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Full Length Research Paper

Cellular and molecular mechanism associated with anticancer activity of *Commelina benghalensis*

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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 ∝g/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^{o}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^{o}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 ∝g/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 reac-tion (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 ∝g) 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^{o}C for 15 min, heated to 95^{o}C for 5 min, and chilled at 4^{o}C for 5 min in a GeneAmp thermal cycler (Applied Biosystems). Two microliters (2 ∝l) 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;

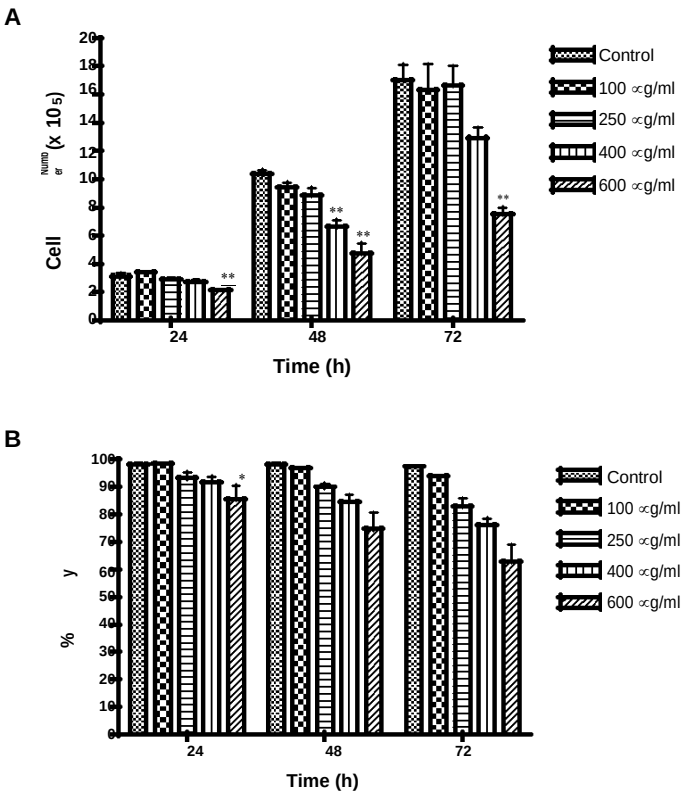

Figure 1. The effects of CMECB on the proliferation (A) and viability (B) of Jurkat T cells. Cells were cultured with or without different concentrations of CMECB for 24, 48 and 72 h. Cell number and percentage cell viability were determined using a Coulter counter and trypan blue dye exclusion assay, 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^{o}C and 40 cycles at 95^{o}C for 15 s, 58^{o}C for 15 s, and 72^{o}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 ∝g/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

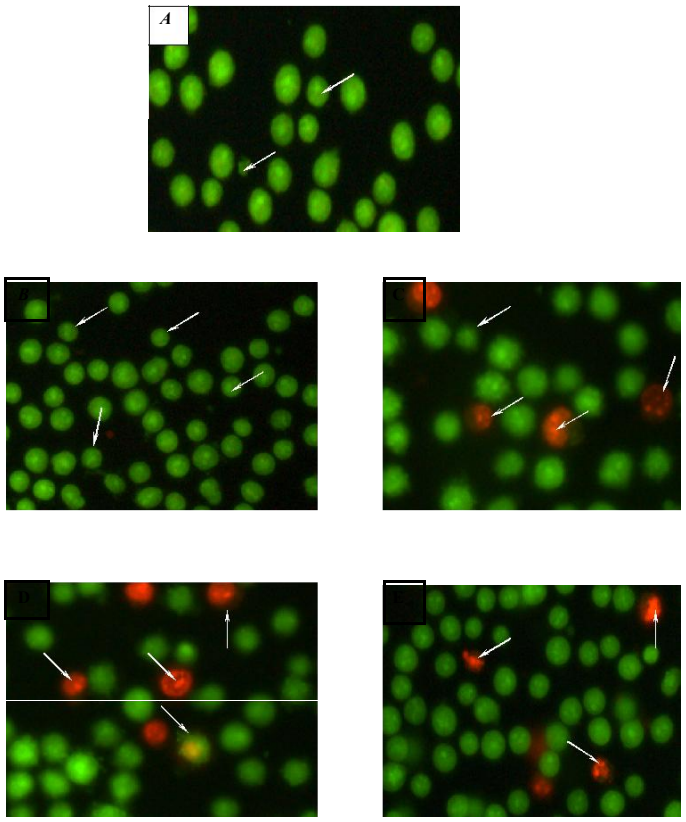

Figure 2. Cellular and nuclear morphological changes of Jurkat T cells following exposure to various concentrations of CMECB for 24 h. Experimental conditions were as follows: A = untreated cells in the presence of DMSO, B = 100 ∝g/ml, C = 250 ∝g/ml, D = 400 ∝g/ml and E = 600 ∝g/ml. Note the morphological 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 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 ∝g/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 ∝g/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 ∝g/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 ∝g/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 expres-sion and a concomitant increased expression of Bax might, in part, contribute to the occurrence of the apoptotic process in CMECB-treated Jurkat T cells. Den-sitometric 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

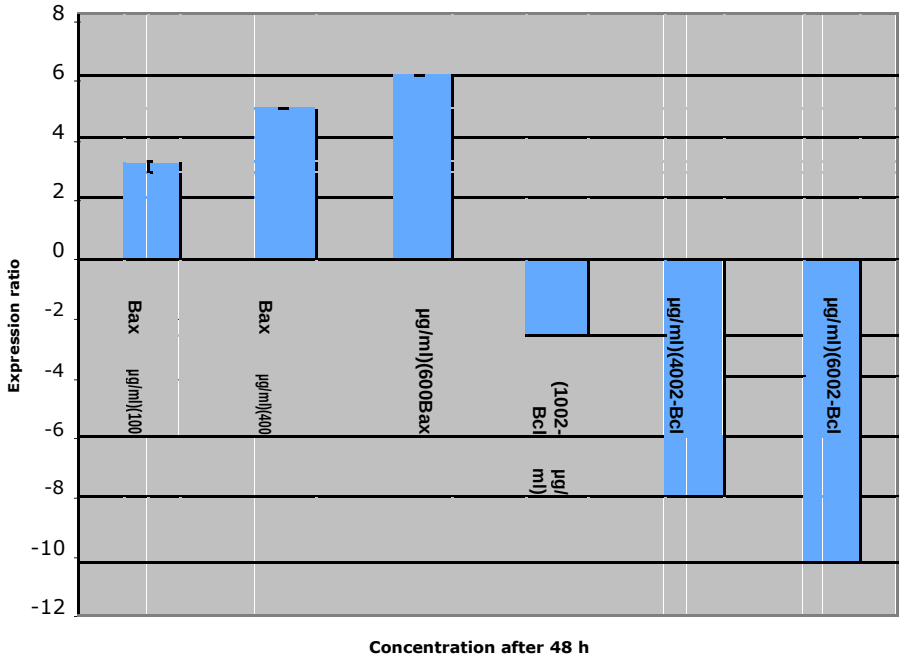

Figure 3. Effects of CMECB on *bax* and *bcl-2* mRNA expression levels in Jurkat T cells after 48 h. Cells were treated with various concentrations of CMECB or vehicle control (DMSO) and mRNA expression levels were determined by quantitative real time RT-PCR.

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-

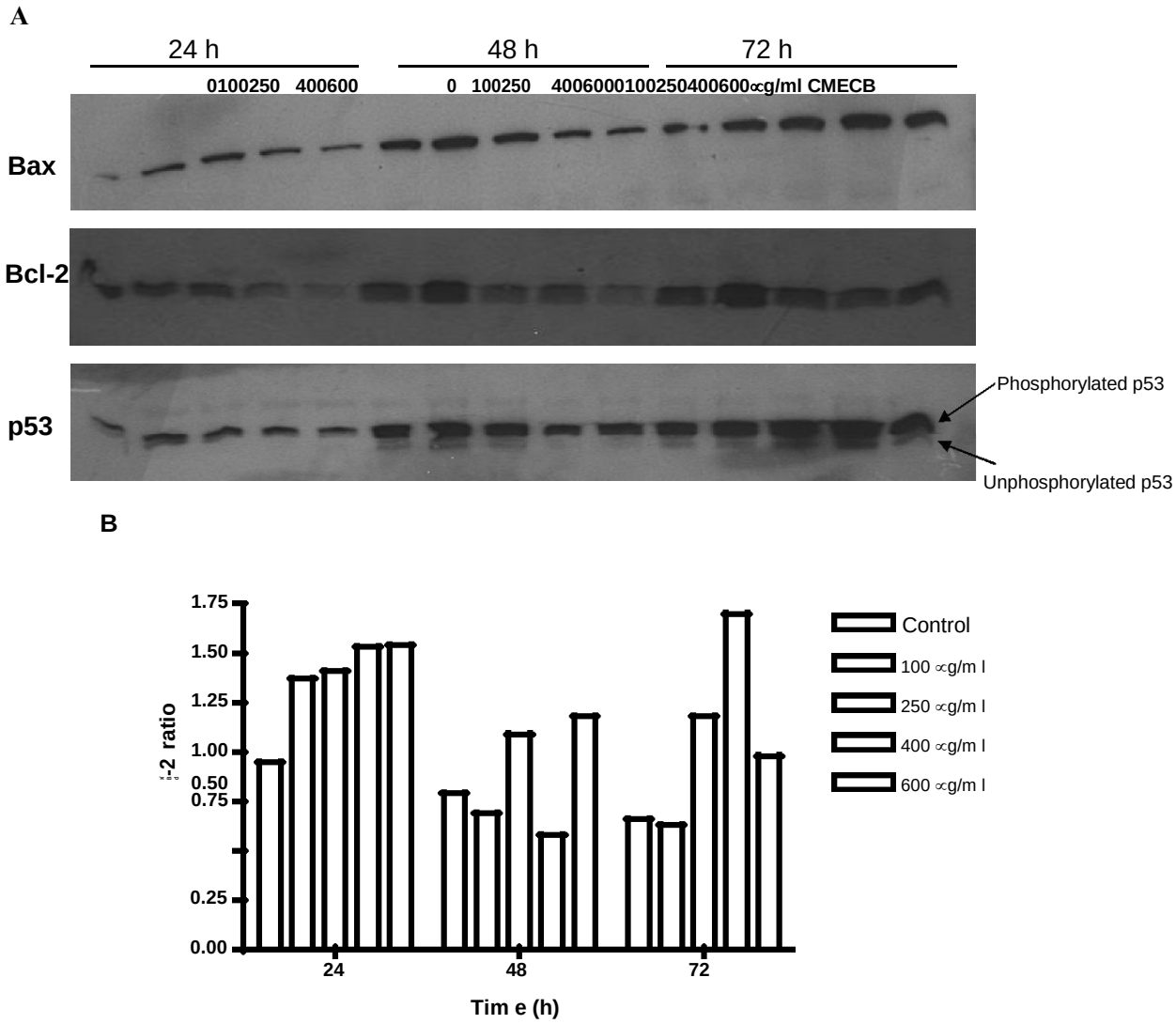

Figure 4. Expression of Bax, Bcl-2 and p53 proteins in CMECB-treated Jurkat T cells (A). Cells were treated with indicated concentrations of CMECB or vehicle control (DMSO) for 24-72 h and protein expression determined by western blotting as described in the materials and methods. The ratio of Bax-to-Bcl-2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