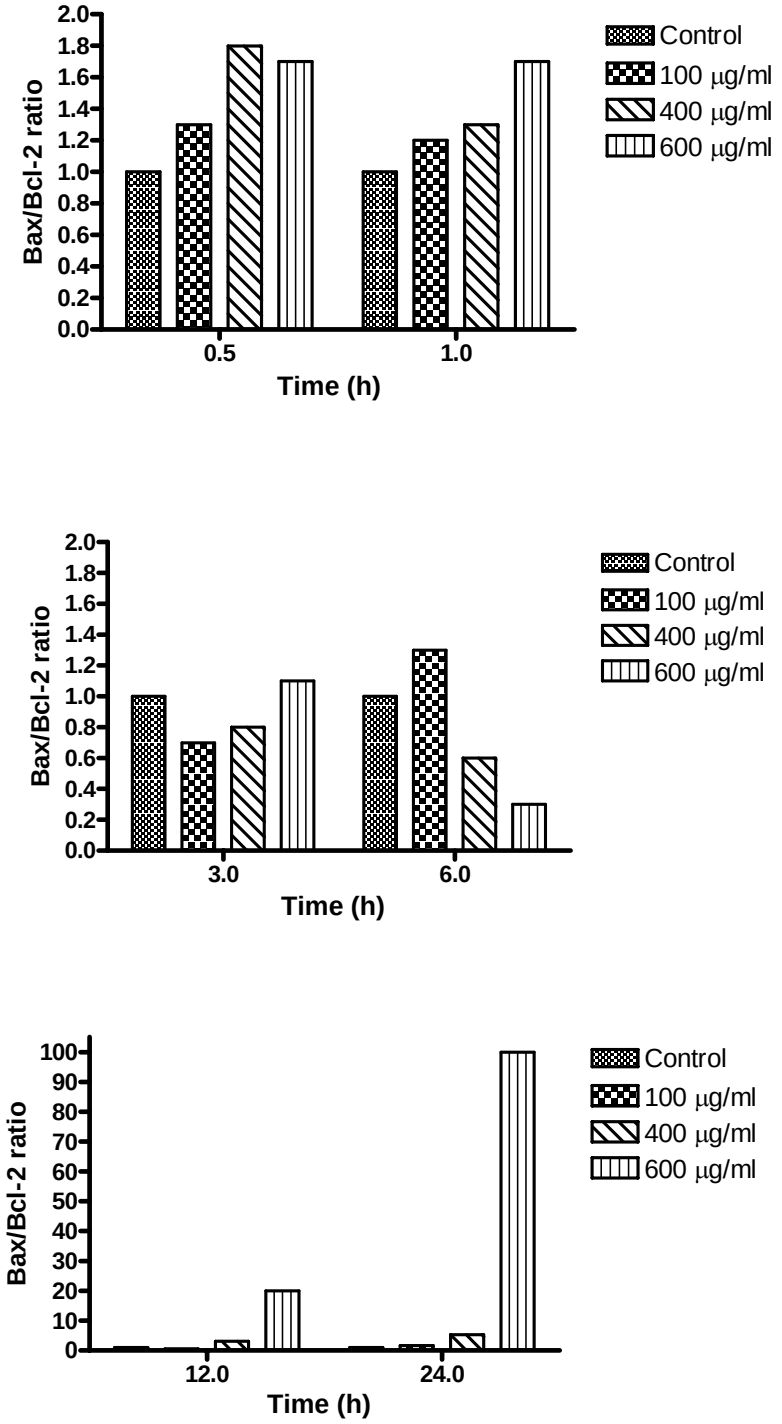



**Figure 3.9.** Bax/Bcl-2 protein expression ratio in CMECB treated Jurkat T cells. Densitometric analysis of Bax and Bcl-2 protein expression of Jurkat T cells exposed to CMECB were used to obtain a Bax/Bcl-2 ratio.

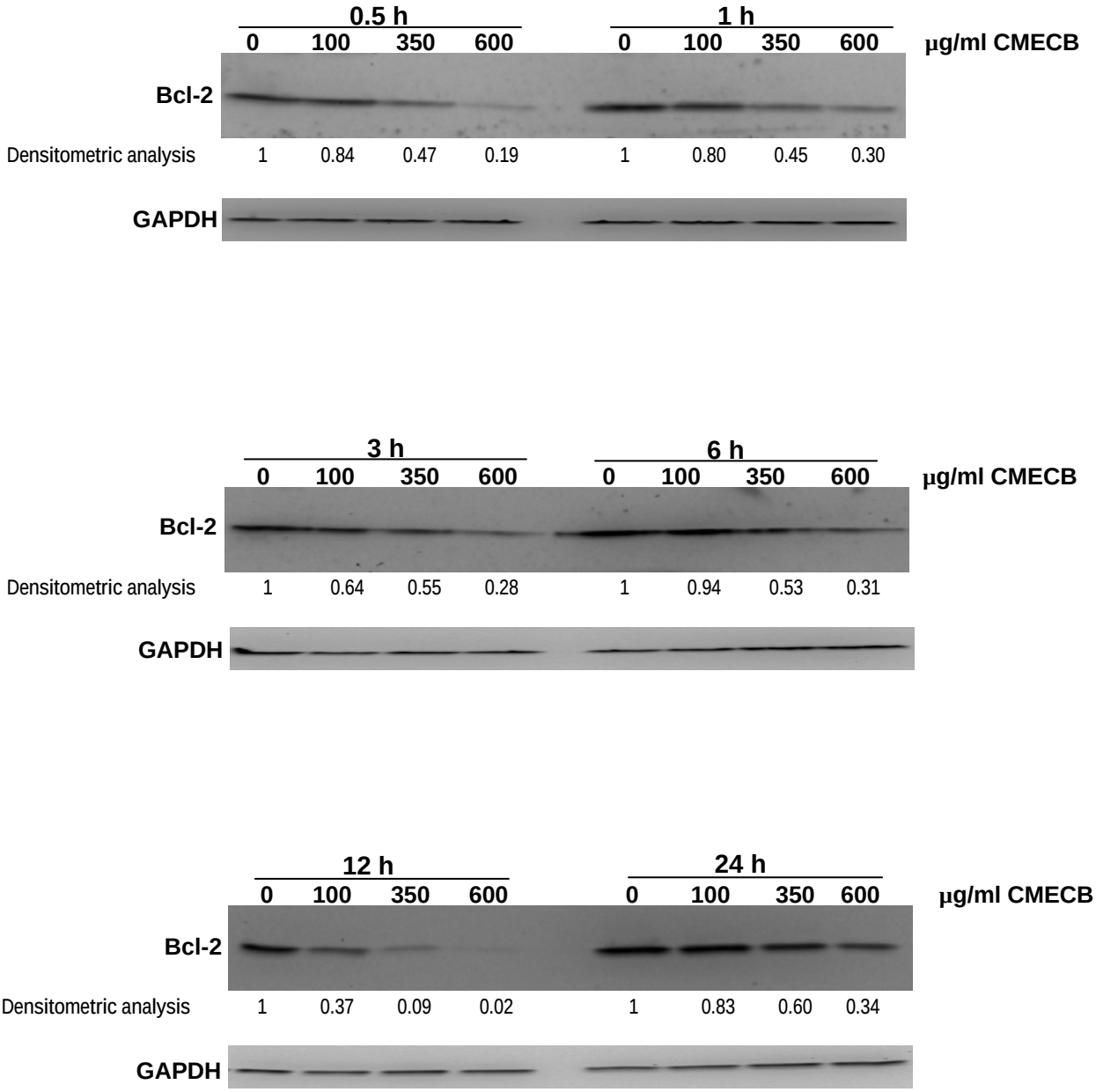



**Figure 3.10.** The effect of CMECB on Bcl-2 protein expression in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 0.5-24 hours and protein expression was analysed by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

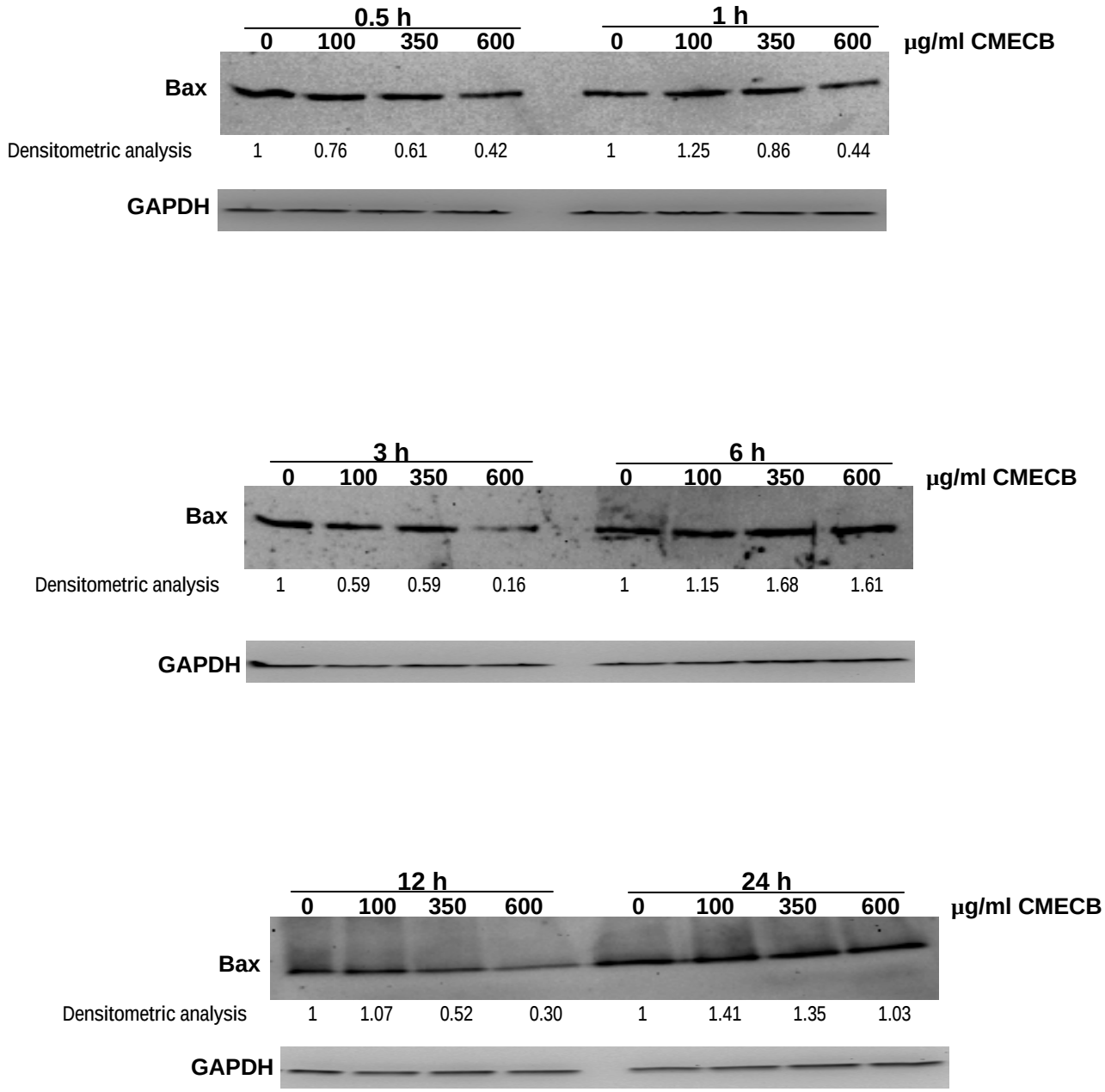



**Figure 3.11.** The effect of CMECB on Bax protein expression in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 0.5-24 hours and protein expression was analysed by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

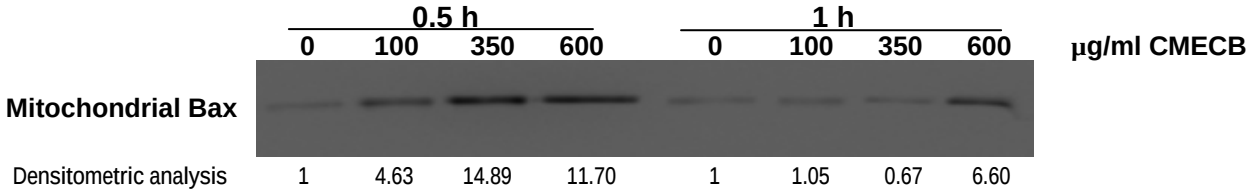



**Figure 3.12.** Effects of CMECB on the translocation of Bax protein to the mitochondria in Wil-2 NS cells as determined by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

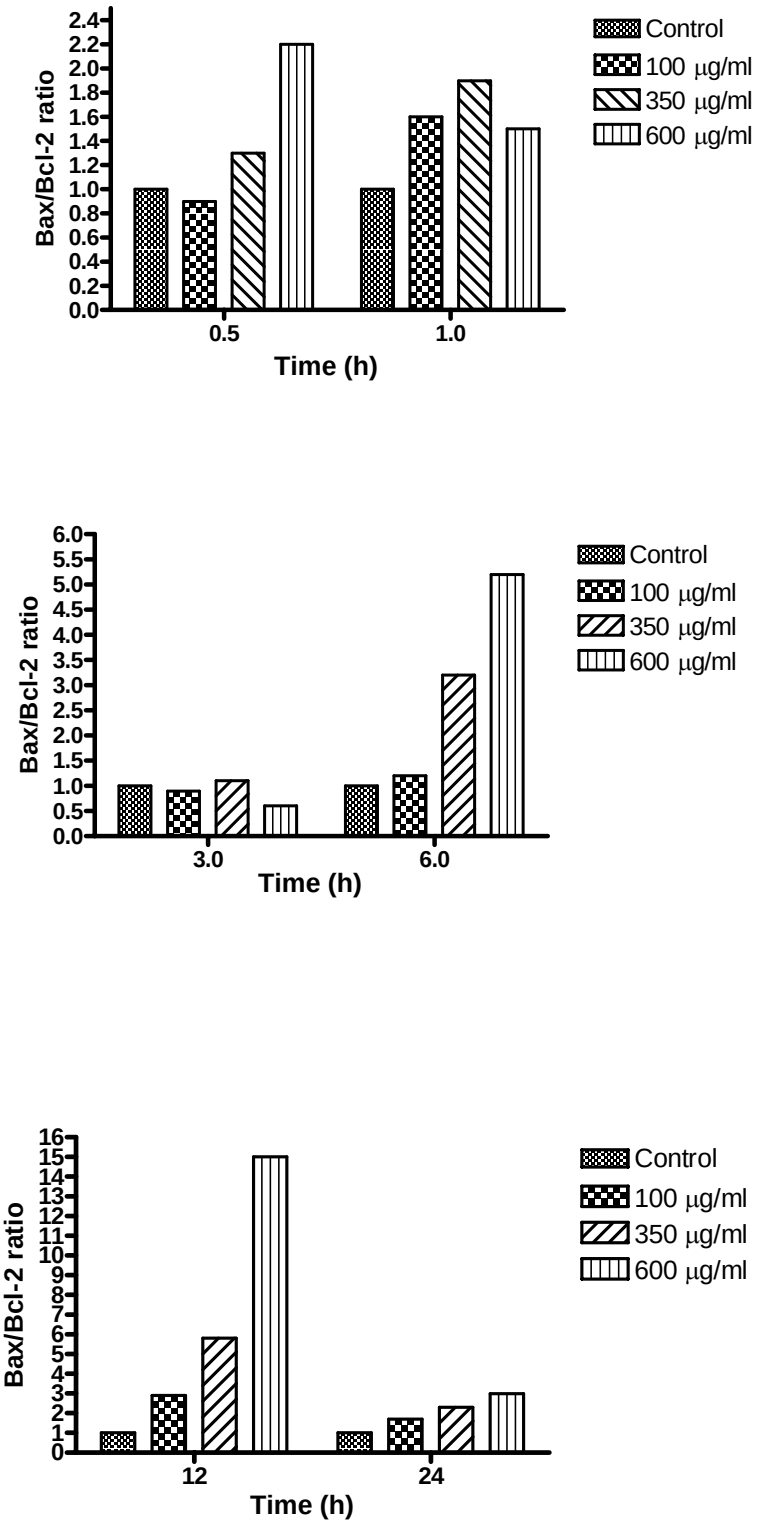



**Figure 3.13.** Bax/Bcl-2 protein expression ratio in CMECB treated Wil-2 NS cells. Densitometric analysis of Bax and Bcl-2 protein expression of Wil-2 NS cells exposed to CMECB were used to obtain a Bax/Bcl-2 ratio.

## 3.4. Effect of CMECB on *bax* and *bcl-2* mRNA expression levels

To investigate whether the observed alteration of Bax and Bcl-2 protein expression levels was as a result of CMECB ability to modulate *bax* and *bcl-2* mRNA expression levels, quantitative real-time PCR was used. No definite up- or down-regulatory trend of *bax* and *bcl-2* mRNA expression was observed in experimental cell cultures exposed to different concentrations of CMECB (data not shown). These results suggest that CMECB regulate the expression of these genes at the protein level and not at transcriptional or mRNA level. In addition, the PCR results emphasises that the translocation of Bax protein from the cytosol to the mitochondria might be the key event in the initiation of CMECB-induced apoptosis in Jurkat T and Wil-2 NS cells rather than dysregulation at the mRNA expression level.

## 3.5. CMECB promotes mitochondrial cytochrome c release in Jurkat T and Wil-2 NS cells

To investigate whether the alteration in the levels of Bax and Bcl-2 protein leads to cytochrome c release, cytochrome c levels were assessed after treatment of cells with CMECB. Western blot results demonstrated an increase in the amount of released cytochrome c into the cytosol in Jurkat T cells treated with different concentrations of CMECB for 1 hour (Fig. 3.14). Further increase in cytosolic cytochrome c levels was also demonstrated in Jurkat T cells exposed to 400 and 600 µg/ml for 6 hours. Furthermore, a significant amount of cytochrome c was released in Jurkat T cells after 12 and 24 hours of exposure to 100, 400 and 600 µg/ml of CMECB (Fig. 3.14). However, no notable change was observed in Jurkat T cells treated with various CMECB concentrations for 0.5 and 3 hours. A dose-dependent mitochondrial release of cytochrome c into the cytosol was observed in Wil-2 NS cells after 0.5, 3, 12 and 24 hours of exposure to various concentrations of CMECB. In addition, exposure of Wil-2 NS cells to 350 and 600 µg/ml for 1 hour and 600 µg/ml for 6 hours also resulted in the elevation of cytochrome c levels in the cytosol (Fig. 3.15). These results suggest that the CMECB-induced release of mitochondrial cytochrome c in Jurkat T and Wil-2 NS cells is mediated through the modulation of the members of the Bcl-2 family of proteins.

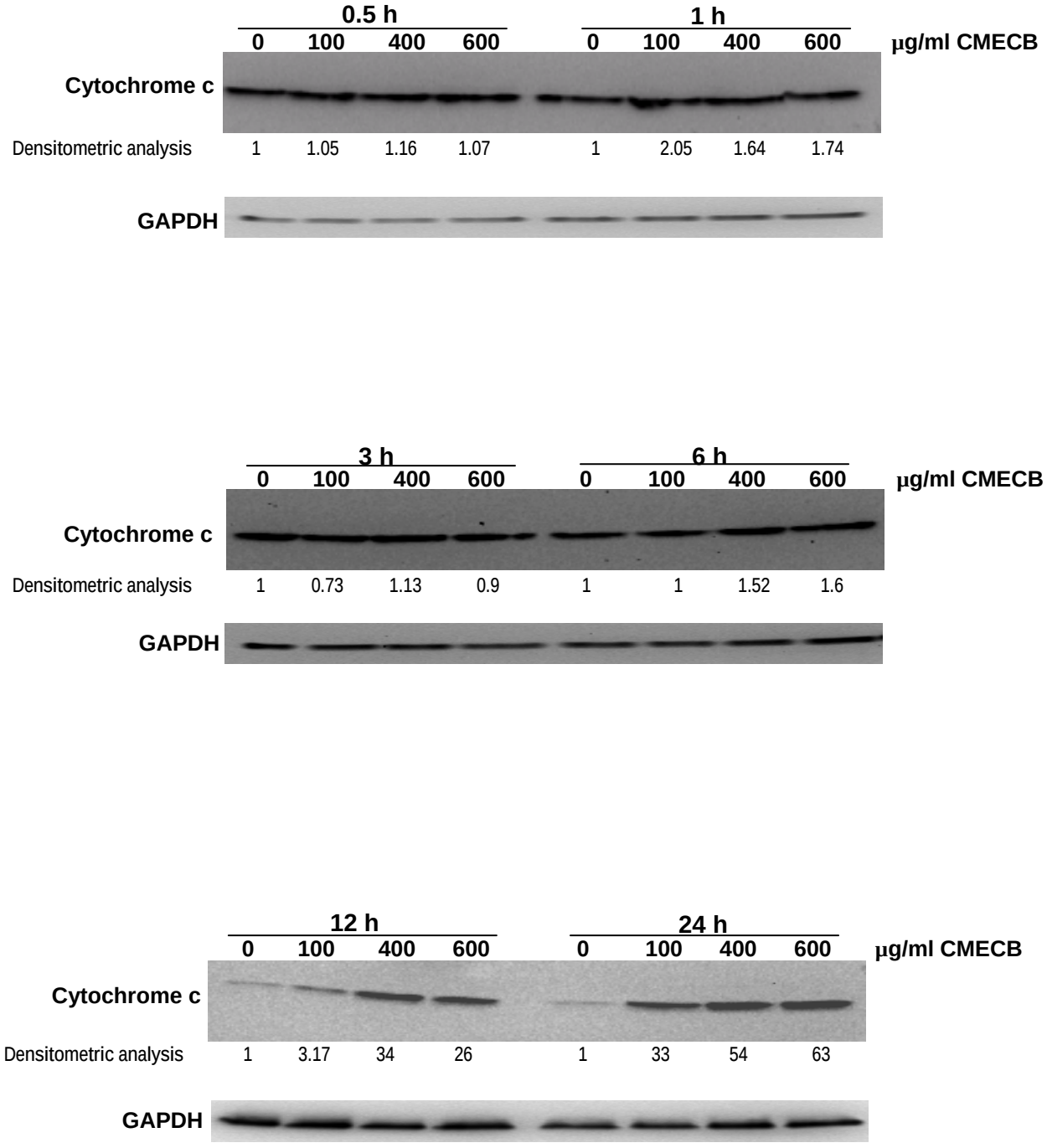



**Figure 3.14.** Western blot analysis of cytochrome c release in Jurkat T cells after treatment with various concentrations of CMECB or vehicle control (DMSO) for 0.5-24 hours. The amount of cytochrome c released into the cytosol was assayed using Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

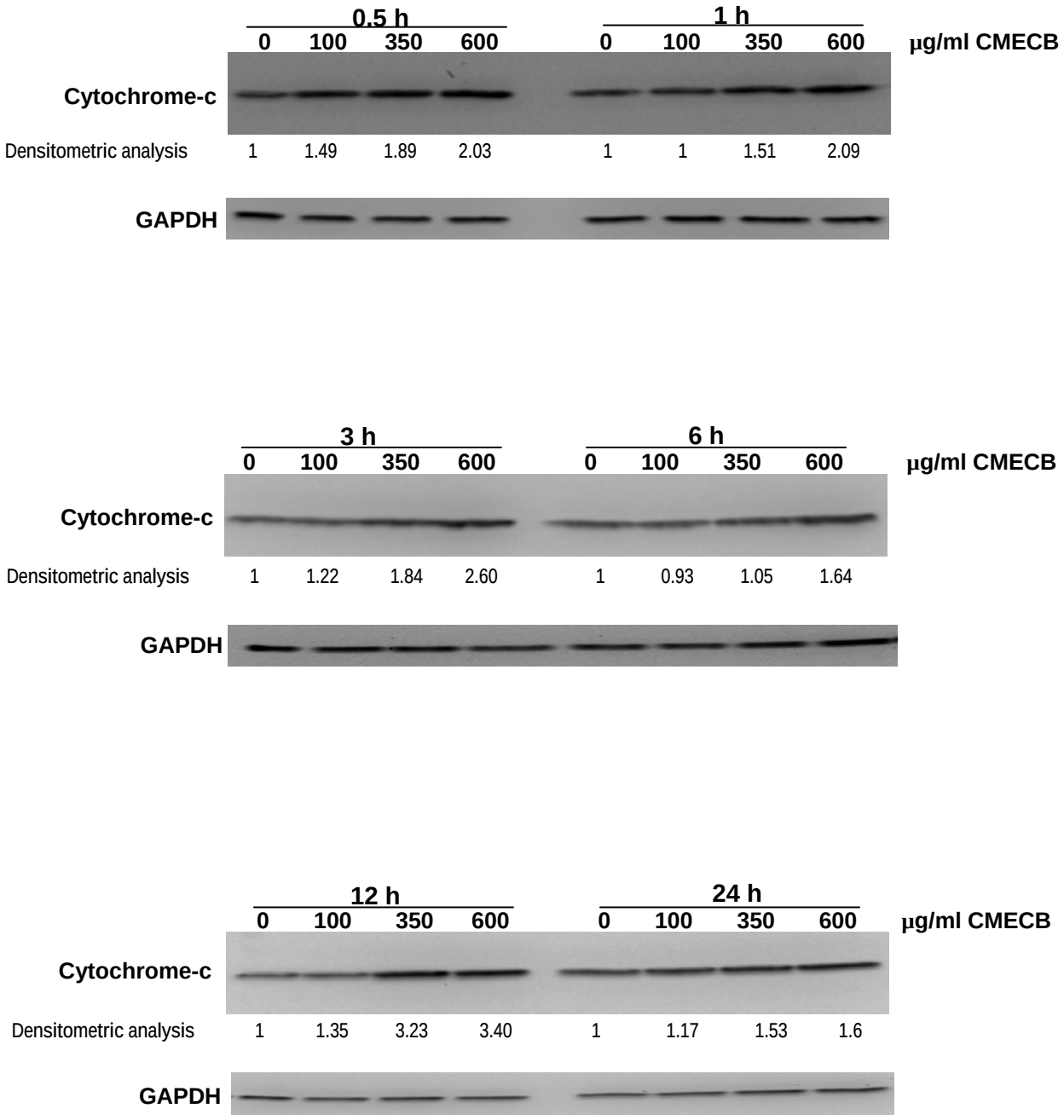



**Figure 3.15.** Western blot analysis of cytochrome c release in Wil-2 NS cells after treatment with various concentrations of CMECB or vehicle control (DMSO) for 0.5-24 hours. The amount of cytochrome c released into the cytosol was assayed using Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

## 3.6. CMECB activates a caspase cascade and PARP cleavage

Mitochondrial release of cytochrome c is reported to trigger the activation of caspases which play a crucial role in the initiation and execution of apoptosis (Gabriel *et al*., 2003). Thus, the involvement of initiator caspase-9 and executioner caspase-3 in Jurkat T and Wil-2 NS cells CMECB-induced apoptosis was investigated. As shown in Fig. 3.16, the exposure of Jurkat T cells to 400 and 600 µg/ml for 1, 3 and 6 hours resulted in caspase-9 activation, as indicated by the decrease in the levels of procaspase-9. Jurkat T cells treated with 100, 400 and 600 µg/ml for 0.5, 12 and 24 hours also demonstrated a decrease in the levels of procaspase-9 (Fig. 3.16). A dose-dependent activation of caspase-3 in CMECB-treated Jurkat T cells was also shown by the decrease in procaspase-3 levels. This effect was observed after 0.5 hour of treatment and lasted up to 24 hours (Fig. 3.18).

Treatment of Wil-2 NS cells with various concentrations of CMECB for different time intervals resulted in a decrease of procaspase-9 protein levels except in cells exposed to 100 µg/ml of CMECB for 1 hour (Fig. 3.21). While there was no notable decrease in the protein levels of procaspase-3 in Wil-2 NS cells exposed to CMECB for 0.5 and 3 hours, a decrease in procaspase-3 levels was observed in cells treated with various concentrations of CMECB at 1, 6, 12 and 24 hours (Fig. 3.23). In addition to Western blot analyses, caspase-9 and caspase-3 activity was also assessed using a colorimetric caspase assay kit. The results revealed that treatment of Jurkat T and Wil-2 NS cells with CMECB resulted in a dose-dependent activation of caspase-9 from 0.5 hour up to 12 hours (Figs. 3.17 and 3.22).

Moreover, CMECB treatment also induced a dose-dependent activation of caspase-3 in both test cell lines after 0.5 hour and persisted up to 12 hours of treatment (Figs. 3.19 and 3.24). Consistent with the activation of caspase-3, a marked increase in the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) was observed in CMECB-treated Jurkat T and Wil-2 NS cells. The cleavage of PARP (a DNA repair enzyme that is cleaved by activated caspase-3 in cells undergoing apoptosis) was evidenced by the decrease of the intact 119 kDa moiety of the protein from 0.5 to 24 hours of exposure to CMECB (Figs. 3.20 and 3.25). The results indicate that the CMECB-induced apoptosis in both experimental cell lines is associated with the activation of caspases-9 and -3 which leads to the degradation of PARP.

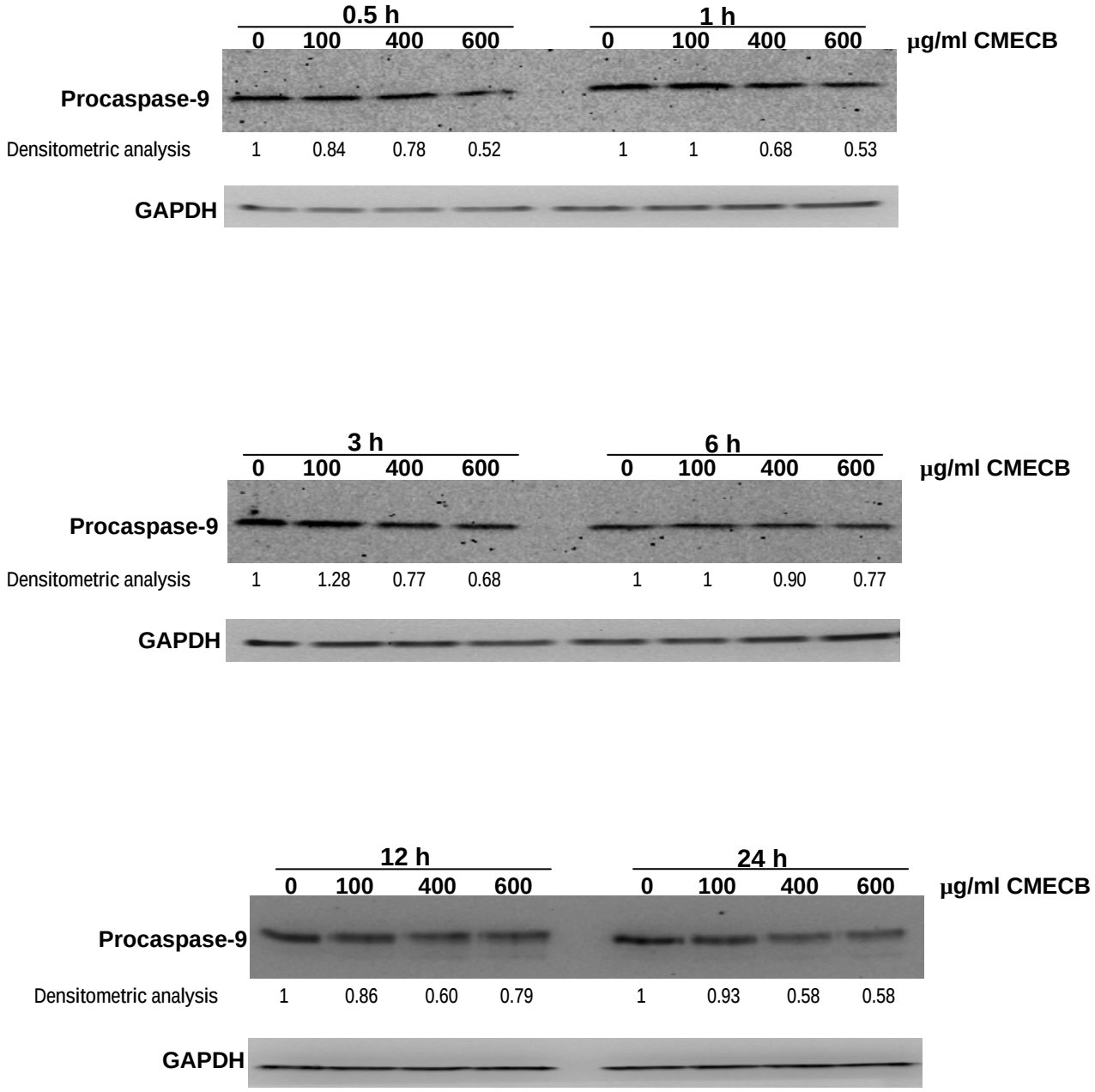



**Figure 3.16.** Effects of CMECB on the expression profile of procaspase-9 in Jurkat T cells. Cells were treated with different concentration of CMECB or vehicle control (DMSO) for 0.5-24 hours and the expression levels of procaspase-9 was determined using Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

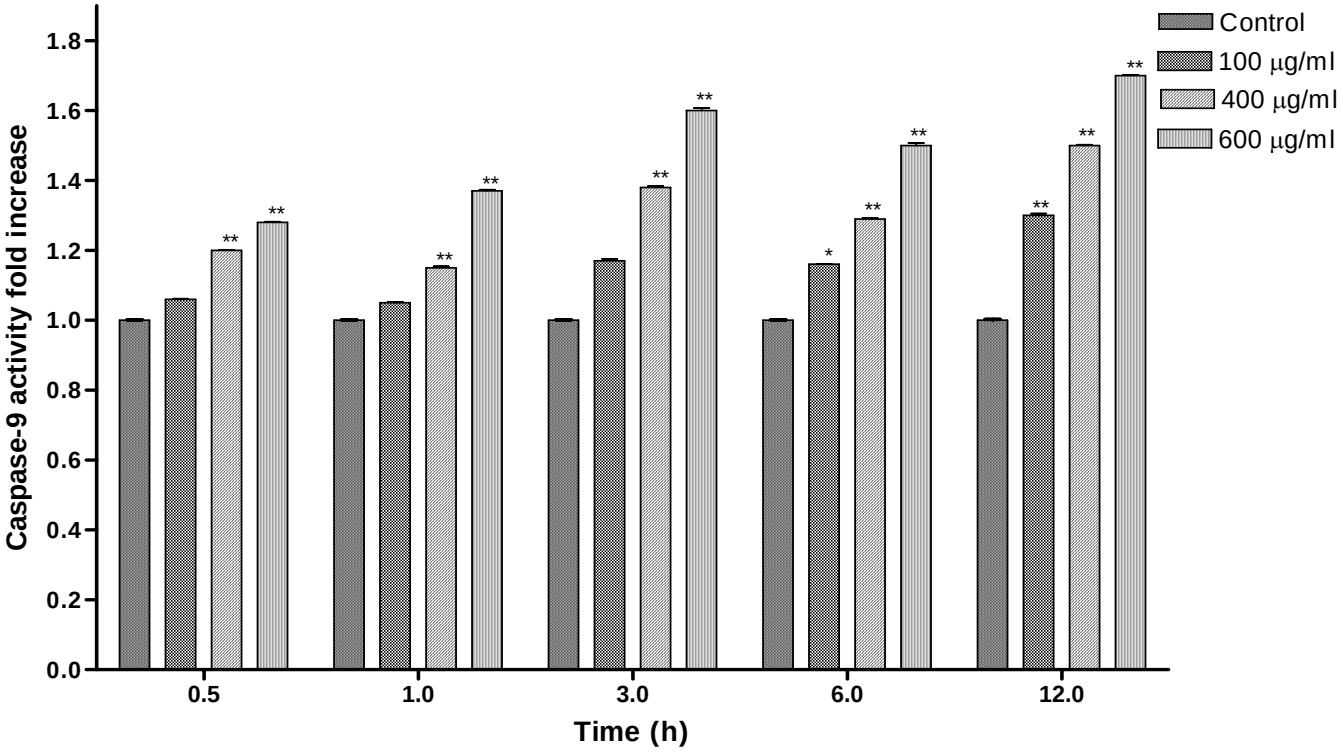



**Figure 3.17.** Activation of caspase-9 in CMECB-treated Jurkat T cells. Cells were exposed to different concentrations of CMECB or vehicle control (DMSO) for the indicated times and caspase-9 activity was determined by the cleavage of a colorimetric caspase-9 substrate monitored at 405 nm using a microtiter plate reader. Each data point represents the mean± S.D. of two independent experiments done in duplicates. *p< 0.05, **p< 0.01.

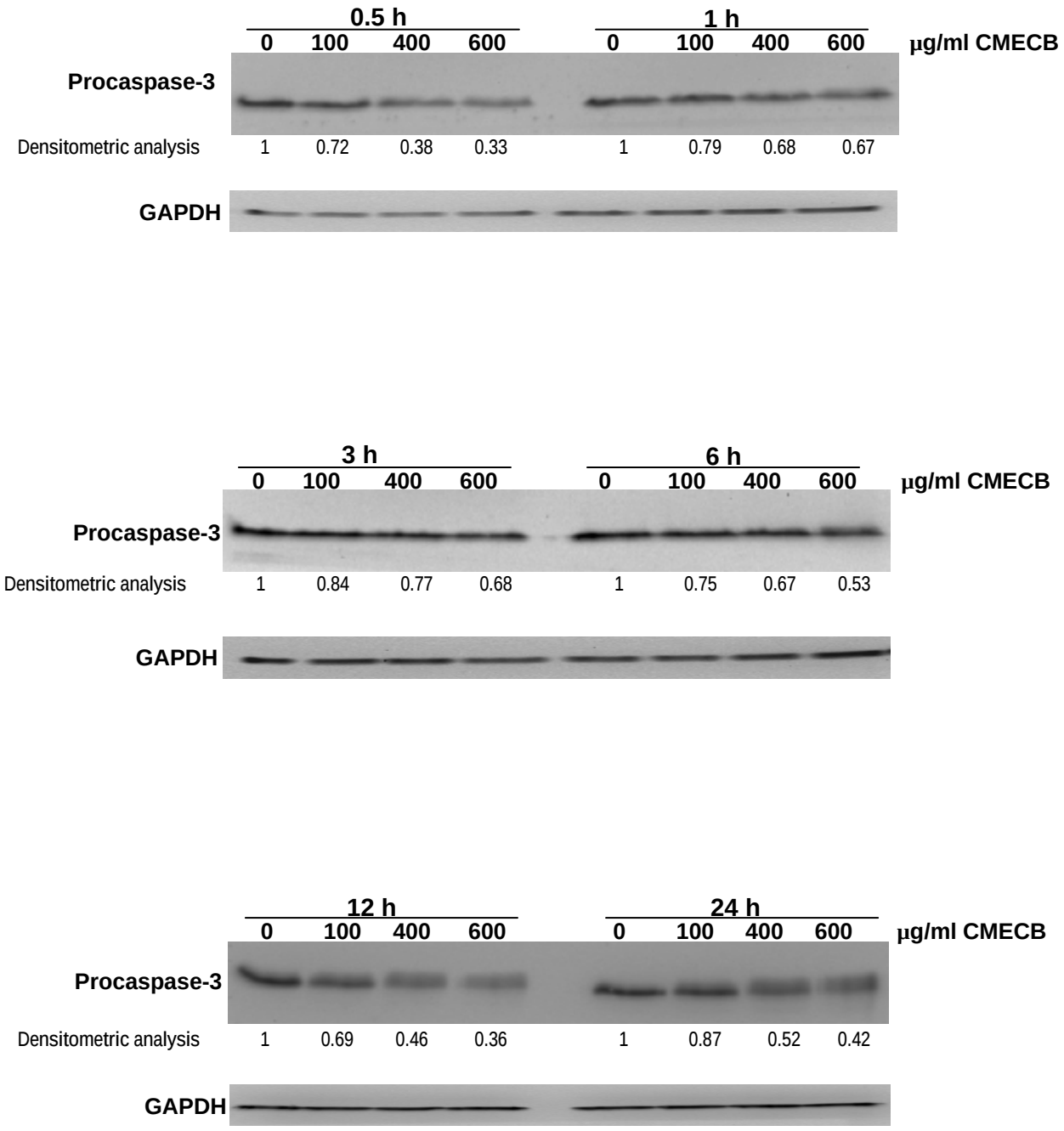



**Figure 3.18.** Effects of CMECB on the expression profile of procaspase-3 in Jurkat T cells. Cells were treated with different concentration of CMECB or vehicle control (DMSO) for 0.5-24 hours and the expression levels of procaspase-3 was determined using Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

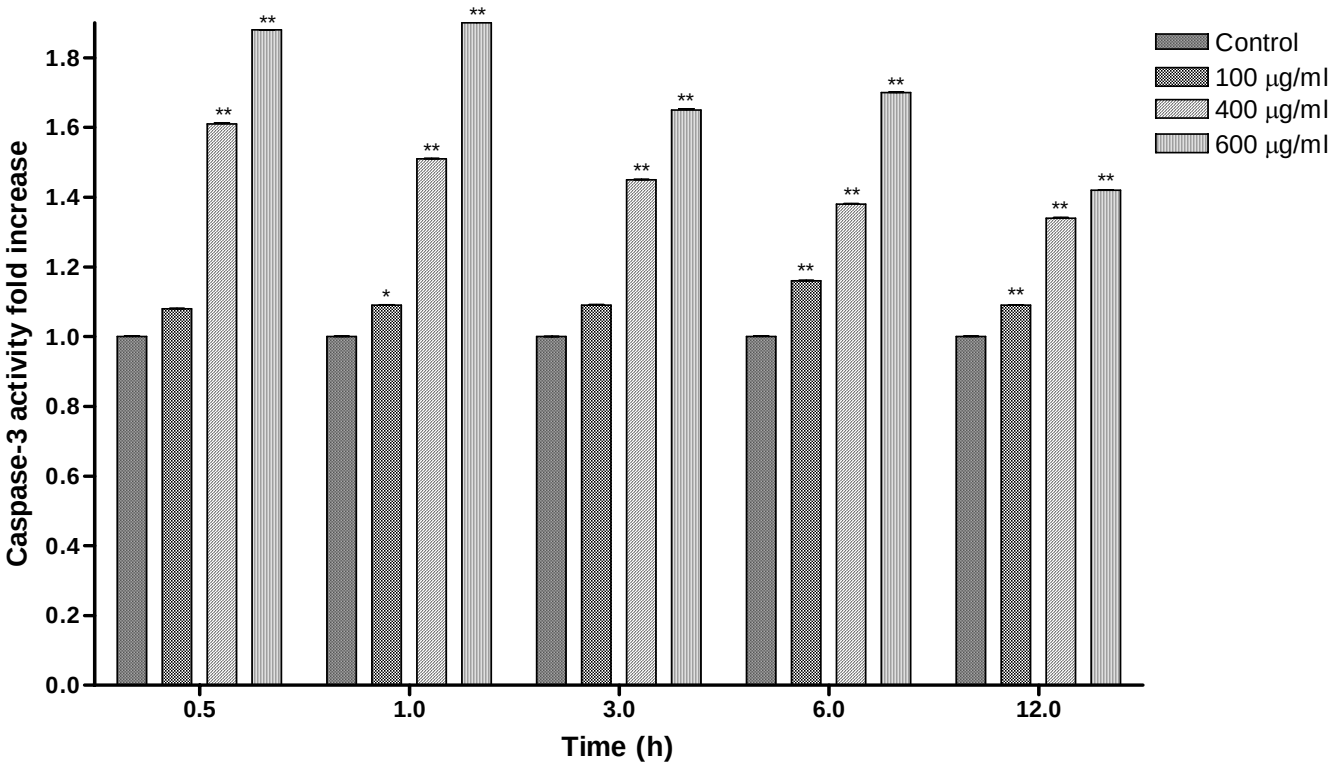



**Figure 3.19.** Activation of caspase-3 in CMECB-treated Jurkat T cells. Cells were exposed to different concentrations of CMECB or vehicle control (DMSO) for the indicated times and caspase-3 activity was determined by the cleavage of a colorimetric caspase-3 substrate monitored at 405 nm using a microtiter plate reader. Each data point represents the mean± S.D. of two independent experiments done in duplicates. *p< 0.05, **p< 0.01.

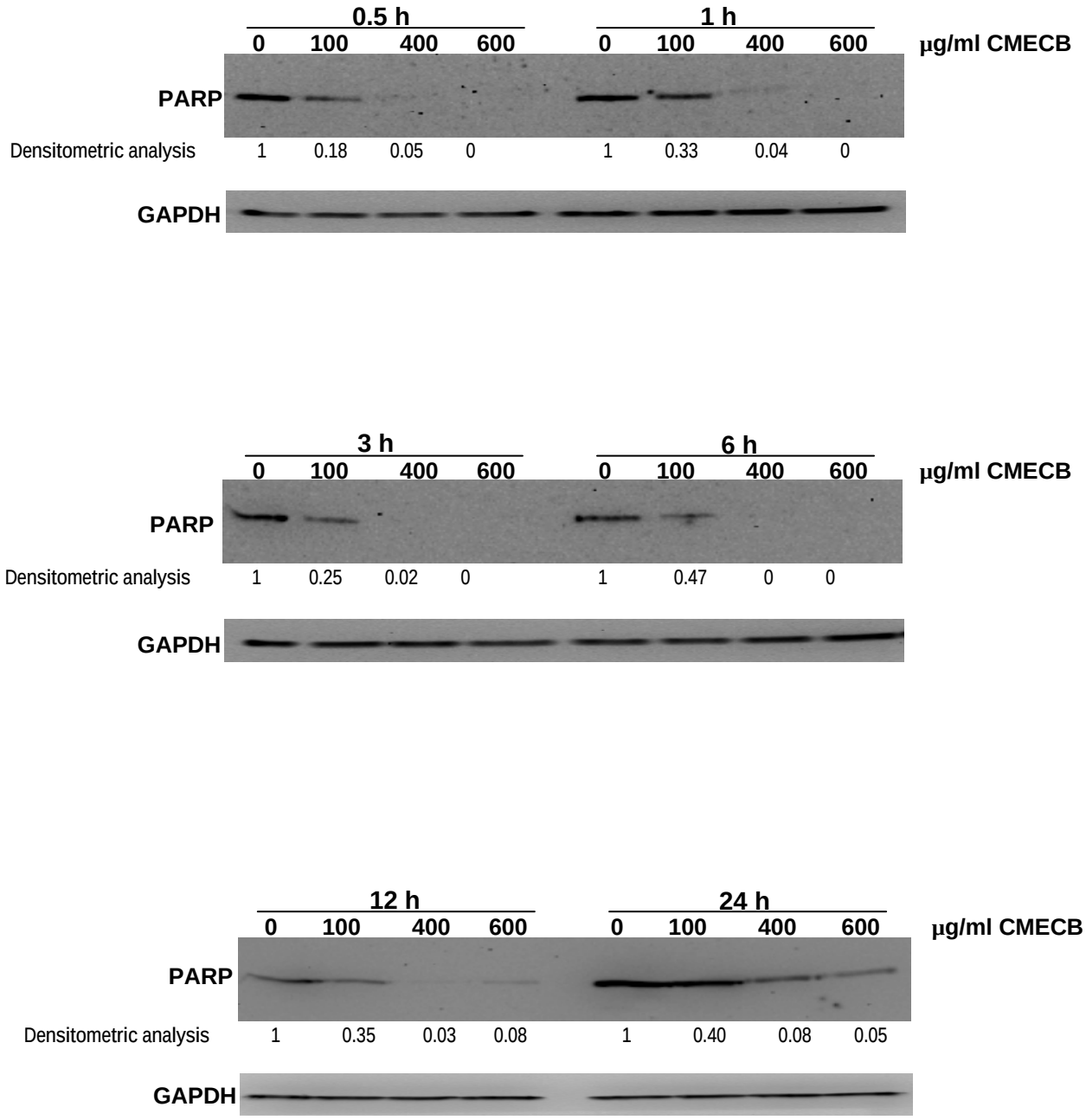



**Figure 3.20.** PARP cleavage in Jurkat T cells exposed to various doses of CMECB or vehicle control (DMSO) for 0.5-24 hours. Protein expression was determined by Western blotting as described in materials and methods. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

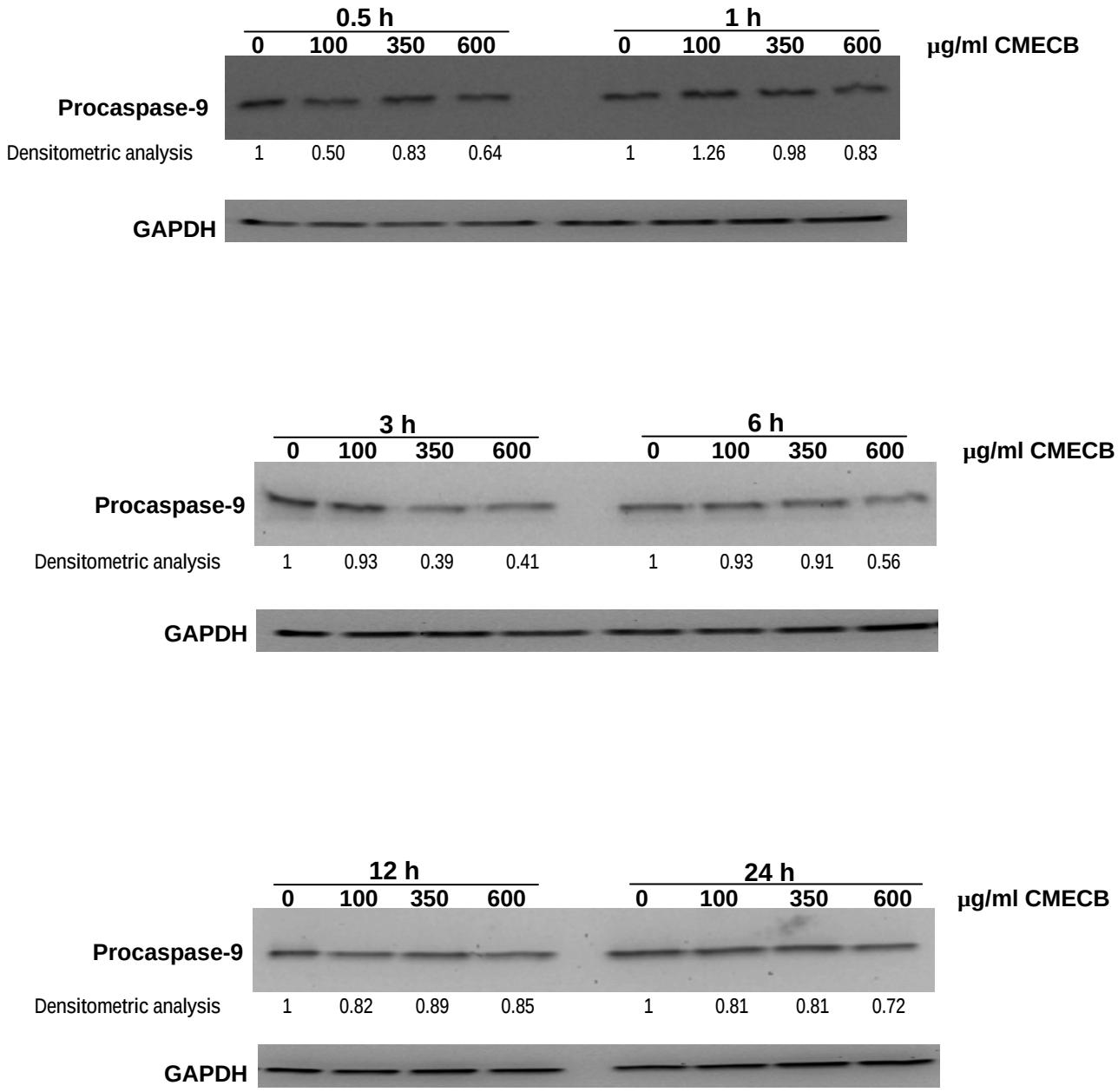



**Figure 3.21.** Effects of CMECB on the expression profile of procaspase-9 in Wil-2 NS cells. Cells were treated with different concentration of CMECB or vehicle control (DMSO) for 0.5-24 hours and the expression levels of procaspase-9 was determined using Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

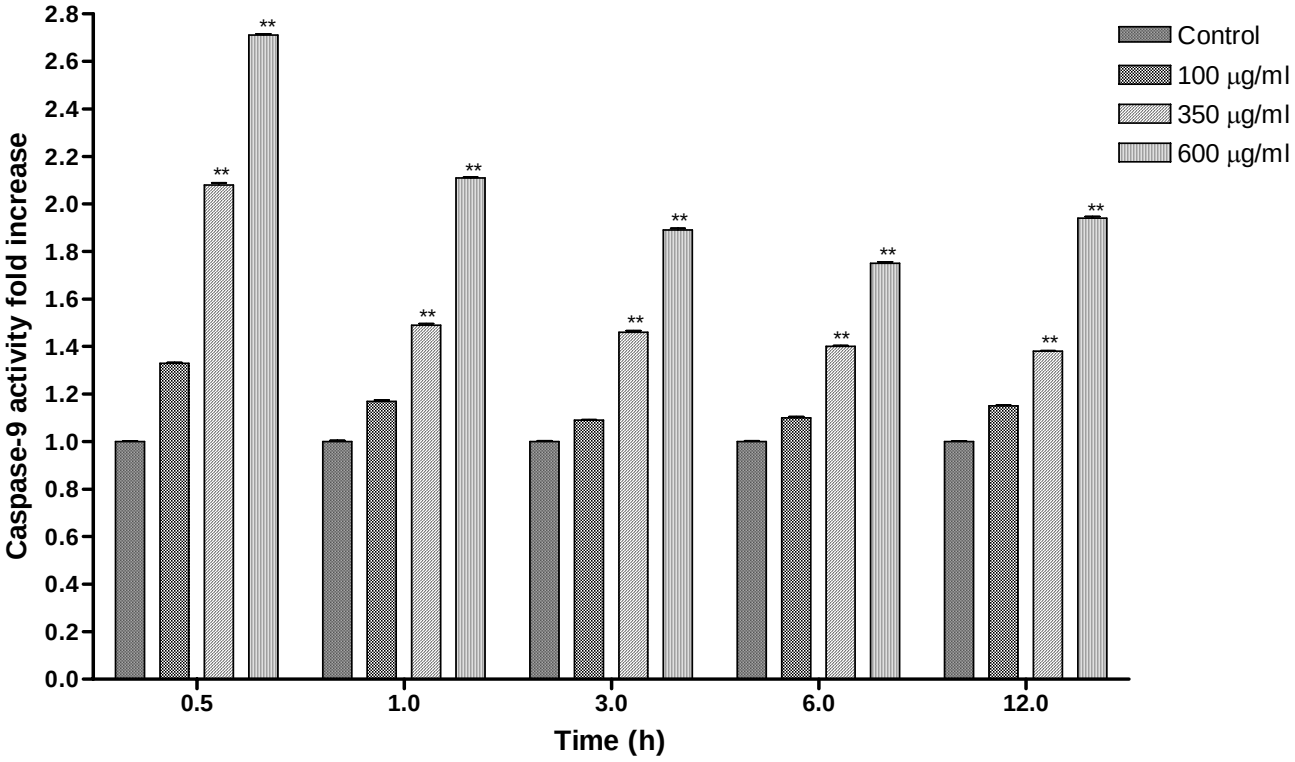



**Figure 3.22**. Activation of caspase-9 in CMECB-treated Wil-2 NS cells. Cells were exposed to different concentrations of CMECB or vehicle control (DMSO) for the indicated times and caspase-9 activity was determined by the cleavage of a colorimetric caspase-9 substrate monitored at 405 nm using a microtiter plate reader. Each data point represents the mean± S.D. of two independent experiments done in duplicates. *p< 0.05, **p< 0.01.

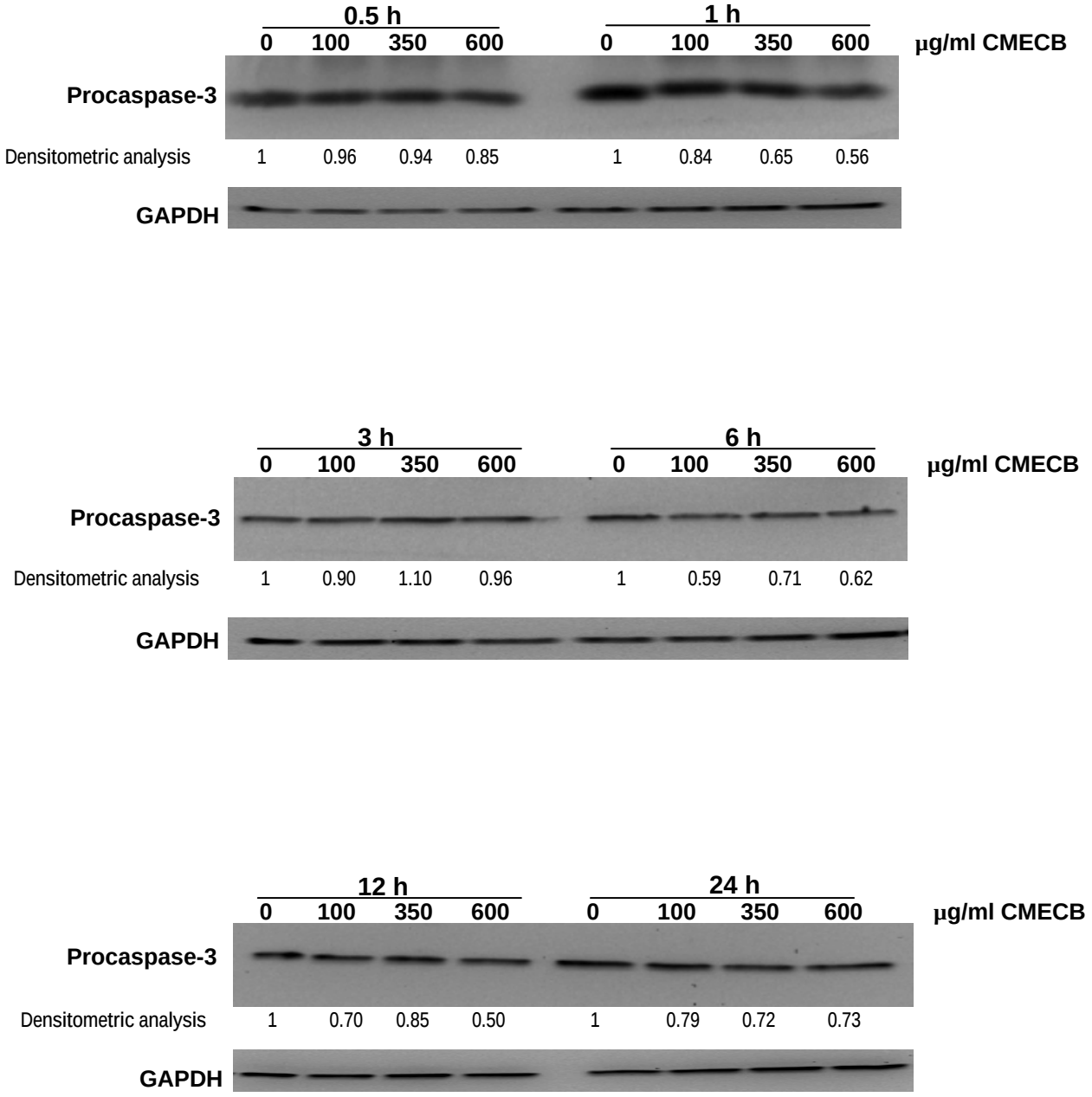



**Figure 3.23.** Effects of CMECB on the expression profile of procaspase-3 in Wil-2 NS cells. Cells were treated with different concentration of CMECB or vehicle control (DMSO) for 0.5-24 hours and the expression levels of procaspase-3 was determined using Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

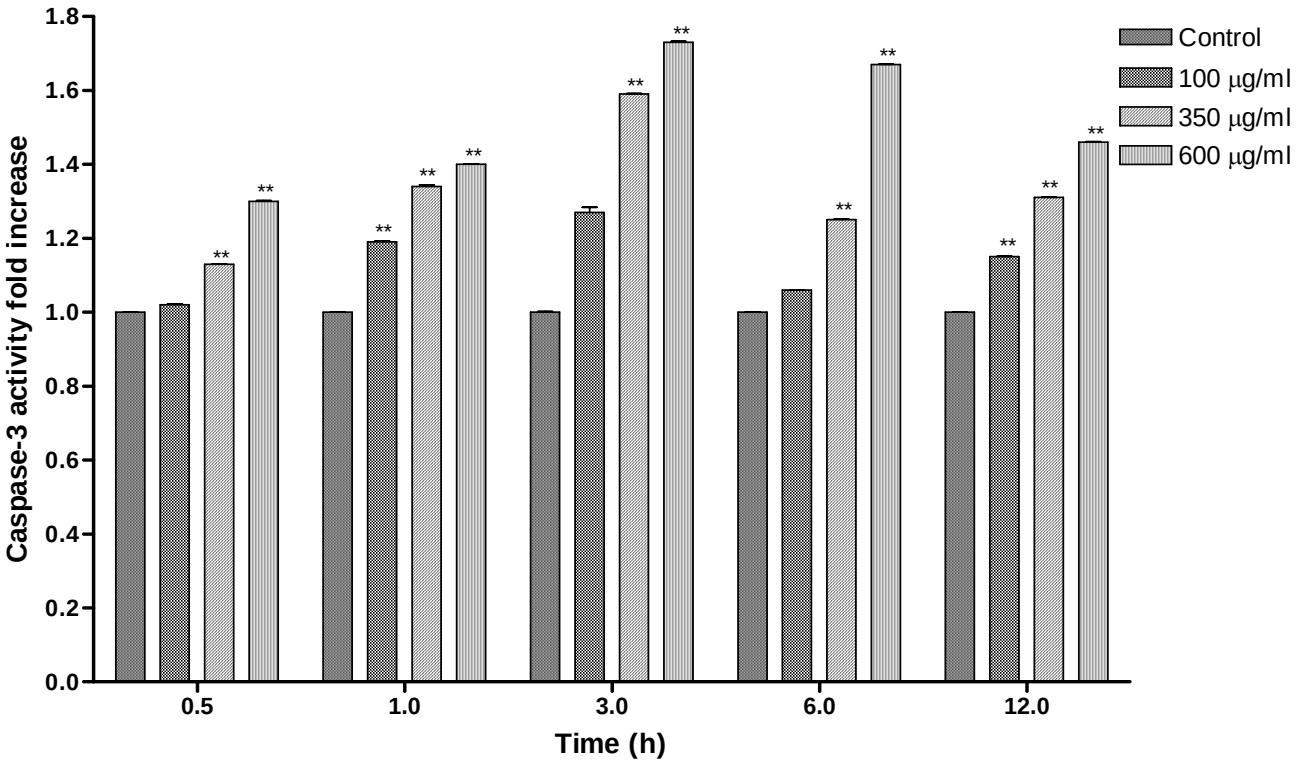



**Figure 3.24.** Activation of caspase-3 in CMECB-treated Wil-2 NS cells. Cells were exposed to different concentrations of CMECB or vehicle control (DMSO) for the indicated times and caspase-3 activity was determined by the cleavage of a colorimetric caspase-3 substrate monitored at 405 nm using a microtiter plate reader. Each data point represents the mean± S.D. of two independent experiments done in duplicates. *p< 0.05, **p< 0.01.

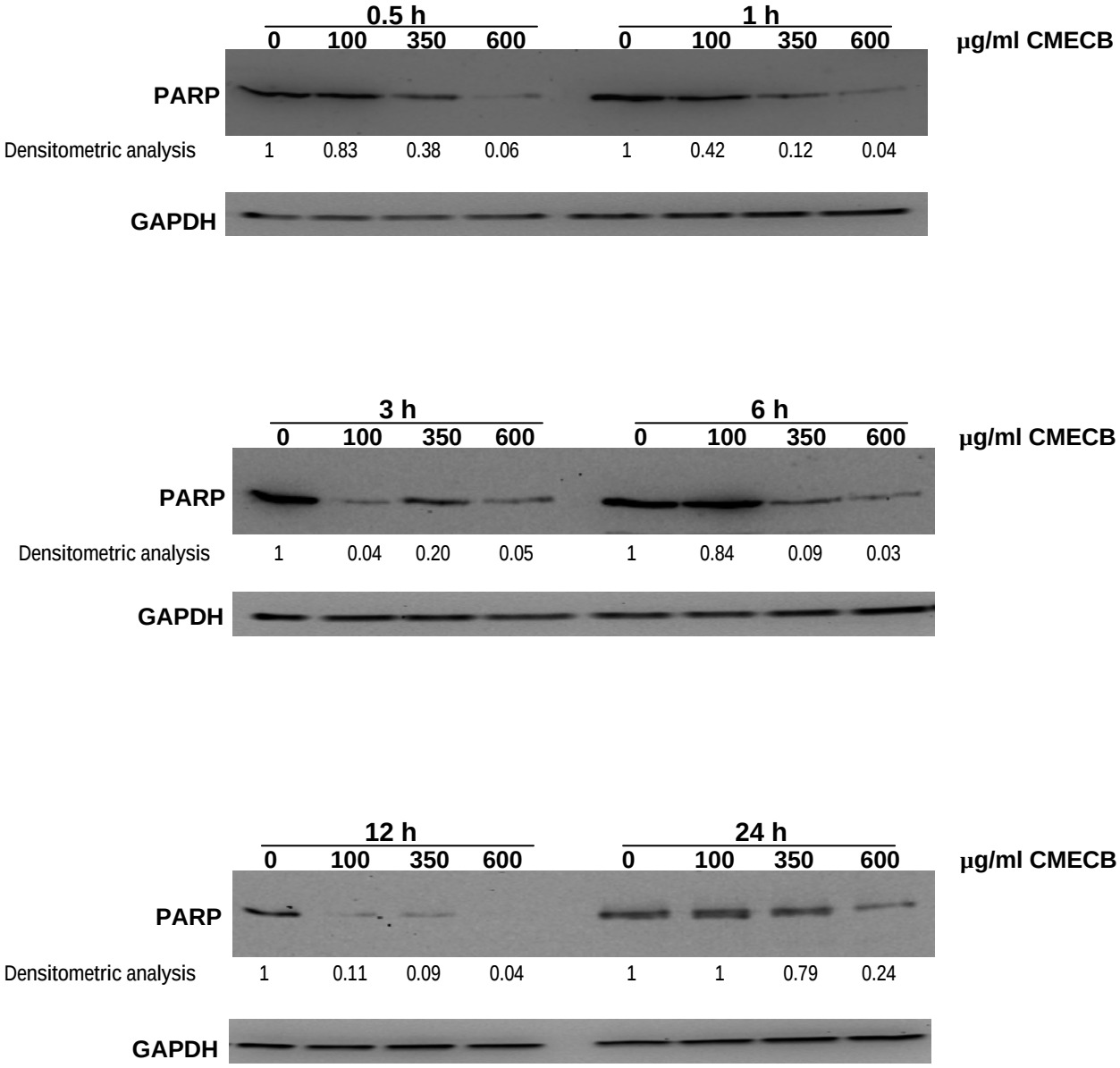



**Figure 3.25.** PARP cleavage in Wil-2 NS cells after treatment with various doses of CMECB or vehicle control (DMSO) for 0.5-24 hours. Protein expression was determined by Western blotting as described in materials and methods. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

## 3.7. CMECB induces $G_2$/M phase cell division cycle arrest

In most cases, the advent of apoptosis is reported to be associated with the arrest of the cell division cycle; thus the effects of CMECB on cell division cycle distribution was evaluated using flow cytometric analysis. No changes in the cell division cycle distribution profiles were observed after 24 hours (Fig. 3.26A) and 48 hours (Fig. 3.26B) in Jurkat T cells and 24 hours (Fig. 3.27A) in Wil-2 NS in response to CMECB exposure. At 72 hours CMECB caused a dose-dependent accumulation of Jurkat T cells in $G_2$/M phase (Fig. 3.26C). CMECB also increased the population of Wil-2 NS cells in the $G_2$/M phase in a dose-dependent manner after 48 and 72 hours as seen by an increase in the population of cells in the $G_2$/M phase (Figs. 3.27B and 3.27C). The results indicate that the exposure of Jurkat T and Wil-2 NS to CMECB leads to $G_2$/M cell division cycle arrest.

**A**

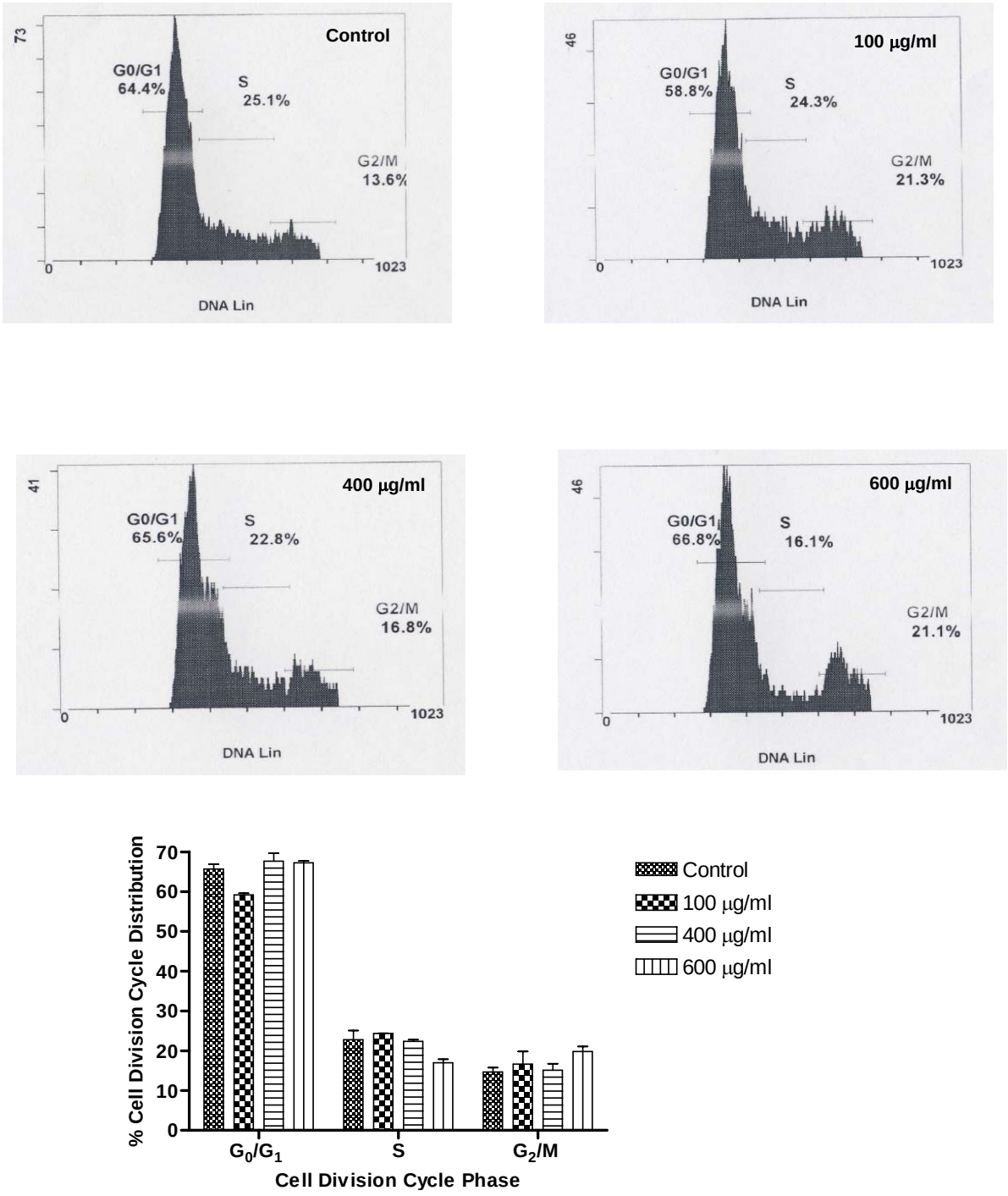



**Figure 3.26A.** The effect of CMECB on cell division cycle distribution in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 24 hours. Cellular DNA content was assayed using a flow cytometer after staining with propidium iodide. Graph shows percentages of cells in various cell division cycle phases. The data shown represent the mean± S.D. of two independent experiments.

**B**

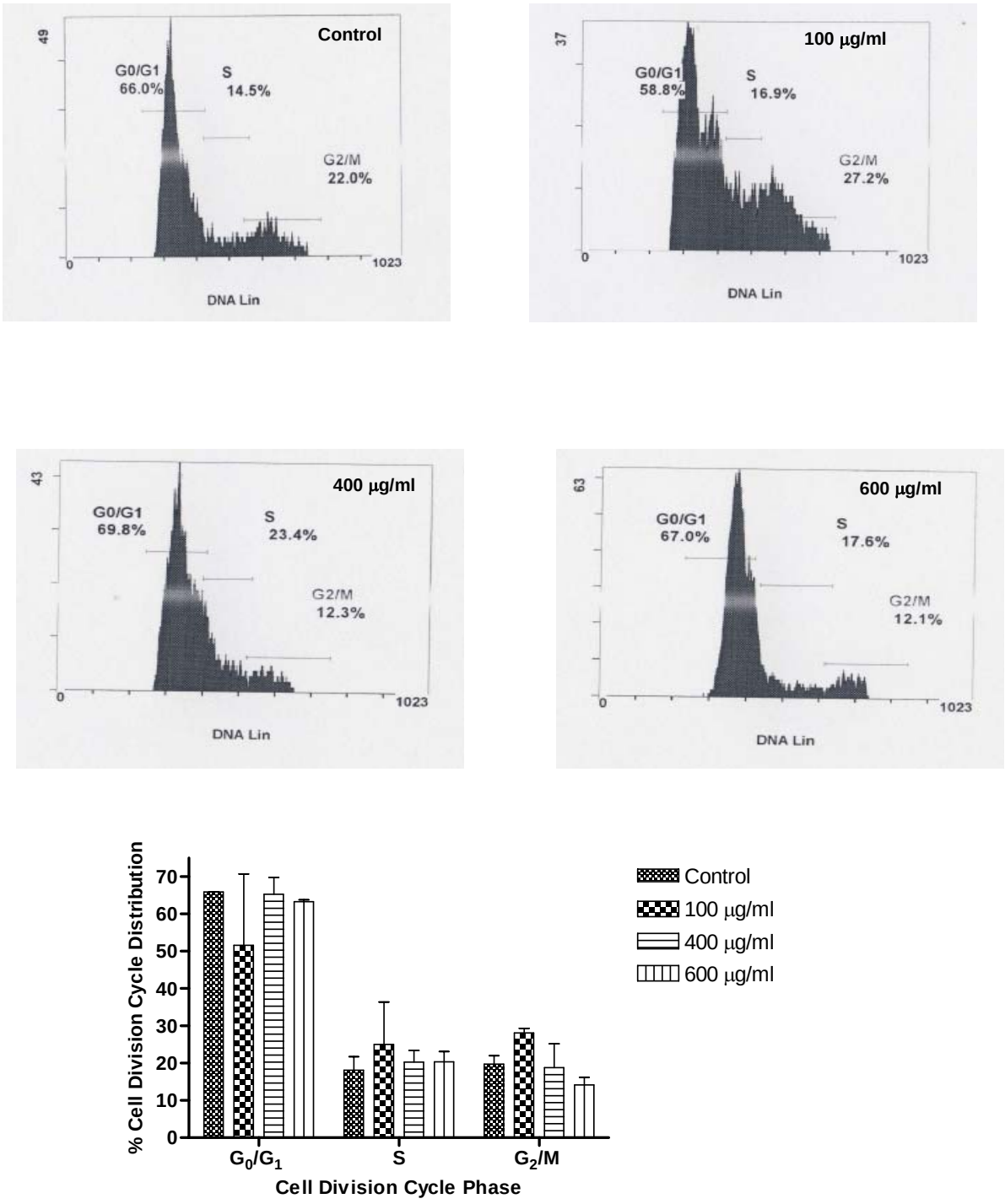



**Figure 3.26B.** The effect of CMECB on cell division cycle distribution in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 48 hours. Cellular DNA content was assayed using a flow cytometer after staining with propidium iodide. Graph shows percentages of cells in various cell division cycle phases. The data shown represent the mean± S.D. of two independent experiments.

**C**

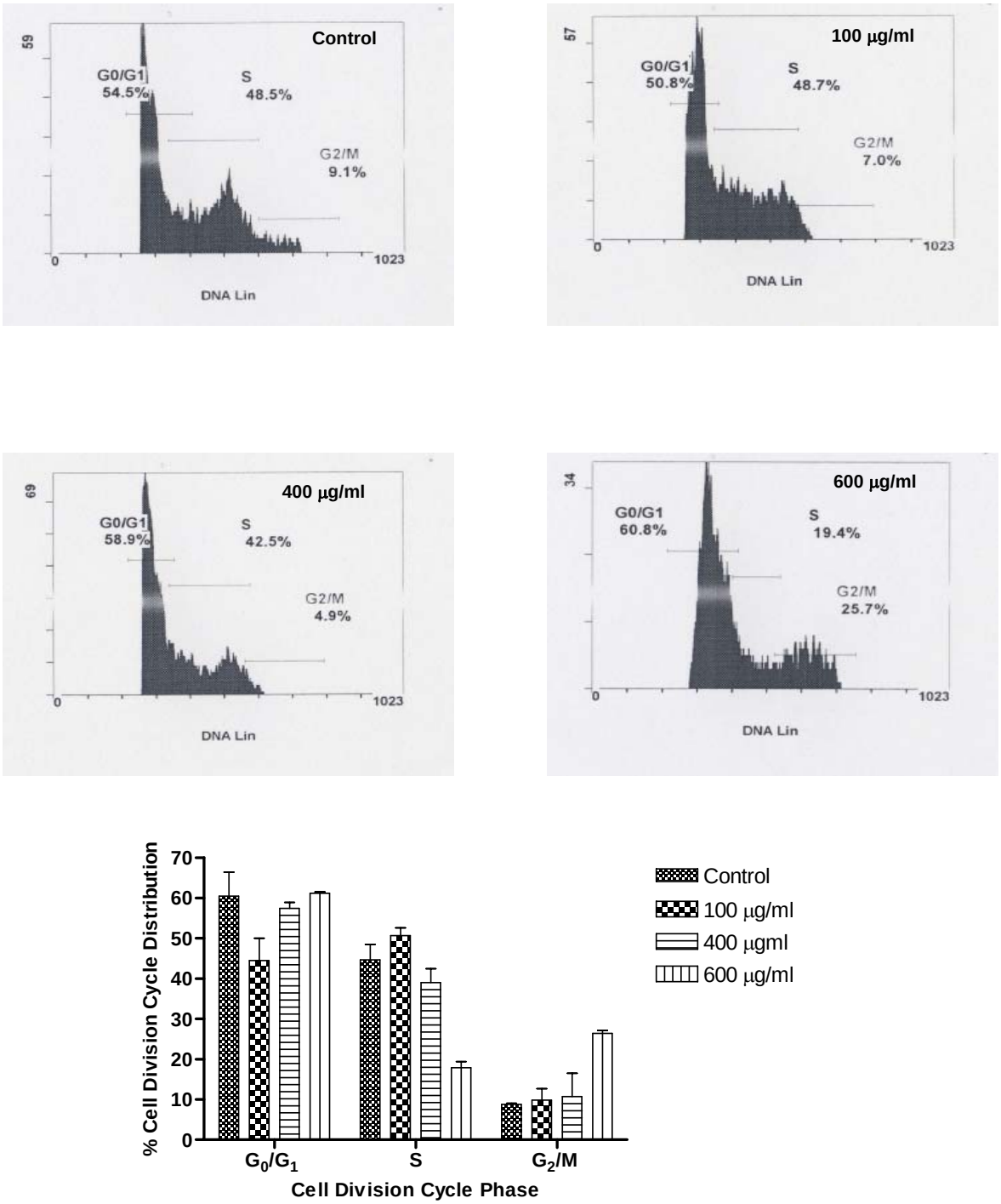
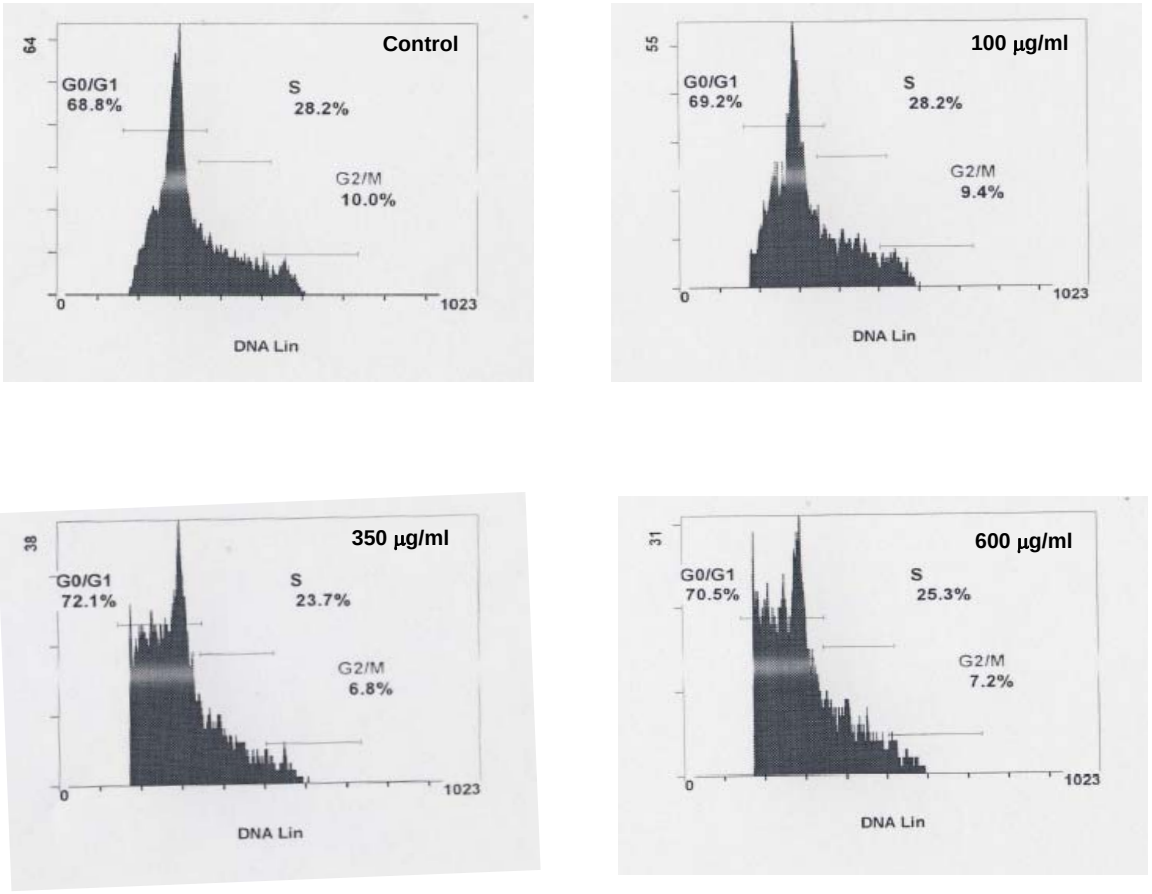



**Figure 3.26C.** The effect of CMECB on cell division cycle distribution in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 72 hours. Cellular DNA content was assayed using a flow cytometer after staining with propidium iodide. Graph shows percentages of cells in various cell division cycle phases. The data shown represent the mean± S.D. of two independent experiments.

**A**



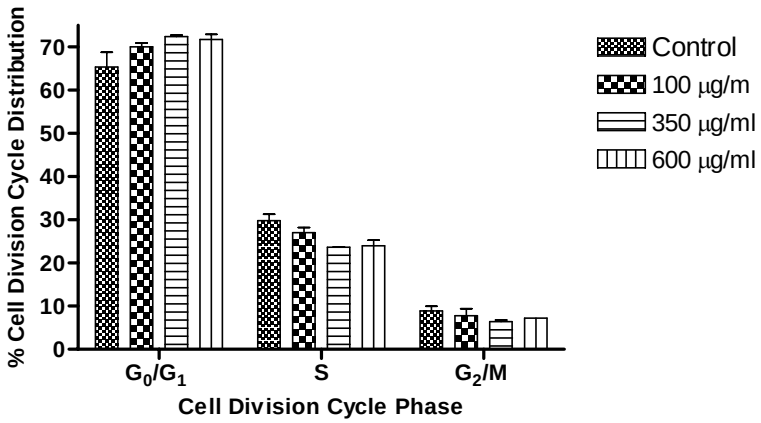
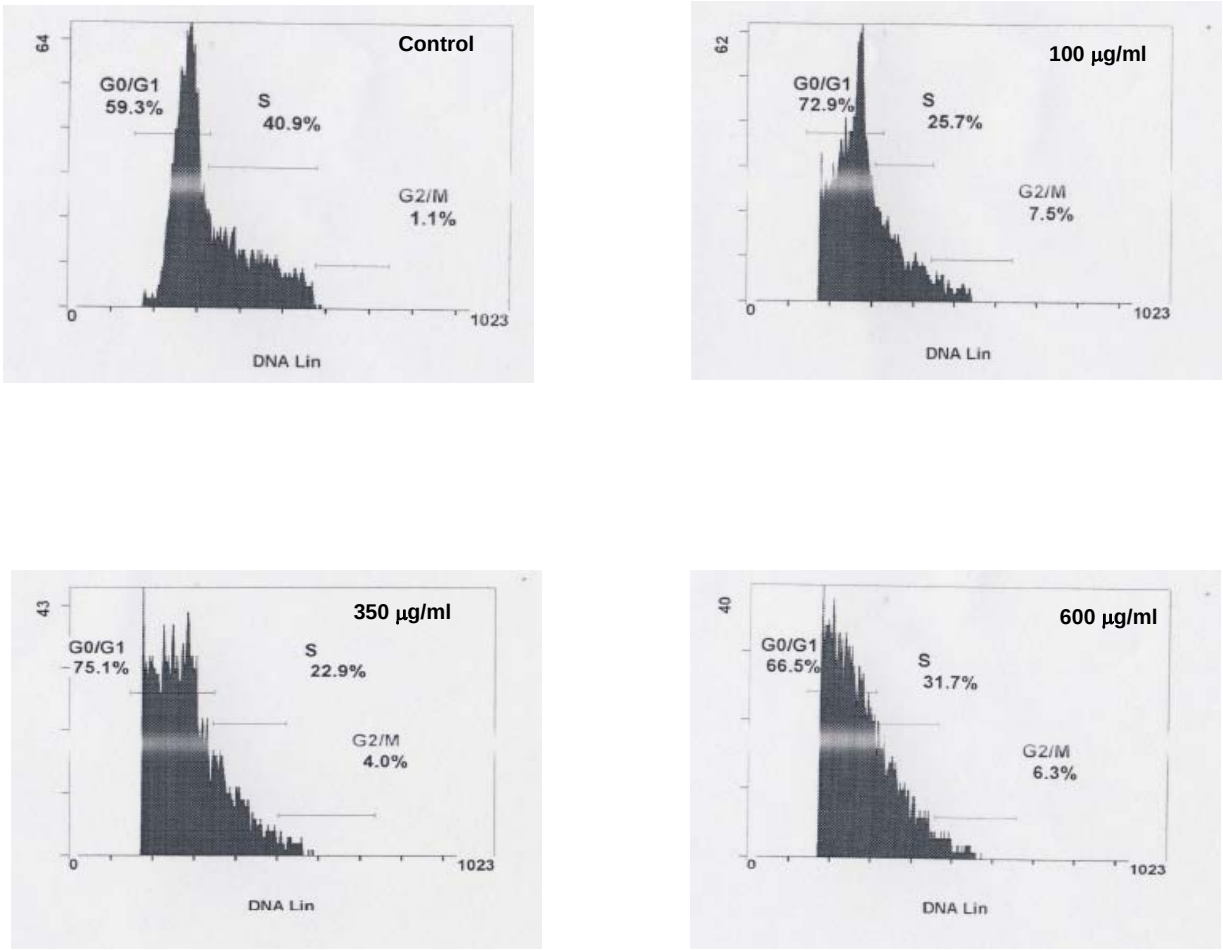




**Figure 3.27A.** The effect of CMECB on cell division cycle distribution in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 24 hours. Cellular DNA content was assayed using a flow cytometer after staining with propidium iodide. Graph shows percentages of cells in various cell division cycle phases. The data shown represent the mean± S.D. of two independent experiments.

**B**



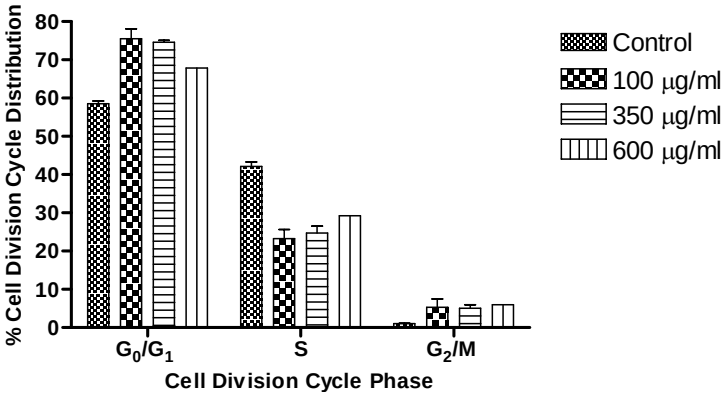
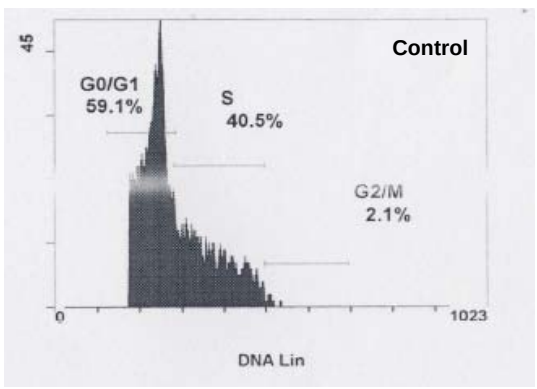
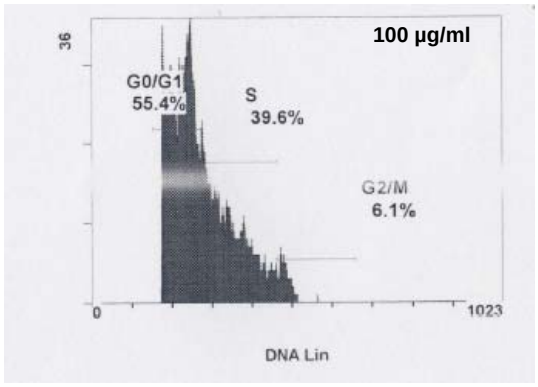
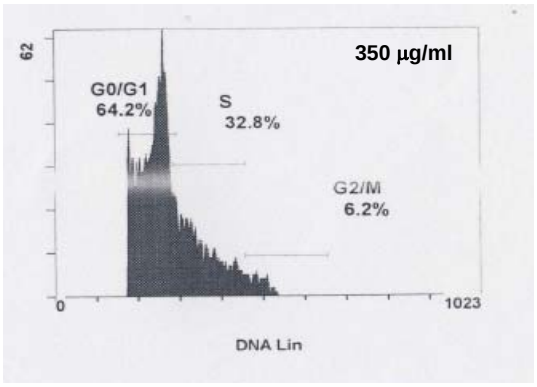



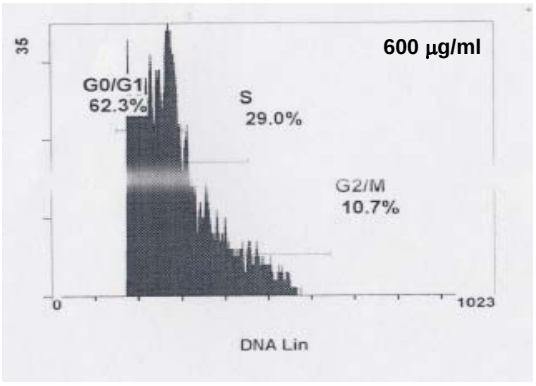

**Figure 3.27B.** The effect of CMECB on cell division cycle distribution in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 48 hours. Cellular DNA content was assayed using a flow cytometer after staining with propidium iodide. Graph shows percentages of cells in various cell division cycle phases. The data shown represent the mean± S.D. of two independent experiments.

**C**



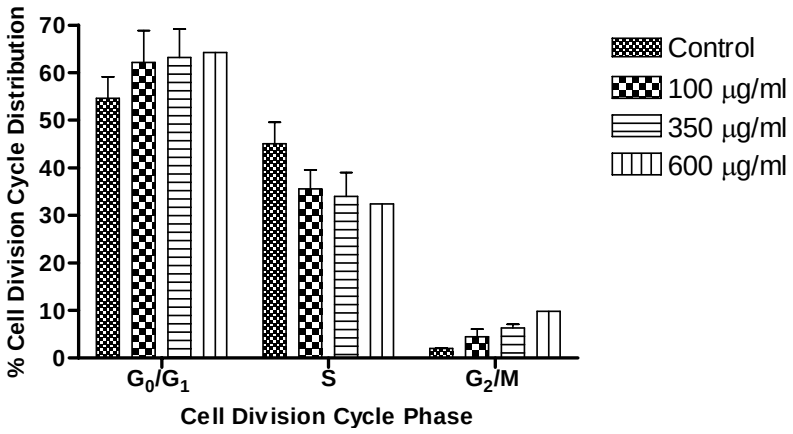




**Figure 3.27C.** The effect of CMECB on cell division cycle distribution in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 72 hours. Cellular DNA content was assayed using a flow cytometer after staining with propidium iodide. Graph shows percentages of cells in various cell division cycle phases. The data shown represent the mean± S.D. of two independent experiments.

## 3.8. CMECB induces $G_2$/M phase arrest by down-regulating cyclin B1 and Cdc2 protein levels

Since the exposure of experimental cultures to CMECB resulted in $G_2$/M phase arrest, the effects of CMECB on $G_2$/M cell division cycle phase regulatory proteins, cyclin B1, Cdc2 and p21, were investigated using Western blotting. Treatment of Jurkat T cells led to an increase in the protein levels of p21 after 12 hours of treatment. In contrast to the results obtained after 12 hours of treatment, a decrease in the protein levels of p21 was seen in Jurkat T cells treated with CMECB for 24 hours (Fig. 3.28). In addition, treatment of Jurkat T cells with 100 to 600 µg/ml of CMECB resulted in a dose-dependent decrease in Cdc2 protein levels which was accompanied by a dose- and time-dependent decrease in cyclin B1 protein expression after 12 and 24 hours (Fig. 3.28). No p21 protein expression levels were detected in response to 12 hours of exposure of Wil-2 NS cells to different concentrations of CMECB. However, a dose-dependent down-regulation in p21 protein levels was observed in Wil-2 NS cells exposed to CMCEB for 24 hours (Fig. 3.29). In addition, a dose-dependent decrease in Cdc2 protein levels in Wil-2 NS cells exposed to CMECB for 12 hours was observed, while no notable change in Cdc2 protein levels was observed in cell treated with CMECB for 24 hours. Furthermore, a dose-dependent decrease in cyclin B1 protein levels was observed in response to CMECB treatment for 24 hours, while no expression of cyclin B1 was detected after 12 hours (Fig. 3.29). These findings suggest that the CMECB-induced $G_2$/M phase arrest is mediated by the down-regulation of cyclin B1 and Cdc2 protein levels.

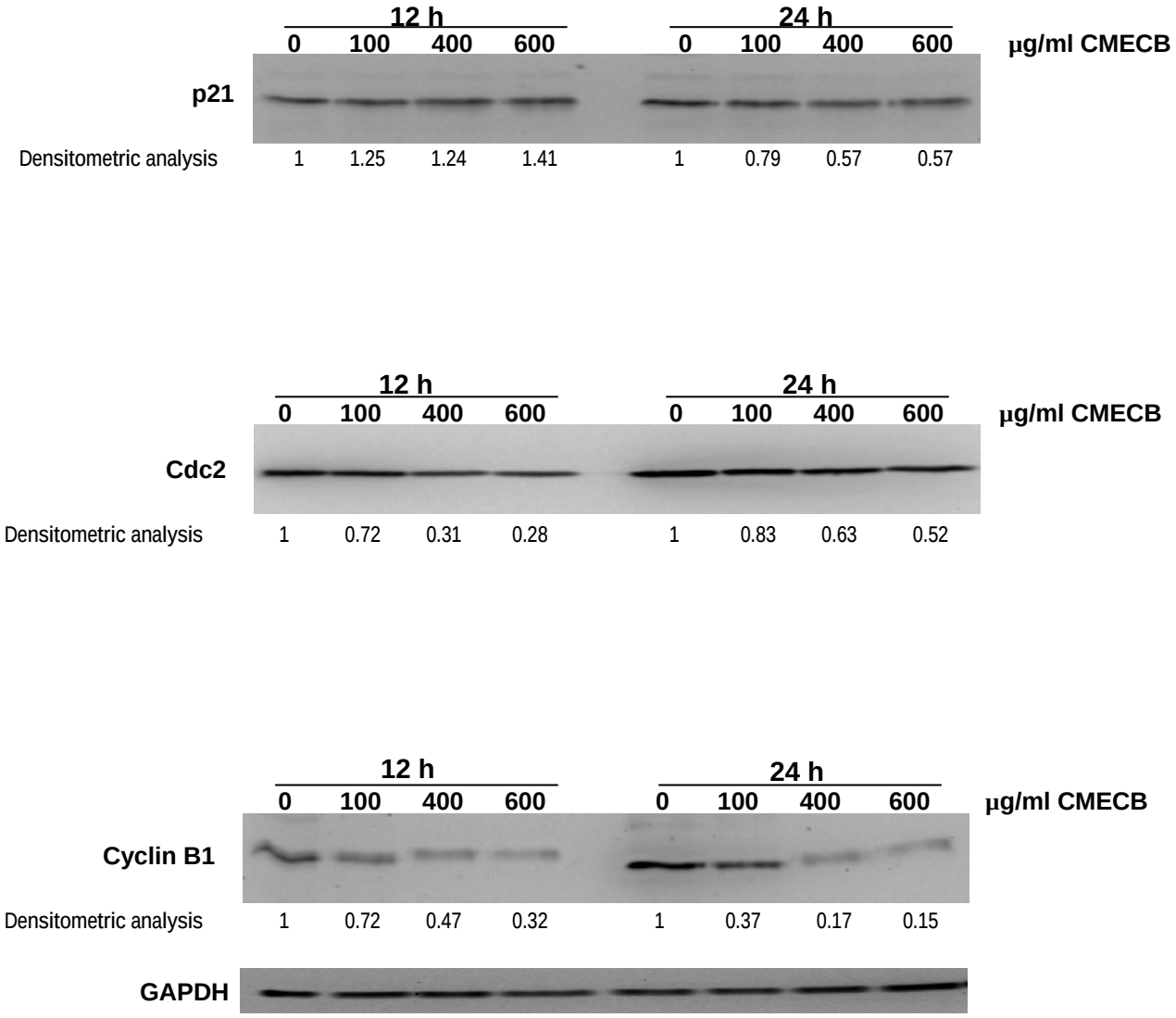



**Figure 3.28.** The effect of CMECB on cell division cycle regulatory proteins in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 12-24 hours and protein expression was analysed by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

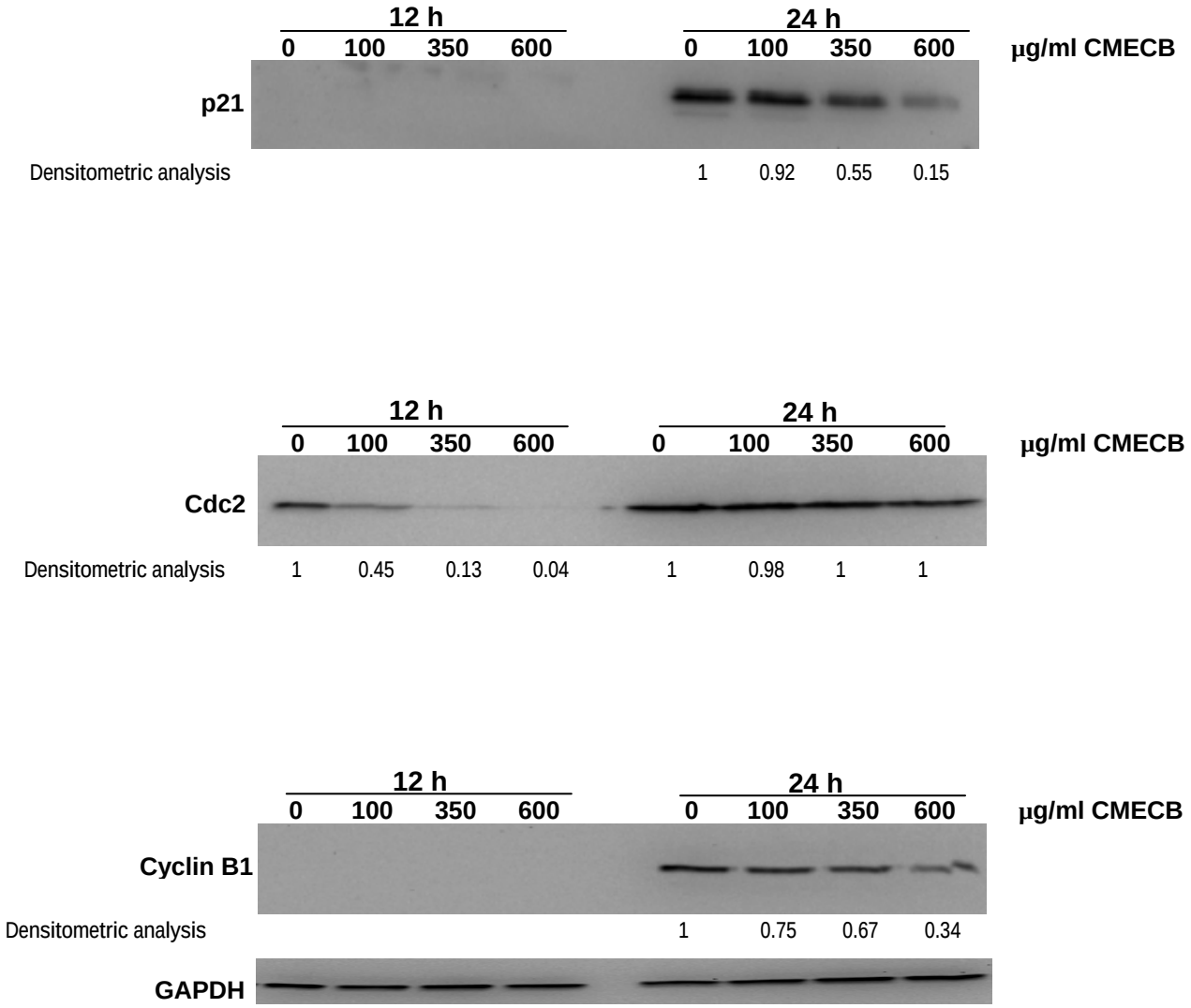



**Figure 3.29.** The effect of CMECB on cell division cycle regulatory proteins in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 12-24 hours and protein expression was analysed by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

## 3.9. The effects of CMECB on the mRNA expression of $G_2$/M phase regulatory genes

To determine the effect of CMECB on the mRNA expression profiles of *p21*, *cdc2* and *cyclin B1*, qrt-PCR was used. The results showed a down-regulation in the expression of *p21* mRNA in Jurkat T cells exposed to different concentrations of CMECB for 12 and 24 hours (Fig. 3.30). In Wil-2 NS cells, an increase in *p21* mRNA expression was observed after 12 and 24 hours in the presence of 100 and 600 µg/ml and different concentrations of CMECB (100 to 600 µg/ml), respectively (Fig. 3.31). Although *cdc2* mRNA expression was up-regulated after 12 hours of exposure of Jurkat T cells to different concentrations of CMECB, treatment of cells with CMECB for 24 hours resulted in a dose-dependent down-regulation of *cdc2* mRNA. (Fig. 3.30). In Wil-2 NS cells, *cdc2* mRNA levels were down-regulated in the presence of 350 and 600 µg/ml while an up-regulation was observed in cells exposed to 100 µg/ml for 12 hours. In contrast, *cdc2* mRNA expression was down-regulated in the presence of 100 µg/ml and up-regulated in cells treated with 350 and 600 µg/ml of CMECB for 24 hours (Fig. 3.31).

Furthermore, treatment of Jurkat T cells with CMECB resulted in the up-regulation of cyclin B1 mRNA expression after 12 and 24 hours (Fig. 3.30). Exposure of Wil-2 NS cells to CMECB led to a decrease in *cyclin B1* mRNA expression in the presence of different CMECB concentrations after 12 hour and 100 µg/ml after 24 hours, while an increase was observed in cells treated with 350 and 600 µg/ml for 24 hours (Fig. 3.31). These results also suggest that CMECB modulates the expression of $G_2$/M phase regulatory genes at the protein level and not at the mRNA level.

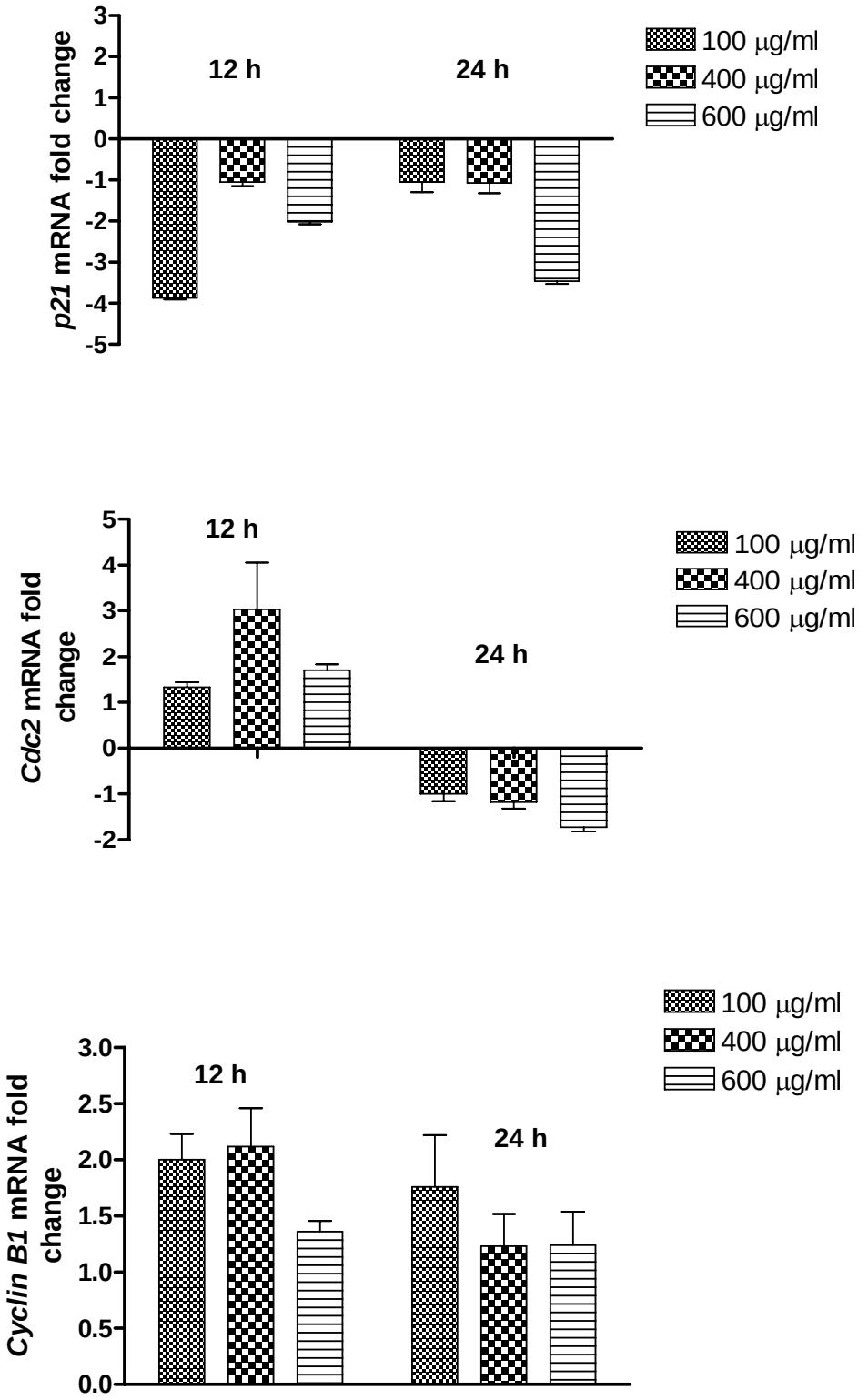



**Figure 3.30.** The effects of CMECB on *p21*, *cdc2* and *cyclin B1* mRNA expression in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for the indicated times and mRNA expression levels were determined by real-time PCR.

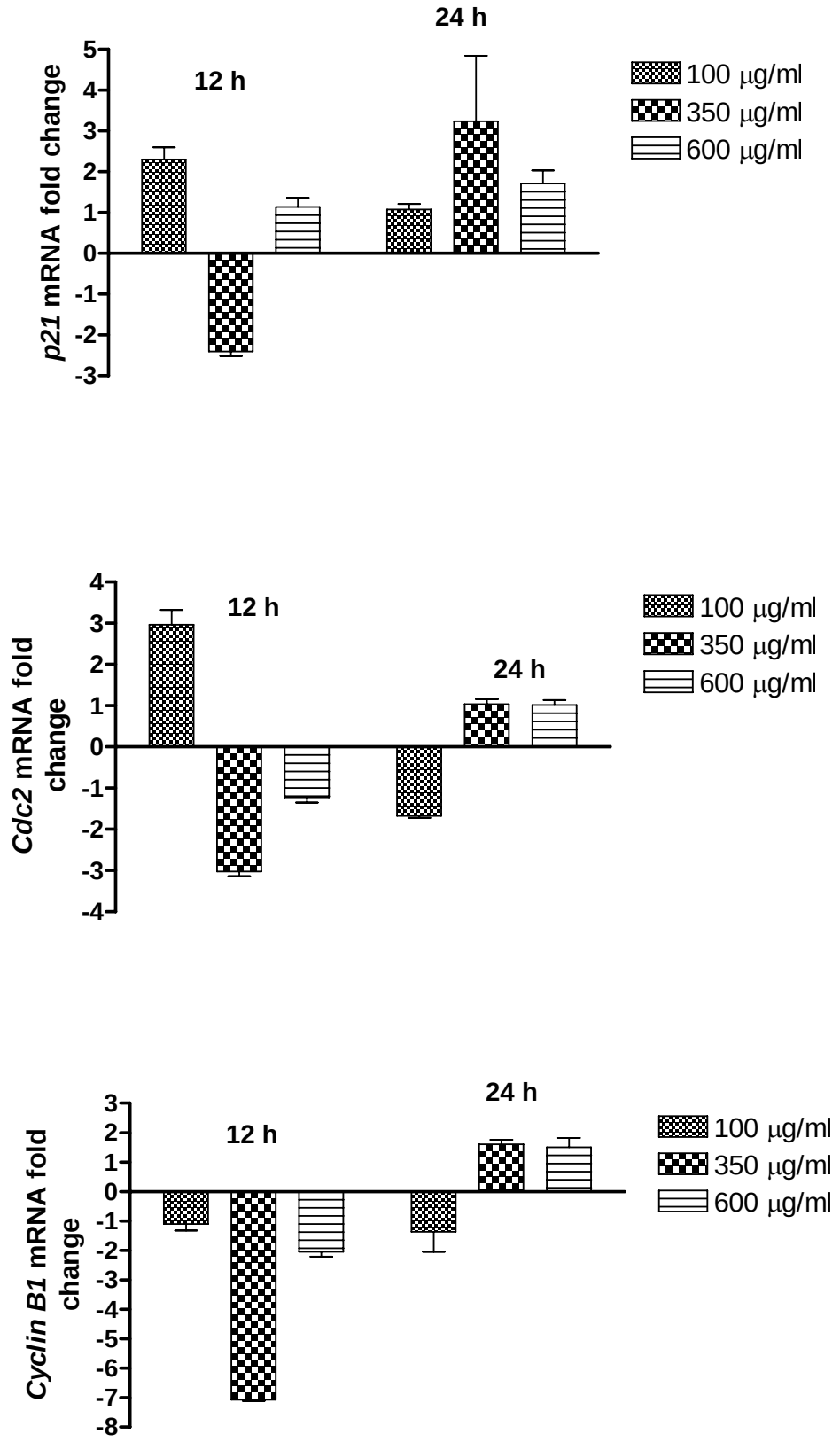



**Figure 3.31.** The effects of CMECB on *p21, cdc2* and *cyclin B1* mRNA expression in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for the indicated times and mRNA expression levels were determined by real-time PCR.

## 3.10. CMECB down-regulates p53 protein expression levels

p53 is known to induce arrest and/or apoptosis in response to various cellular stresses and anticancer agents by regulating the cell division cycle and apoptosis regulatory genes. The involvement of p53 in CMECB-induced cell division cycle arrest and apoptosis was investigated using Western blotting. No p53 protein expression was detected in Jurkat T cells in response to CMECB treatment at all experimental time intervals evaluated. In Wil-2 NS cells, p53 protein levels were down-regulated in response to CMECB after 12 and 24 hours (Fig. 3.32). These results suggest that the observed CMECB-induced apoptosis and $G_2$/M phase arrest are mediated in a p53-independent manner.

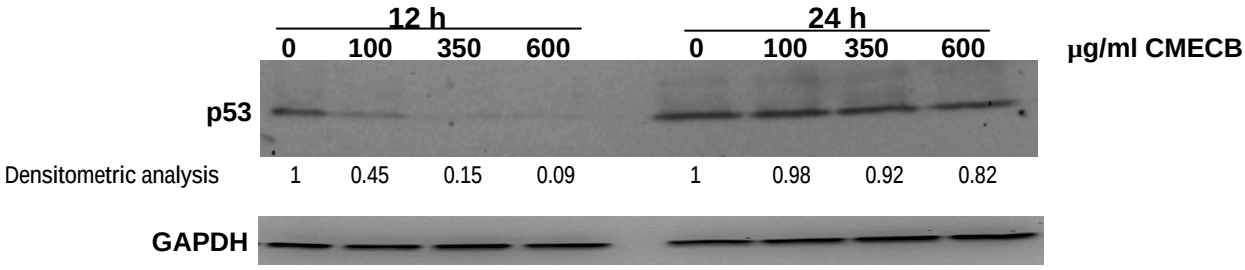



**Figure 3.32.** The effect of CMECB on p53 protein expression in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for 12-24 hours and protein expression was analysed by Western blotting. Densitometric band analysis was done using Quantity One Software. The intensity of each band relative to control is shown beneath the bands.

## 3.11. The effects of CMECB on *p53* mRNA expression

Using qrt-PCR the effects of CMECB on *p53* mRNA expression of the experimental cultures were investigated. At 12 hours Jurkat T cells exposed to 100 and 600 µg/ml showed a decrease in *p53* mRNA expression which was accompanied by an increase in *p53* mRNA expression in the presence of 400 µg/ml. This was followed by a dose-dependent down-regulation of *p53* mRNA expression in Jurkat T cells exposed to different concentrations of CMECB for 24 hours (Fig. 3.33). Treatment of Wil-2 NS cells resulted in the up-regulation of *p53* mRNA expression in the presence of 100 and 600 µg/ml after 12 hours and 350 and 600 µg/ml of CMECB for 24 hours (Fig. 3.34). The results suggest that CMECB down-regulates p53 gene expression at protein level and not at the mRNA level.

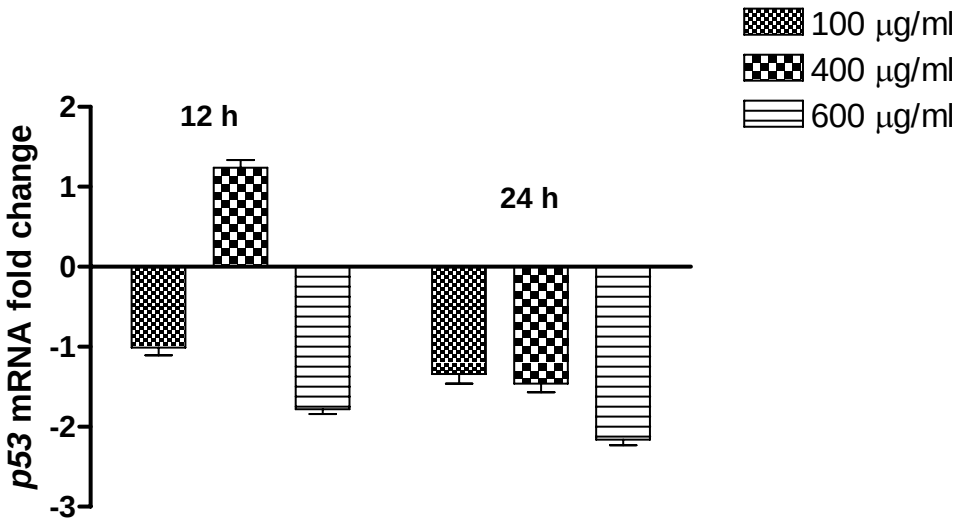



**Figure 3.33.** The effects of CMECB on *p53* mRNA expression in Jurkat T cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for the indicated times and mRNA expression levels were determined by real-time PCR.

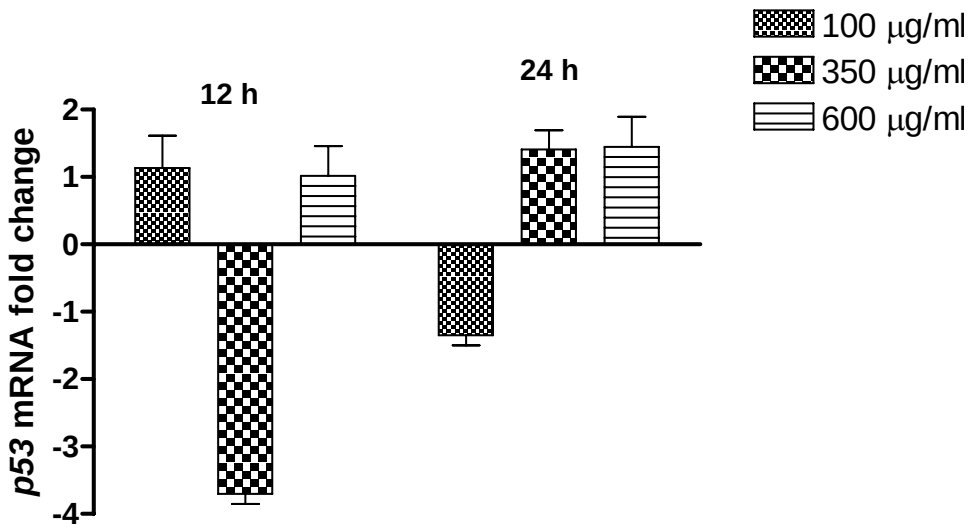



**Figure 3.34.** The effects of CMECB on *p53* mRNA expression in Wil-2 NS cells. Cells were exposed to various concentrations of CMECB or vehicle control (DMSO) for the indicated times and mRNA expression levels were determined by real-time PCR.

# Chapter 4

## Discussion and Conclusion

Plant extracts and/or plant-derived compounds have attracted a lot of interest as potential anticancer agents. This is attributed to their ability to induce cancer cell division cycle arrest and apoptosis with less or no harm to the surrounding normal cells (Hsu *et al*., 2006; Srivastava and Gupta, 2006). Indeed, apoptosis is a preferred way of eliminating cancerous cells since apoptotic cells are engulfed by macrophages and thus removed from the tissue without eliciting or invoking an inflammatory response (Brown and Wouters, 1999; Seraste and Pulkki, 2000; Zimmerman *et al*., 2001). The development of anticancer agents such as vinblastine from *Catharanthus roseus,* podo-phyllotoxin from *Podollum emody* and paclitaxel from *Taxus brevifolia* in the recent past has fuelled the search for more safe and effective plant-derived compounds and/or extracts that can be used as anticancer agents (Schwartsmann *et al*., 2002).

This renewed interest in traditional pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for the plants usage but also with the understanding of the molecular mechanisms and signaling pathways that are involved in their anticancer activity as well as the discovery of novel compounds of pharmaceutical value (Fennel *et al*., 2004). Previous studies from our laboratory demonstrated CMECB to possess anti-proliferative effects and also to induce apoptosis by a p53-dependent modulation of Bax and Bcl-2 protein expression levels in

Jurkat T and Wil-2 NS cells (Mbazima, 2005). Thus, it was against this background that the pro-apoptotic molecular events that are induced by CMECB, downstream of the mitochondria, and its effect on the cell division cycle distribution in Jurkat T and Wil-2 NS cancer cell lines were investigated in this study.

The first objective of the study was to evaluate the effect of CMECB on Jurkat T and Wil-2 NS cancer cell growth. The results showed a dose-dependent inhibition of cell proliferation and a dose- and time-dependent decrease in cell viability. Cell death is known to occur in two distinct modes: necrosis, an unordered and accidental form of cell death that occurs in response to a number of independent biochemical events that are activated by severe cell injury and depletion of cell energy (Kanduc *et al.*, 2002) and apoptosis, a controlled type of cell death that can be induced by a variety of physiological stimuli and pharmacological agents (Kerr *et al.*, 1972). Apoptotic cells are characterised by several cellular and nuclear morphological features such as cell shrinkage, condensation and margination of chromatin at the nuclear membrane, plasma membrane blebbing and apoptotic body formation. Therefore, to characterise the mode of cell death associated with the CMECB-induced growth inhibitory activity in Jurkat T and Wil-2 NS cells, the morphological changes associated with the induction of apoptosis were assessed. Similar to our previous findings (Mbazima, 2005), CMECB-treated Jurkat T and Wil-2 NS cells clearly demonstrated apoptotic nuclear morphological features such as the condensation and margination of chromatin (Figs. 3.4 and 3.5). These results indicate that the growth inhibitory

activity of CMECB in Jurkat T and Wil-2 NS cells is indeed associated with the induction of an apoptotic cell death programme.

Apoptosis is known to occur through two signaling pathways: the extrinsic and intrinsic pathways. The extrinsic pathway is mediated by death receptor protein members of the tumour necrosis factor (TNF) receptor superfamily. The intrinsic apoptosis pathway leads to cytochrome c release from the mitochondria (Fan *et al*., 2005; Kim, 2005). The commitment of cells to apoptosis initiated by either pathway is dependent on the balance between pro- and anti-apoptotic protein members of the Bcl-2 family. This is due to their ability to regulate the permeability of the outer mitochondrial membrane, which when disturbed leads to the release of apoptotic factors such as cytochrome c into the cytosol. The anti-apoptotic protein Bcl-2 resides on the outer membrane of the mitochondria and promotes cell survival by inhibiting the formation of pores and the subsequent release of cytochrome c which is required for caspase activation. In contrast, the pro-apoptotic protein Bax is located in the cytosol and becomes activated in response to various death stimuli. Once active, Bax translocates to the mitochondria where it is reported to antagonise the anti-apoptotic activity of Bcl-2 or forms channels that eventually lead to mitochondrial cytochrome c release (Hervouet *et al*., 2007; Youle and Strasser, 2008). The presence of cytochrome c in the cytosol is reported to initiate the formation of a complex called the apoptosome which is required for the recruitment and activation of initiator caspase-9. In turn, the active caspase-9 proteolytically cleaves and activates the executioner caspase-3 which further cleaves and inactivates various cellular proteins

including PARP (Letai, 2005). Although CMECB failed to influence the mRNA expression levels of *bax* and *bcl-2* (data not shown), western blot results showed a definite translocation and subsequent accumulation of Bax protein levels in the mitochondria (Figs. 3.8 and 3.11); this was accompanied by a decrease in Bcl-2 protein levels (Figs. 3.6 and 3.9) and an increase in cytosolic cytochrome c levels (Figs. 3.14 and 3.15) in both CMECB-treated cell lines. Based on these observations, we concluded that CMECB modulates *bax* and *bcl-2* gene expression at the protein level. In addition, the activation of initiator caspase-9 and executioner caspase-3, as well as PARP cleavage, was observed in response to CMECB exposure to Jurkat T and Wil-2 NS cells. These results suggest that CMECB induces modulation of the Bcl-2 family of proteins which in turn mediates mitochondrial cytochrome c release into the cytosol; this subsequently led to the activation of caspase-9 and caspase-3 and eventual PARP degradation. The modulation of Bcl-2 family members, release of cytochrome c and activation of caspase-9 strongly suggests that CMECB induces mitochondrial-mediated apoptosis signaling pathway in these cellular systems.

Since it has been reported in various studies that apoptosis occurs in close association with cell division cycle arrest, it was pertinent to investigate the effect of CMECB on the cell division cycle distribution profiles and cell division cycle regulatory proteins in both Jurkat T and Wil-2 NS cells. Clearly, our results demonstrated that CMECB treatment of Jurkat T and Wil-2 NS cells leads to the arrest of the cell division cycle at $G_2$/M phase (Figs. 3.26 and 3.27). Since cell proliferation is tightly linked to the cell division cycle, these

results suggest that the observed CMECB-induced anti-proliferative activity might be associated with cell division cycle arrest. The progression of the cell division cycle is positively regulated by catalytic complexes formed between cdks and cyclins. The cyclin B1/Cdc2 complex is crucial for the transition of the cell division cycle from the $G_2$ to the M phase. In addition, the kinase activity of the Cdc2 protein is dependent on its association with cyclin B1 (Johnson and Walker, 1999). Thus, the down-regulation of cyclin B1 and/or the inhibition of cyclin B1/Cdc2 complex formation may lead to $G_2$/M phase cell division cycle arrest. Accordingly, CMECB treatment caused a decrease in the protein expression levels of cyclin B1 and Cdc2 in both the test cell lines (Figs. 3.28 and 3.29). Consistent with the CMECB s inability to affect the mRNA levels of *bax* and *bcl-2* genes, no clear trend or notable effect was observed in the expression of *cdc2* and *cyclin B1* mRNA levels in Jurkat T and Wil-2 NS cells exposed to CMECB (Figs. 3.30 and 3.31). This may suggest that CMECB down-regulate $G_2$/M phase regulatory genes at the protein level rather than at the mRNA level.

Considering that protein p21 is a cell division cycle inhibitor which exerts its inhibitory effects by binding cyclin/cdk complexes and inactivating the kinase activity of cdks, its role in the CMECB-induced cell division cycle arrest was investigated. Despite the reduction in cyclin B1 and Cdc2 protein levels in Wil-2 NS cells, a decrease in the protein levels of p21 was observed. In contrast, a slight up-regulation of p21 protein was detected in Jurkat T cells after 12 hours of exposure to CMECB. These results, therefore, suggest that CMECB treatment causes Jurkat T and Wil-2 NS cells, $G_2$/M phase arrest by down-

regulating cyclin B1 and Cdc2 protein levels; this subsequently led to the inhibition of cyclin B1/Cdc2 complex formation, a key event for the transition from the $G_2$ to the M phase. Furthermore, CMECB-induced $G_2$/M phase arrest in Wil-2 NS cells is thought to be mediated by other mechanisms or molecules independent of p21 activity. On the other hand, our observations suggest that the CMECB-induced $G_2$/M phase arrest in Jurkat T cells might be mediated, in part, by the p21 protein. Again, there was no notable change in the expression levels of *p21* mRNA (Figs. 3.30 and 3.31), thus further confirming that CMECB exerts its anticancer effects by modulating these apoptosis and cell cycle regulatory genes at the protein level rather than at the level of the mRNA expression.

Since apoptosis and cell division cycle are closely linked and are regulated by the phosphoprotein p53, the role of the p53 protein in the observed CMECB-induced $G_2$/M cell division cycle arrest and apoptosis was investigated. p53 is reported to play an important role in deciding whether a cell undergoes reversible cell division cycle arrest and/or apoptosis in response to DNA damage, oxidative stress and other cellular stress signals (Marchetti ***et al***., 2004; Michael and Oren, 2002). The decision to induce cell division cycle arrest and/or apoptosis by p53 depends on the intensity of the cellular stress or DNA damage and the available amount of the p53 protein in the cell. Less intense stress signals lead to p53-mediated cell division cycle arrest while highly intense signals lead to p53-mediated apoptotic cell death. p53 is also reported to carry out its dual role as a cell division cycle and apoptosis

regulator by transcriptionally activating and/or repressing several cell division cycle and apoptosis regulatory genes (Fuster *et al*., 2007).

Most notably, p53 has been demonstrated to induce the transcription of the universal cyclin-dependent kinase inhibitor, p21 (Kanemitsu *et al*., 2009). The induction of p21 protein leads to the inhibition of Cdc2 activity, a critical event for the transition of cells from the $G_2$ to the M phase. As a result of Cdc2 inhibition, the cells get arrested at the $G_2$/M phase to give time to repair themselves or undergo apoptosis (Taylor and Stark, 2001). Although a slight up-regulation in the p21 protein levels was observed in CEMCB-treated Jurkat T cells (Fig. 3.28), no p53 protein (data not shown) as well as appreciable *p53* mRNA expression levels (Fig.3.30) were detected at all experimental time points evaluated. Our results therefore, suggest that the slight increase in p21 protein expression levels and the $G_2$/M phase arrest in CMECB-treated Jurkat T cells are mediated in a p53-independent manner. In addition, CMECB also induced a p53-independent $G_2$/M phase arrest in Wil-2 NS cells. This was shown by the down-regulation of Cdc2, cyclin B1 and p21 protein levels (Fig. 3.29) which were accompanied by a decrease in p53 protein levels after 12 and 24 hours of exposure to CMECB (Fig. 3.32). The decrease in p53 protein expression levels in Wil-2 NS cells treated with CMECB as compared to the control cells (Fig. 3.32) suggests that the extract might be enhancing the degradation of p53 protein through a proteosome/ubiquitin pathway. In summary, the results suggest that CMECB induces $G_2$/M phase arrest in Jurkat T and Wil-2 NS cells independent of p53 protein activity. Therefore, the

mechanism(s) involved in the CMECB-induced $G_2$/M phase arrest in both experimental cultures remains to be identified.

p53 is reported to regulate apoptosis by regulating the expression levels of Bax and Bcl-2, both in a transcription-dependent and/or-independent manner (Fuster *et al*., 2007; Hofseth *et al*., 2004). Upon sensing stress signals, p53 translocates to the nucleus where it plays a role as a transcription factor by activating Bax protein expression and suppressing Bcl-2 protein levels (Yee and Vousden, 2005). In transcription-independent mechanism, p53 has also been shown to translocate to the mitochondria where it directly binds to Bcl-2 proteins. The interaction between the p53 and Bcl-2 proteins is believed to lead to the inactivation of Bcl-2 s anti-apoptotic activity and the promotion of mitochondrial release of cytochrome c into the cytosol (Lavin and Gueven, 2006; Liu *et al*., 2007; Johnstone *et al*., 2002). In contrast to these reports, our results gave an indication that p53 was not involved in the events leading to the alteration of Bax and Bcl-2 protein expression levels in both experimental culture systems. This is because the down-regulation of Bcl-2 protein levels and the increase in Bax protein levels in the mitochondria, as a result of Bax translocation, were detected as early as 0.5 hour after treatment; and at that point no p53 expression was detected both in the total cellular and mitochondrial protein lysates (data not shown). These results are also in contrast with the previous findings (Mbazima, 2005; Mbazima *et al*., 2008) that showed a p53-mediated alteration of *bcl-2* and *bax* gene expression levels in Jurkat T cells. This discrepancy might be due to the difference in the time intervals used in the present and the previous studies. The current

observations suggest that other mechanisms or molecules may be involved in the CMECB-induced activation and subsequent translocation of Bax from the cytosol to the mitochondria and the down-regulation of Bcl-2 protein levels. These mechanisms or molecules remain to be delineated or elucidated.

In summary, CMECB exerts its anticancer activity by inducing cell division cycle arrest that is accompanied by mitochondrial-mediated apoptosis in Jurkat and Wil-2 NS cells. This was demonstrated by the modulation of Bax and Bcl-2, mitochondrial cytochrome c release and activation of caspases-9 and -3, as well as PARP degradation. Judging from the early activation of Bax and caspases -9 and -3, the CMECB-induced apoptosis appears to precede cell division cycle arrest. Cell division cycle arrest was demonstrated by the accumulation of cell population at the $G_2$/M phase which was also confirmed by the down-regulation of cyclin B1 and Cdc2 proteins. It is possible that the late $G_2$/M phase arrest may subsequently trigger more cells to undergo apoptosis in an attempt to eliminate the severely stressed or damaged cells. Our data also revealed that the CMECB-induced cell division cycle arrest and apoptosis were independent of p53 activity in both Jurkat T and Wil-2 NS cells. Based on these findings, two apoptotic pathway models that are induced by CMECB in both Jurkat T and Wil-2 NS cells are proposed (Figs. 4.1 and 4.2, respectively). Since we do not know the identity of the active component(s) in CMECB that are responsible for the plant's inherent anticancer activity, a follow-up study is underway in an effort to purify and identify these bioactive components.

In conclusion, the ability of CMECB to induce cell cycle arrest associated with apoptosis in a p53-independent manner makes it a potential candidate for the treatment of tumours that occur due to the lack of p53 activity. Additionally, since most cancers occur as a consequence of a rampant or non-functional p53 gene or gene products, the findings of this study are particularly interesting. Furthermore, these findings show the potential of *C. benghalensis* L as an anticancer agent. However, further studies are warranted to evaluate the molecules or mechanism(s) of Bax translocation induced by CMECB. Furthermore, there is also a need to evaluate the potential *in vivo* anticancer activity of CMECB using animal models.

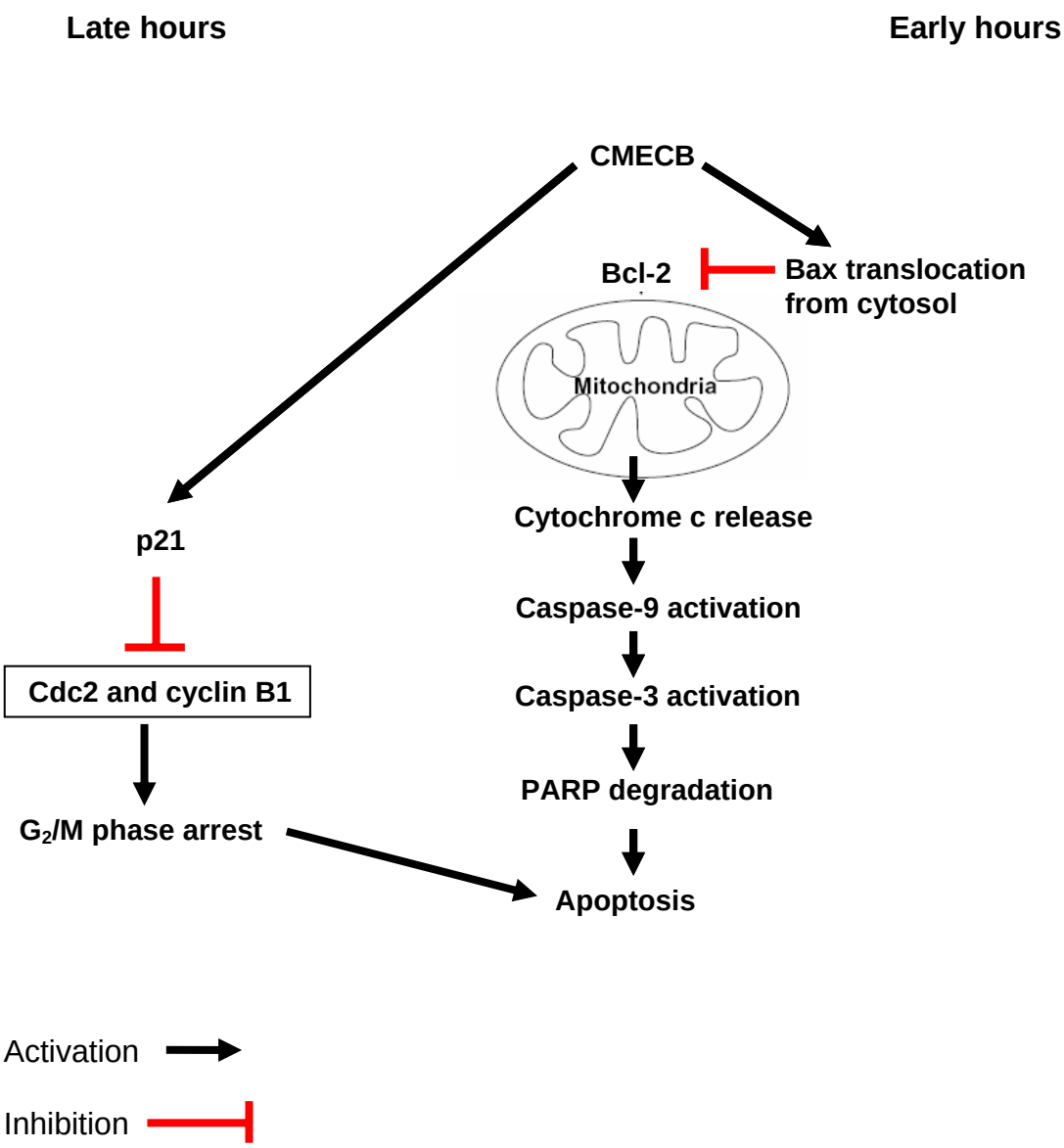



**Figure 4.1. A proposed model of an apoptotic pathway induced by CMECB in Jurkat T cells.** At early hours, CMECB induces Bax protein translocation from the cytosol to the outer membrane of the mitochondria (OMM). On the OMM, Bax protein antagonises the anti-apoptotic activity of Bcl-2 and form pores that facilitate the release of mitochondrial cytochrome c into the cytosol. When in the cytosol, cytochrome c activates a caspase cascade which leads to the degradation of PARP and eventual apoptosis. After 12 and 24 hours of exposure, CMECB induces a p21 which in turn arrests the cell division cycle at the $G_2$/M phase by inhibiting cyclin B1/Cdc2 complex formation, thus leading to apoptosis.

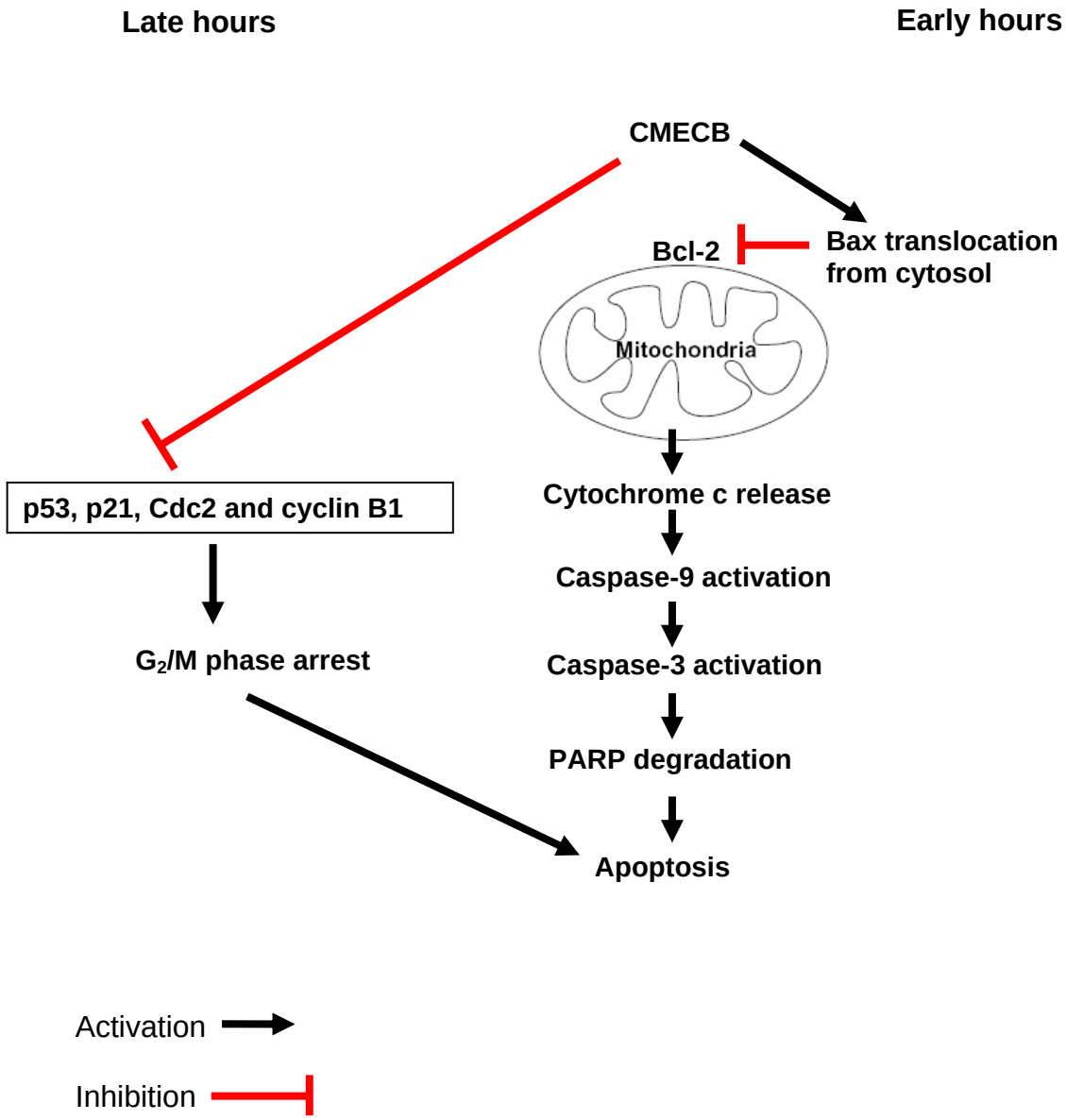



**Figure 4.2. A proposed model of an apoptotic pathway induced by CMECB in Wil-2 NS cells.** At early hours, CMECB induces Bax protein translocation from the cytosol to the outer membrane of the mitochondria (OMM). On the OMM, Bax protein antagonises the anti-apoptotic activity of Bcl-2 and form pores that facilitate the release of mitochondrial cytochrome c into the cytosol. When in the cytosol, cytochrome c activates a caspase cascade which leads to the degradation of PARP and eventual apoptosis. After 12 and 24 hours of exposure, CMECB induces cell division cycle arrest at the $G_2$/M phase by inhibiting cyclin B1/Cdc2 complex formation and induce apoptosis in a p53- and p21-independent manner.

# Chapter 5

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# Appendix

## A1: Abstract for conference oral presentation

### 1. Growth inhibitory and apoptosis inducing effects of crude methanolic extracts of *Commelina* species through modulation of apoptosis regulatory genes in Wil-2 NS and Jurkat T cancer cell lines.

Mbazima, V.G and Mampuru, L.J.

*Biochemistry Discipline, School of Life and Molecular Sciences, University of the North, Private Bag X 1106, Sovenga, 0727, Limpopo Province, South Africa.*

Extracts of *Commelina* species used in our study, although not extensively documented, are frequently used in traditional medicine for the treatment of ailments such as skin ulcers and skin lumps. In this study, we investigated the effects of CMEC on cell proliferation and the induction of apoptosis in cultured Wil-2 NS and Jurkat T cancer cell lines. CMEC inhibited cell proliferation and decreased cell viability in a time and dose dependent manner, as determined by the Coulter counter, trypan blue dye exclusion assay, respectively. Other results revealed that CMEC induced classical morphological changes associated with apoptosis and modulated the expression levels of apoptosis regulatory genes, such as, *bax*, *bcl-2* and *p53*, as demonstrated by Western blot analysis and RT-PCR. Taken together, the data strongly suggest that the crude extracts of *Commelina* species contain bioactive compounds that may be beneficial in the treatment of cancerous growths and this apparent anti-neoplastic activity is a consequence of dysregulated expression of apoptosis-responsive genes.

- Presented at the South African Society of Biochemistry and Molecular Biology (SASBMB) XIX$^{th}$ conference held at the University of Stellenbosch from 16-20 January 2005.

# A2: Abstracts for conference poster presentations

## 1. Pro-apoptotic effects of *Commelina* species on Jurkat T cancer cell line

Mbazima, V.G and Mampuru, L.J

*Department of Biochemistry, Microbiology and Biotechnology, School of Life and Molecular Sciences, University of Limpopo, Turfloop Campus, Private Bag X 1106, Sovenga, 0727, Limpopo Province, South Africa.*

Extracts of *Commelina* species used in our study, although not extensively documented, are frequently used in traditional medicine for the treatment of ailments such as skin ulcers and skin lumps. In this study, we investigated the effects of crude methanolic extracts of *Commelina* (CMEC) on cell proliferation and the induction of apoptosis in cultured Jurkat T cancer cell lines. CMEC inhibited cell proliferation and decreased cell viability in a time and dose-dependent manner, as determined by the Coulter counter and trypan blue dye exclusion assay, respectively. Other results revealed that CMEC induced classical morphological changes associated with apoptosis and modulated the expression levels of apoptosis regulatory genes, such as, *bax*, *bcl-2* and *p53*, as demonstrated by Western blot analysis and RT-PCR. Taken together, the data strongly suggest that the crude extracts of *Commelina* species contain bioactive compounds that may be beneficial in the treatment of cancerous growths and this apparent anti-neoplastic activity is a consequence of dysregulated expression of apoptosis-responsive genes.

- Presented at the Indigenous plant use forum (IPUF) conference held at the University of Botswana from 3-6 July 2006.

- Presented at the Molecular and Cell Biology Group (MCBG) annual symposium held at the University of Witwatersrand on October 12, 2006.

## 2. The crude methanol extract of *Commelina benghalensis* induces $G_2$/M phase arrest and apoptosis in Wil-2 NS cells

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*Commelina* species used in this study is used in traditional medicine in the eastern and northern parts of South Africa. It is used for the treatment of skin outgrowth, epilepsy, stomach disorders. We have evaluated CMECB as a cancer chemopreventative agent *in vitro* by studying its role in the regulation of proliferation, cell division cycle and apoptosis in Wil-2 NS cancer cell line. CMECB inhibited cell proliferation and induced $G_2$/M arrest and apoptosis in Wil-2 NS cells. CMECB-induced apoptosis in Wil-2 NS cells is mediated via the modulation of the intrinsic apoptotic pathway. Upregulation and phosphorylation of p53 resulted in the down regulation of the anti-apoptotic protein Bcl-2. The altered expression of Bcl-2 triggered the release of cytochrome c and activation of caspase-9 followed by activation of effector caspase-3 leading to PARP cleavage and apoptosis. In summary, inhibition of Wil-2 NS cells proliferation is related to cell cycle arrest at the $G_2$/M and induction of caspase-mediated apoptosis.

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# INHIBITION OF WIL-2 NS CELLS PROLIFERATION IS RELATED TO G2/M ARREST AND INDUCTION OF CASPASE-MEDIATED APOPTOSIS

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## Abstract

*Commelina benghalensis* L is an annual or perennial herb with fleshy creeping stems that root readily at the nodes. The plant is used in the eastern and northern parts of South Africa as a crude medicinal agent in the treatment of stomach disorders, skin lumps and/or on cancerous skin outgrowths. We have evaluated CMECB for its possible chemopreventative application in vitro by studying its role in the regulation of proliferation, cell division cycle and apoptosis in Wil-2 NS cancer cell line. CMECB inhibited cell proliferation and induced $G_2$/M arrest and apoptosis in Wil-2 NS cells. CMECB-induced apoptosis of Wil-2 NS cells is mediated via the modulation of the intrinsic apoptotic pathway. Up-regulation and phosphorylation of p53 resulted in the down regulation of the anti-apoptotic protein Bcl-2. The altered expression of Bcl-2 triggered the release of cytochrome c and activation of caspase-9 followed by activation of effector caspase-3 thus leading to PARP cleavage and apoptosis. In conclusion, CMECB exerts its anti-neoplastic effects by modulating the cell cycle and apoptosis regulatory genes. Acknowledgements: National Research Foundation (NRF) and the University of Limpopo senate research fund for financial support.

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*Full Length Research Paper*

# Alteration of Bax-to-Bcl-2 ratio modulates the anticancer activity of methanolic extract of *Commelina benghalensis* (Commelinaceae) in Jurkat T cells

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**Stem extracts of *Commelina benghalensis* (Linn.), although not extensively documented, are frequently used in traditional medicine for the treatment of ailments such as skin malformations and outgrowths. Accordingly, the study was aimed to investigate possible molecular mechanisms that are associated with the potential anti-carcinogenic property of this agrofield weed. Jurkat T cells were exposed to different concentrations (0-600 µg/ml) of the crude methanolic extract of *C. benghalensis* to evaluate their growth inhibitory and apoptosis inducing effects. The extract elicited a dose- and time-dependent inhibition of cell proliferation, followed by a concomitant decrease in cell viability. The observed cytotoxicity was linked to the induction of apoptosis as determined by morphological and biochemical features known to be associated with the advent of apoptosis. Real time quantitative RT-PCR and Western blot analyses of *Bax*, *Bcl*-2 and p53 exhibited aberrant expression profiles of these genes under various treatment conditions. Taken together, the data suggest that the crude methanolic extract of *C. benghalensis* contains bioactive compounds that may be beneficial in the treatment of malignant growths, and that this apparent antineoplastic activity is a consequence of dysregulated expression of apoptosis-responsive genes. These observations could provide a credible scientific justification upon which the ethnopharmacological utilisation of *C. benghalensis* is founded.**

**Key words:** Apoptosis, *Bcl*-2, *Bax*, p53, *Commelina benghalensis*; Jurkat T cells.

## INTRODUCTION

Plant-derived compounds and extracts have traditionally played important roles in the treatment of a number of human diseases (Farnsworth, 1984; Cox, 1994; Taraphdar et al., 2001). Recent studies suggest that a number of anticancer drugs have side effects and encounter resistance from slow growing cancers. Thus, there is a need to develop new, safe and effective prototype anticancer drugs from natural products (Yang et al., 2000; Taylor et al., 2001). Indeed, recent studies on tumour inhibitory compounds of plant origin have yielded an impressive array of novel structures, with notable drugs such as taxol. These new prototypes could serve as templates in the design of potential chemo-therapeutic agents. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells (Yi et al., 2003). This ideal situation is achievable by inducing apoptosis in cancer cells.

Apoptosis is a defined form of cell death, which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the

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populations in tissues under physiological and pathological conditions (Leist and Jaattela, 2001). Apoptotic cells are characterised by a number of morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and the formation of apoptotic bodies (Zimmerman et al., 2001; Orienius, 2004). Some of the morphological changes associated with apoptosis occur as a result of the activation of endogenous endonucleolytic and proteolytic (caspases) enzymes that, in turn, mediate the cleavage of DNA into fragments as well as protein substrates, which usually determine the integrity and shape of the cytoplasm and organelles (Seraste and Pulkki, 2000; Denault and Salvesen, 2002; Kasibhatla and Tseng, 2003). Furthermore, apoptosis is regulated and executed by different interplay of many genes responsive to various stimuli (Huang and Cidlowski, 2002). The most widely studied genes involved in apoptosis are the *bcl-2* family members and *p53*; these are major regulators of the apoptotic process, whereas caspases are the major executioners (Cohen, 1997).

The tumour suppressor protein p53 is a transcription factor that regulates various genes involved in the regulation of cellular response to DNA-damage, cell cycle control and induction of apoptosis (Xie and Shaikh, 2006; Liu et al., 2007). p53 is reported to induce apoptotic cell death by directly or indirectly modulating the expression of the Bcl-2 family of proteins (Bax and Bcl-2). The Bcl-2 family of proteins play an important role in the control of apoptosis by regulating mitochondrial membrane permeability (Hofseth et al., 2004). The family includes a number of anti-apoptotic members such as Bcl-2 and Bcl-$X_L$, as well as pro-apoptotic members including Bax and Bad (Scatena et al., 1998). Bax is a pro-apoptotic member of the Bcl-2 family that resides in the cytosol. Together with other pro-apoptotic family members, Bax promotes apoptosis by forming homodimeric and heterodimeric complexes that result in the formation of channels or pores on the mitochondrial membrane to facilitate the release of cytochrome c and apoptosis-inducing factors (AIFs) from mitochondrial intermembrane spaces into the cytosol. The formation of these pores results in the loss of the selective ion permeability across the mitochondrial membrane. The intracisternal contents that are released into the cytosol triggers a cascade of events that culminate in the execution of apoptosis through activation of caspases (Israels and Israels, 1999).

In contrast, the anti-apoptotic Bcl-2 protein localises the outer mitochondrial membrane and mediates its anti-apoptotic effects by stabilising the integrity of the mitochondrial membrane. The stabilisation of the mitochondrial membrane leads to the inhibition of the permeability transition pore and the release of cytochrome c (Vander and Thompson, 1999), and this is achieved by formation of heterodimers with pro-apoptotic members of such as Bax and Bak, thus neutralising their activity (Zörnig et al., 2001). Indeed, heterodimerisation of pro- and anti-apoptotic proteins was shown to antagonise apoptosis (Zörnig et al., 2001). Thus, the sensitivity of cells to apoptotic stimuli will depend upon the relative ratios of Bax/Bax homodimers, Bax/Bcl-2 heterodimers and Bcl-2/Bcl-2 homodimers (Israels and Israels, 1999). Additionally, over expression of Bcl-2 prevents the activation of caspases in apoptosis and hypoxia-induced necrosis, suggesting that Bcl-2 prevents cell death by acting upstream of the activation of caspases (Chinnaiyan and Dixit, 1996).

*Commelina benghalensis* Linn. [family: Commelinaceae] commonly known as the Benghal dayflower or tropical spiderwort, is an annual or perennial herb native to the tropical and subtropical Asia and Africa. It is widely distributed in the eastern and northern parts of South Africa, including the Limpopo province. The stem sap squeezed from *C. benghalensis* is topically used, in local traditional medicine, for the treatment of skin lumps or skin outgrowths (*K. Mabela*, personal communication). In the Chhattisgarh province of India, *C. benghalensis* (locally called common Rice weed or *Kaua-Kaini*) is used by senior traditional healers in the treatment of cancer (http://botanical.com/site/column_poudhia/articles/_11840.html). Additionally, the plant is used medicinally in China as a diuretic, febrifuge and an anti-inflammatory agent (Hong and DeFillipps, 2000). The plant is also used as animal fodder and vegetable, as a laxative and to cure inflammations of the skin as well as leprosy (Qaiser and Jafri, 1975). However, there is virtually no scientific substantiation to support and validate the above claims. It was, therefore, the objective of this study to try to delineate and validate some of the cellular and molecular mechanism(s) associated with the anticancer activity of *C. benghalensis* L.

## MATERIALS AND METHODS

### Reagents

RPMI-1640 media and foetal bovine serum (FBS) were purchased from Highveld Biologicals (Pty) Ltd, Lyndhurst, South Africa. Penicillin, streptomycin and neomycin, (PSN) antibiotic cocktail from Gibco, Auckland, New Zealand. Monoclonal IgG primary antibodies, Goat anti-mouse IgG-HRP conjugated secondary antibodies, and Western blotting luminol reagent from Santa-Cruz, Biotechnology Inc., USA. DMSO and Tween-20 from Merck Laboratory Suppliers (Pty). Sodium dodecyl-sulphate (SDS), BDH Laboratory Suppliers. Phenylmethylsulfonyl fluoride (PMSF) from Boehringer-Mannheim, Germany. Bromophenol blue, Coomassie blue R200, Sodium chloride, Ethanol, and Methanol from Saarchem (Pty) Ltd, South Africa.

### Preparation of crude extract

Frozen stems of *C. benghalensis* (CB) were minced in liquid nitrogen using a wharing blendor. Ground stems were weighed and exhaustively extracted with absolute methanol (1 g/10 ml, w/v) at room temperature for 24 h. The resultant methanolic extract was

**Table 1.** The primers used for the amplification of the respective genes in the real time quantitative RT-PCR.

| Gene | Primer Sequence |
|---|---|
| Bax | Forward primer: 5'-GCCCTTTTGCTTCAGGGTTT-3' |
| | Reverse primer: 5'-TCC AAT GTC CAG CCC ATG AT-3' |
| Bcl-2 | Forward primer: 5'-GACAGAAGATCATGCCGTCC-3' |
| | Reverse primer: 5'-GGTACCAATGGCACTTCAAG-3' |
| GAPDH | Forward primer: 5'- ACCCACTCCTCCACCTTTG-3' |
| | Reverse primer: 5'- CTCTTGTGCTCTTGCTGGG-3' |
| HPRT | Forward primer: 5'-TGACACTGGCAAAACAATGCA-3' |
| | Reverse primer: 5'-GGTCCTTTTCACCAGCAAGCT-3' |

then filtered through No.2 Whatman filter paper and the filtrate was dried at 40°C under low pressure using a Büchi rotavapor R-205 (Büchi Labortechnik AG, Switzerland). The dried extract was weighed and dissolved in 100% dimethyl sulphoxide (DMSO) to an appropriate concentration and stored as a stock solution under dark conditions at -20°C until required.

**Cell culture and treatment**

Jurkat T cells were obtained from the American type culture collection (ATCC, Baltimore, USA). The cultures were grown and maintained in tissue culture flasks at 37°C in a humidified atmosphere at 95% air/5% $CO_2$ in RPMI-1640 supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS) and 1% antibiotic (Penicillin, Streptomycin and Neomycin, PSN) cocktail. For the treatment of experimental cultures, the extract solution (stock) was diluted with RPMI-1640 medium supplemented with 10% FBS to give final concentrations ranging from 0 to 600 µg/ml. The prepared concentrations were filter sterilised through a 0.22 µm pre-sterilised GP express plus steritop™ filter (Millipore Corporation, MA, USA) before testing. Both solvent controls (DMSO) and experimental cultures received equivalent amounts of DMSO at concentrations less than 0.1%. Normal Monkey Vero cells were used as normal control cells.

**Cell proliferation and assessment of cell viability**

To determine the effect of the crude methanolic extract of *C. benghalensis* (CMECB) in respect of dosage and time on cell growth, Jurkat T cells ($1x10^5$ cells/ml) were treated with different concentrations (0, 100, 250, 400 and 600 µg/ml) of the extract and incubated for 24, 48 and 72 h. To assess the effect of the extract on cell proliferation after each treatment, cells were sampled and counted at different times of incubation using a Coulter counter model $Z_1$ (Coulter Electronics, (Pty), Ltd, UK). Cell viability was assessed by staining cells with 0.4% trypan blue dye. Numbers of dead and viable cells were counted using a haemocytometer under a light microscope (Carl Zeiss, Germany); cell viability was expressed as percentage per 100 cells counted.

**Morphological examination of apoptosis**

The morphology of experimental cultures was analysed by fluorescence microscopy after 24 h of treatment. CMECB-treated and untreated cells were harvested by centrifugation (100 x g), washed twice in ice-cold phosphate-buffered saline, PBS (pH 7.4), and re-suspended in 300 µg/ml acridine orange/1 mg/ml ethidium bromide dual stain in PBS (pH 7.4) for 10 min. Cellular and nuclear morphological changes indicative of the occurrence of apoptosis were then evaluated under fluorescence microscopy (Axioplan, Zeiss, Germany). Acridine orange stains early stage apoptotic cells, whereas late-stage apoptotic cells (that have lost their membrane integrity) stain with ethidium bromide.

**Real time quantitative RT-PCR analysis of apoptosis-regulatory genes**

Expression levels of *Bax* and *Bcl-2* mRNA were analysed by the real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique. Experimental cells were treated with various concentrations of CMECB and then harvested at 24, 48 and 72 h. The cells were washed twice with ice-cold PBS. Total RNA was isolated using the high pure RNA isolation kit according to the manufacturer′s instructions (Perkin-Elmer, Roche Diagnostics). The integrity and purity of RNA was electrophoretically verified by formaldehyde agarose gel stained with ethidium bromide and optical density (OD) absorption ratio $OD_{260nm}/OD_{280nm}$, respectively.

One microgram (1 µg) of RNA was reverse transcribed into cDNA in a reverse transcription reaction mixture containing 1x PCR buffer, 0.5 mM deoxy-nucleoside triphosphates (dNTPs), one unit of RNase inhibitor, 2.5 µM of oligo $d(T)_{16}$, and 2.5 units of MuLV reverse transcriptase (Perkin-Elmer, Roche Molecular Systems, Inc, New Jersey, USA). After 10 min of incubation at room temperature to allow primer annealing, the reaction mixture was incubated at 42°C for 15 min, heated to 95°C for 5 min, and chilled at 4°C for 5 min in a GeneAmp thermal cycler (Applied Biosystems). Two microliters (2 µl) of the resultant cDNA products was used for PCR amplification.

Real time quantitative RT-PCR was performed on a Lightcycler 2.0 system (Roche Applied Systems) to analyse the expression levels of *Bax* and *Bcl-2* relative to the housekeeping genes, *HPRT* and *GAPDH*. Primers sets for *HPRT, GAPDH, Bax* and *Bcl-2*, were designed using the Real-Time Quantitative PCR probe design software (Roche Applied Systems). The primers used for the amplification of the respective genes are listed in Table 1. PCR reactions for these primers were first optimised using conventional PCR and product sizes were verified by electrophoresis on a 2% agarose gel.

For quantitative Real-Time PCR, 20 µl amplification mixtures (LightCycler Faststart DNA Master$^{PLUS}$ SYBR Green Reaction Mix;

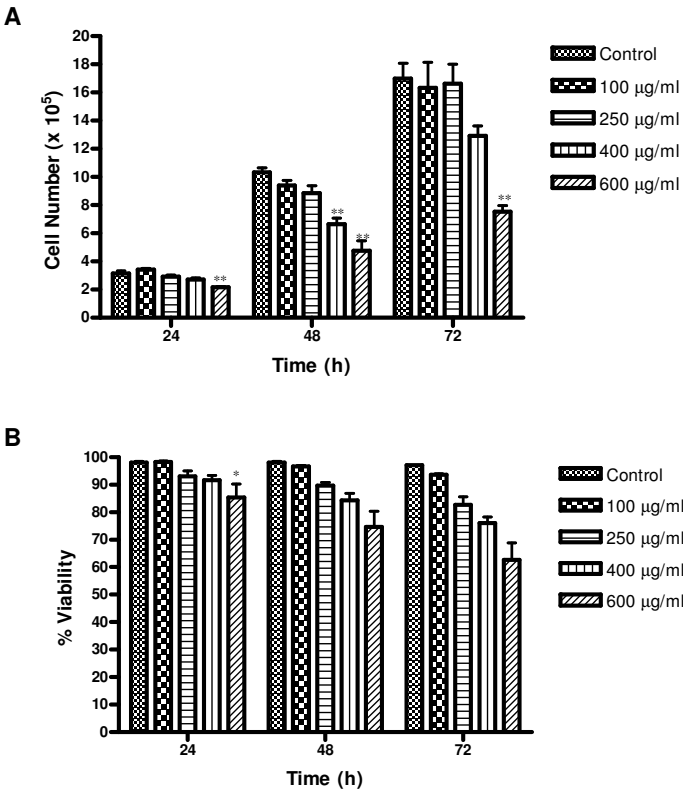


**Figure 1.** The effects of CMECB on the proliferation (A) and viability (B) of Jurkat T cells. Cells were cultured with or without different concentrations of CMECB for 24, 48 and 72 h. Cell number and percentage cell viability were determined using a Coulter counter and trypan blue dye exclusion assay, respectively. Each data point represents the mean ± S.D value of three independent experiments performed in triplicates. *Indicates the statistical difference between control and CMECB treated cells. *p<0.05, **p<0.01.

Roche Applied Science) were prepared as per manufacturer's instructions, containing cDNA (equivalent to 100 ng reverse-transcribed RNA) and 0.5 µM of each primer (Table 1). The cycling conditions were: 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s.

Quantification of the molecular concentration of template cDNA was performed with the standard curve method for relative quantification. The Real-Time PCR efficiencies (E) were calculated for each gene. The baseline and the threshold were automatically set by the software. The crossing point of the amplification curve with the threshold represents the cycle threshold (Ct). In addition, the real-time reaction of the products was checked by melting point analysis after each reaction.

The expression levels of *Bax* and *Bcl-2* was determined relative to *HPRT* and *GAPDH* using both the geNORM excel spreadsheet (Vandesompele et al., 2002) and the Relative expression software tool (REST-384) (Pfaffl et al., 2002).

### Western blotting

After treatment with various concentrations of CMECB, cells were collected by centrifuging at 100 x g and washed twice with ice-cold PBS (pH 7.4) and lysed in 1 ml lysis buffer (2 mM Tris-HCl, pH 8.0, 1% Nonident P-40, 13.7 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate ($NaVO_3$), 1 mM phenylmethyl-sulfonyl fluoride and 10 µg/ml Aprotinin) for 20 min on ice. Lysates were centrifuged at19 000 x g for 15 min at 4°C, and aliquots of the supernatants were used to determine protein concentration using the BCA assay (Pierce, Rockford, USA). Aliquots containing equal amounts of proteins (20 - 30 µg) were boiled in 2 x sodium dodecyl sulfate (SDS) sample loading buffer (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8) before being resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Proteins on the gels were then electro-blotted onto Immobilon-P transfer membrane (Millipore Corporation, Bedford, USA) using a blotting buffer (10% methanol, 10 mM CAPS, pH 11.0) at 200 mA for 2 h at 4°C. Following electro-blotting, the membranes were blocked with 0.05% TBS-Tween (20 mM Tris-HCl, 200 mM NaCl, pH 7.4) containing 5% non-fat dry milk for 1 h at room temperature. After blocking, the membranes were washed three times for 10 min each with wash buffer (0.05% TBS-Tween without milk), and then incubated with specific primary goat anti-mouse Bcl-2 antibody (1: 1000), goat anti-mouse Bax antibody (1: 500), and goat anti-mouse p53 antibody (1: 1000) for overnight at 4°C. After washing three times with washing buffer for 10 min each, membranes were further incubated for 1 h in the presence of a peroxidase (HRP)-conjugated secondary antibody (1: 10 000) diluted with blocking buffer. The membranes were washed again as described above and immunoreactive proteins were then detected using the Western blotting luminol reagent (Santa-Cruz Biotechnology Inc., Santa-Cruz, CA, USA) following the manufacturer's protocol.

### Statistical analysis

The results of each series of experiments (performed in triplicates) are expressed as the mean values ± standard deviation of the mean (SD). Levels of the statistical significance were calculated using the paired student *t-test* when comparing two groups, or by analysis of variance (ANOVA). *P*-values of ≤ 0.05 were considered significant.

## RESULTS

### CMECB inhibits cancer cell growth

To evaluate the potential use of CMECB as a chemotherapeutic agent, Jurkat T cells were incubated in the absence or presence of different concentrations of the extract for 24, 48 and 72 h. CMECB was demonstrated to inhibit cellular proliferation of Jurkat T cells in a dose- and time-dependent manner (Figure 1A). This anti-proliferative property of CMECB suggested cell death in the experimental cultures. This was confirmed by cell viability assay using the trypan blue dye exclusion method. The results demonstrated that CMECB not only inhibited cellular proliferation of Jurkat T cells but also decreased their viability in a dose- and time-dependent manner (Figure 1B). Statistically significant (p-value ≤0.05) inhibition of cell proliferation and reduction in cell viability was observed at high doses of the extract. Normal Monkey Vero cells were used as normal control cells. Extract

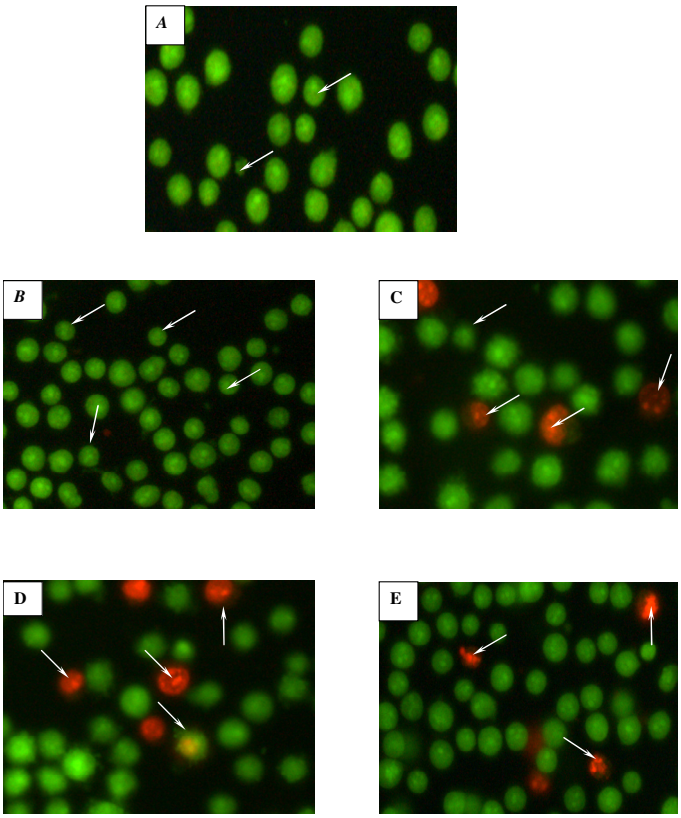


**Figure 2.** Cellular and nuclear morphological changes of Jurkat T cells following exposure to various concentrations of CMECB for 24 h. Experimental conditions were as follows: A = untreated cells in the presence of DMSO, B = 100 µg/ml, C = 250 µg/ml, D = 400 µg/ml and E = 600 µg/ml. Note the morphological features that are indicative of cells undergoing apoptosis (see arrows).

concentrations of up to 500 µg/ml were demonstrated to be non-cytotoxic to normal Monkey Vero cells tested (data not shown).

## CMECB induces morphologic changes indicative of apoptosis

Cellular and nuclear morphological changes of CMECB-treated cells were assessed to determine whether the observed anti-proliferative effects, and the concomitant decrease in cell viability, were a consequence of an apoptotic process. Extract-treated cells displayed morphological changes typical of an apoptotic process including cellular shrinkage, condensation and margination of the chromatin, and the formation of apoptotic bodies (Figure 2B-2E). On the other hand, untreated cells showed intact chromatin and the normal round-to-oval cellular shapes (Figure 2A). These results suggest that CMECB induces Jurkat T cell death through the process of apoptosis.

## CMECB modulates Bax-to-Bcl-2 mRNA expression ratio

The Bcl-2 family of proteins plays an important role in the regulation of apoptosis. Thus, in order to understand the molecular or biochemical mechanisms by which CMECB induces apoptosis in Jurkat T cells, we analysed the expression levels of Bax and Bcl-2 after CMECB treatment using real time quantitative RT-PCR. The real time quantitative RT-PCR data demonstrated that treatment of Jurkat T cells with CMECB did not have any effect on the expression levels of Bcl-2 mRNA after 24 h of treatment (data not shown). However, there was decreased expression of Bcl-2 mRNA levels in the presence of 100, 400 and 600 µg/ml of the extract after 48 and 72 h of treatment (Figure 3 shows expression data for treatment after 48 h). Bcl-2 mRNA expression was down-regulated by factors of 2.5 (p-value = 0.022) and 6.8 (p-value = 0.034) in samples treated with 100 and 400 µg/ml CMECB, respectively, for 48 h in comparison with the untreated samples. On the other hand, Bax mRNA expression levels showed 3.1 (p-value = 0.046) and 5.2 (p-value = 0.047) fold increases after treatment with 100 and 400 µg/ml CMECB, respectively, for 48 h in comparison with the untreated samples.

## CMECB induces apoptosis by regulating the expression of Bax, Bcl-2 and p53 proteins

Western blot analysis of Bcl-2 and Bax proteins showed that CMECB down-regulated Bcl-2 protein levels in a dose-dependent manner (Figure 4A). Increased Bax protein levels were observed in cultures treated with 100 and 250 µg/ml of the extract after 24 and 48 h of treatment. We also observed increased Bax protein levels in all treated cells after 72 h (Figure 4A). These results suggest that the down-regulation of Bcl-2 expression and a concomitant increased expression of Bax might, in part, contribute to the occurrence of the apoptotic process in CMECB-treated Jurkat T cells. Densitometric analysis was subsequently used to corroborate the observed down-regulation of Bcl-2 and the up-regulation of Bax (Figure 4B).

The possible role of p53 in the CMECB-induced apoptosis of Jurkat T cells was also investigated. Western blot analysis demonstrated a dose- and time- dependent up-regulation of p53, and phosphorylation at 48 and 72 h (Figure 4A). The results suggested that CMECB-induced apoptosis of Jurkat T cells could be mediated through a p53-dependent pathway.

## DISCUSSION

The aim of the present study was to evaluate the effect of

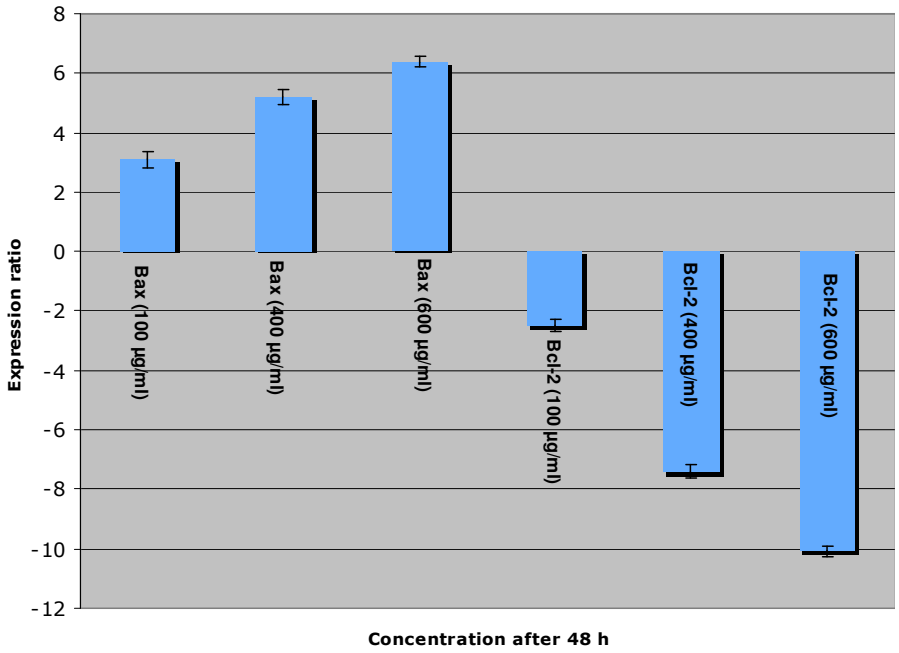


**Figure 3.** Effects of CMECB on *bax* and *bcl-2* mRNA expression levels in Jurkat T cells after 48 h. Cells were treated with various concentrations of CMECB or vehicle control (DMSO) and mRNA expression levels were determined by quantitative real time RT-PCR.

the crude methanolic extract of *C. benghalensis* (CMECB) in Jurkat T cell proliferation, and to further elucidate possible molecular and biochemical mechanisms that may occur as a result of challenging Jurkat T cells with CMECB. This study has demonstrated that the treatment of Jurkat T cells with CMECB resulted in a dose- and time-dependent inhibition of cell proliferation and a concurrent decrease in cell viability (Figure 1). These results suggest that CMECB induced cell death of Jurkat T cells. Consequently, morphological changes known to be associated with apoptosis were investigated. The advent of an apoptotic process is confirmed by the presence of defined and systematic morphological changes such as rapid reduction in the cellular volume (cell shrinkage), chromatin condensation and fragmentation, followed by the formation of apoptotic bodies (Lau et al., 2004). Our results indeed demonstrated that Jurkat T cells treated with different concentrations of the CMECB displayed the aforementioned morphological hallmarks of apoptosis (Figure 2).

The Bcl-2 family of genes consists of the pro-apoptotic and anti-apoptotic members such as *bax* and *bcl-2*, respectively (Miyoshi et al., 2003). Bcl-2 as a key regulator of apoptosis, promotes cell survival either by inhibiting factors for the activation of caspases (Ling et al., 2002) or by regulation of apoptosis through functional antagonism through the formation of heterodimers with other Bcl-2 family members. Bax, a pro-apoptotic member, on the other hand, binds to the anti-apoptotic Bcl-2 protein and thus acts by antagonising the function of Bcl-2 to abrogate apoptosis. Induction of Bax is also reported to promote cytochrome c release from the mitochondria, which eventually leads to apoptosis (Thomas et al., 2000). In this study, an apparent increase in the expression of Bax and a concomitant decrease in Bcl-2 mRNA expression levels, as determined by real time quantitative RT-PCR, were observed in the CMECB-treated Jurkat T cells (Figure 3). In agreement with the real time quantitative RT-PCR analysis, an increase in Bax protein expression was observed in CMECB-treated Jurkat T cells (Figure 4A). More importantly, the expression levels of Bcl-2 protein were concurrently down-regulated after CMECB treatment (Figure 4A); hence the ratio of pro-apoptotic proteins to the anti-apoptotic proteins was altered in favour of apoptosis (Figure 4B). Thus, the results suggest that an up- regulation of Bax and the corresponding down-regulation of Bcl-2 proteins observed in this study may be one of the critical mechanisms through which CMECB induces apoptosis in Jurkat T cells.

Furthermore, our results showed that treatment of Jurkat T cells resulted in an increased expression and phosphorylation levels of p53 (Figure 4A). p53 protein is one of the key regulators of apoptosis and plays a crucial role in cell division cycle arrest to allow for the repair of damaged cellular DNA. In addition, post-translational modifications, such as phosphorylation, play an important role in the stabilisation, up-regulation and functional acti-

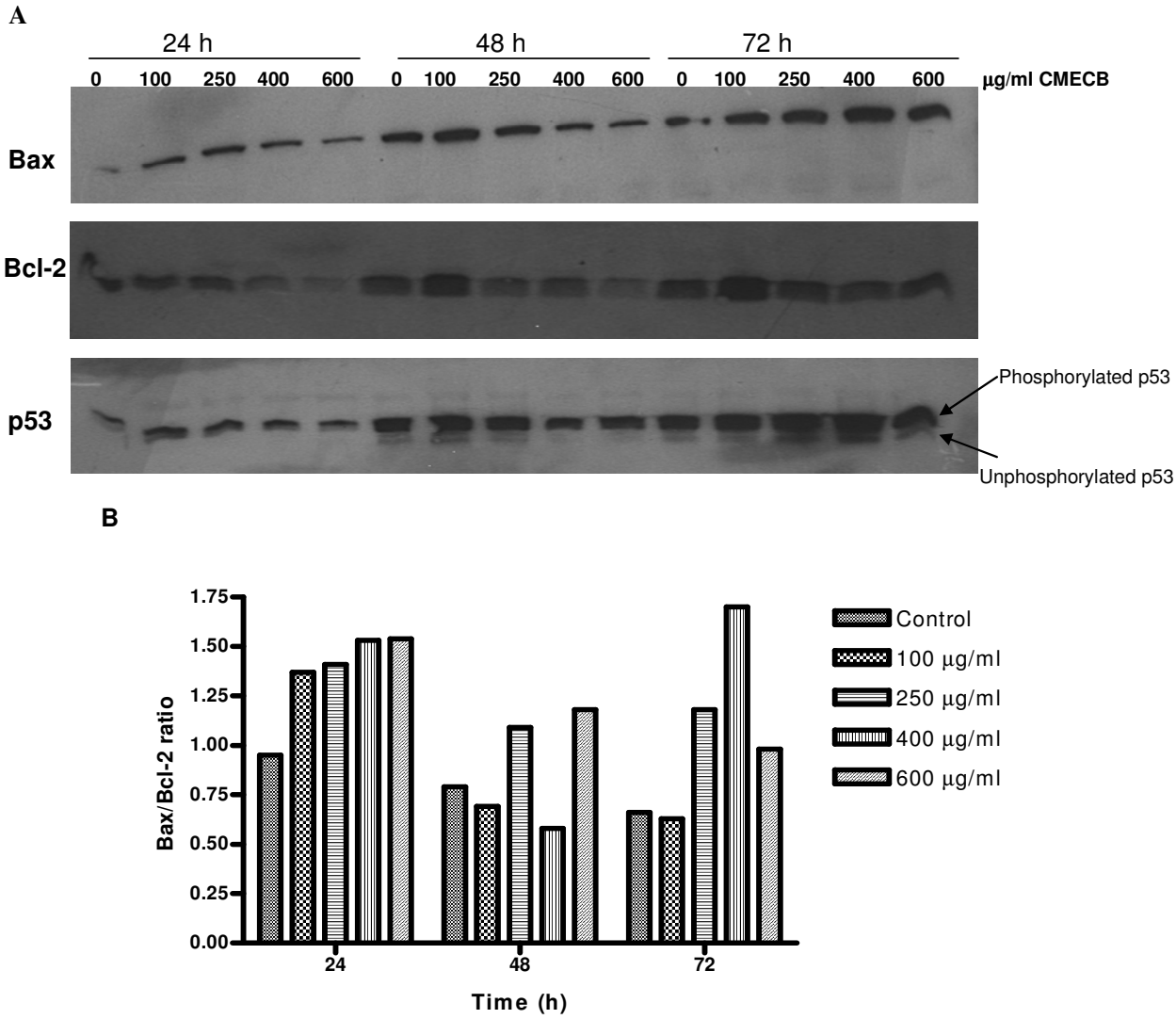


**Figure 4.** Expression of Bax, Bcl-2 and p53 proteins in CMECB-treated Jurkat T cells (A). Cells were treated with indicated concentrations of CMECB or vehicle control (DMSO) for 24-72 h and protein expression determined by western blotting as described in the materials and methods. The ratio of Bax-to-Bcl-2 expression was determined by densitometric analysis (B).

vity of p53 protein (Sutcliffe and Brehm, 2004). Although the mechanism of p53-mediated apoptosis, after cellular stress, remains incompletely characterised, substantial evidence suggests that p53 may induce apoptosis, at least in part, by regulating the transcription of a number of target genes involved in the regulation of apoptosis (*Bax*, *Bcl-2*, Fas/APO1, KILLER/DR5, Tsp1, etc) and cell division cycle ($p21^{WAF1/CIP1}$, GADD45, MDM2, 14-3-3σ, etc) (Lee et al., 2003). Therefore, the data seem to suggest that CMECB up-regulates the expression of p53, with a simultaneous down-regulation of Bcl-2 protein and an up-regulation of Bax, thus altering their ratio in favour of an apoptotic cell death of Jurkat T cells.

## Conclusion

The study suggests that both the Bcl-2 family of proteins (Bax and Bcl-2) and the p53 tumour suppressor protein play a pivotal role in CMECB-induced apoptotic cell death of Jurkat T cells. These findings might help explain and accentuate the purported anticancer properties of CMECB and may further provide scientific evidence that validates the ethnopharmacological use of *C. benghalensis*. Further investigations regarding the purification and identification of the active compounds, the effect of CMECB on the regulation of cell division cycle genes, as well as the elucidation of both the up- and down-stream

apoptotic regulators and effectors that may facilitate the events leading to CMECB-induced apoptosis are warranted.

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