Anti-leukemic activity of COR-D on U937 and HL-60 cells
INTRODUCTION

The aerial parts *Corchorus acutangulus* Lam. (synonym- *C. aestuans*) contain triterpenoidal glycosides and corchorusins (Mahato *et al.*, 1987). Corchorusins have structural similarity with saikosaponins (Mahato *et al.*, 1987; Hsu *et al.*, 2000), which have been reported to possess potent anti-tumor activity (Hsu *et al.*, 2000; Hsu *et al.*, 2004a, 2004b; Bachran *et al.*, 2008; Sun *et al.*, 2009). Therefore the *in vitro* anti-leukemic activity of the methanolic extract (ME) of aerial parts of *C. acutangulus* was investigated in human leukemic cell lines U937 and HL-60. ME demonstrated significant inhibition of the leukemic cell lines which encouraged further polarity based fractionation into three fractions, namely ethyl acetate fraction (EAF), n-butanol fraction (NBF) and aqueous fraction (AF). All the three fractions were evaluated for their potential to inhibit the leukemic cell lines to find that the maximum inhibition lies in NBF. Further silica gel column chromatography followed by HPLC purification led to isolation of the most active triterpenoid glycoside which was identified as COR-D (Chapter II).

COR-D produced apoptotic cell death via mitochondrial dysfunction and was found to pursue the intrinsic pathway by inciting the release of apoptosis-inducing factors (AIFs) from mitochondria. It induced translocation of Bax from cytosol to mitochondria facilitating caspase-9 activation as well as up-regulation of downstream pathways leading to caspase-3 activation and PARP cleavage, which resulted in subsequent accumulation of cells in the sub-G0 phase followed by DNA fragmentation. The compound possesses significant anti-leukemic activity in U937 and HL-60 cell lines by acting on the mitochondrial apoptotic pathways. Since necrotic body formation is low after COR-D treatment, the occurrence of inflammation in *in-vivo* systems could be reduced, which represents a positive indication in favour of therapeutic application.
RESULTS

In vitro cell proliferation assay

In MTT assay, ME and its butanolic fraction inhibited cell growth and produced significant cytotoxicity in the leukemic cell lines in a concentration-dependent manner [Table 4.1]. While ME exerted 50% growth inhibition (IC$_{50}$) of U937 and HL-60 cell lines at concentrations of 235 µg/ml (305.5 µM) and 212.5 µg/ml (276.25 µM), respectively, the n-butanol fraction showed IC$_{50}$ values of 150 µg/ml (195 µM) and 155 µg/ml (201.5 µM) in U937 and HL-60, respectively. The IC$_{50}$ value of COR-D in U937 and HL-60 cell lines were 80.64 µg/ml (104.83 µM) and 86.99 µg/ml (113.09 µM), respectively.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ME (µg/ml)</th>
<th>NBF (µg/ml)</th>
<th>COR-D (µg/ml)</th>
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<tbody>
<tr>
<td>U937</td>
<td>235 ± 6.42</td>
<td>150 ± 3.6</td>
<td>80.64 ± 1.12</td>
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<tr>
<td>HL-60</td>
<td>212.5 ± 2.92</td>
<td>155 ± 4.1</td>
<td>86.99 ± 1.8</td>
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*Mean ± SEM

Effect of COR-D in HL-60 and U937 cell lines as deciphered by cell viability and cytotoxicity assays and by confocal microscopy

COR-D inhibited cell proliferation of U937 and HL-60 cells in a concentration- and time-dependent manner. At the concentrations of 25 µg/ml (32.5 µM), 50 µg/ml (65 µM), 75 µg/ml (97.5 µM), 100 µg/ml (130 µM), 125 µg/ml (162.5 µM) and 150 µg/ml (195 µM), it produced a significant inhibition of cell proliferation [Fig 4.1A] and caused statistically significant reduction in OD value in MTT assay [Fig 4.1B]. At similar concentrations the compound possessed insignificant toxicity in the cellular metabolism of normal PBMC [Fig 4.1C]. Morphological changes such as cell shrinkage, membrane blebbing, condensation and fragmentation of chromatin, which are usually associated with
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PBMC .M i i assay (24 h) ! ®3onar 1
□Donar2
Concentration of COR-D
MTT assay .24 h e<J53?j
50 75 100 125 150

Concentration of COR-D in two leukemic cell lines. HL-60 and U937 cells (1 × 10^6/well) were incubated with 25 μg/ml (32.5 μM), 50 μg/ml (65 μM), 75 μg/ml (97.5 μM), 100 μg/ml (130 μM), 125 μg/ml (162.5 μM) and 150 μg/ml (195 μM) of COR-D in triplicate for 24 h (A). Cell count was taken using trypan blue (B). At the end of treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for another 4 h. The supernatant in each well was replaced with 100 μl of DMSO to solubilize the MTT formazan precipitate and optical density (OD) was measured immediately at 490 nm. Ara-C at its IC_{50} value was used as the standard reference drug (C). COR-D treated normal PBMC was evaluated using MTT assay. Each sample was performed in triplicate and in three independent experiments. Percentage inhibition of the growth was calculated and expressed as mean ± SEM. * P < 0.05 vs control.

Effect of COR-D on DNA fragmentation

DNA fragmentation is a typical feature of apoptosis (Cohen et al., 1994). Cellular DNA fragmentation was studied by gel electrophoresis, and a ladder-like pattern, typical of DNA cleavage between nucleosomes, was visible [Fig 4.2B].
Results

**Effect of COR-D on U937 & HL60 Cells**

Fig 4.2: COR-D induced DNA fragmentation and externalization of phosphatidylserine.

(A) Confocal microscopic images showing apoptosis induced by COR-D in HL-60 (a and b) and U937 (c and d) cells using nuclear staining dye DAPI (4, 6-diamidino-2-phenylindole). Arrows indicate the nuclear fragmentation in COR-D treated cells. (B) Gel pattern showing intact DNA band in control and degraded or fragmented DNA after treatment with COR-D in HL-60 and U937 cell lines. (C) HL-60 and U937 cells were incubated with different concentrations- 50 μg/ml (65 μM), 75 μg/ml (97.5 μM) and 100 μg/ml (130 μM)- of COR-D for 18 h and stained with annexin-V FITC and PI. Live statistics were used to align the X and Y values of the annexin-V FITC or PI stained quadrant populations by compensation.

COR-D induces apoptosis, rather than necrosis, as depicted by FACS quantification

To understand the nature of cell death, the double labeling technique using annexin-V and PI was utilized to distinguish between apoptotic and necrotic cells. The flowcytometric data revealed that most of the COR-D treated cells, in contrast to the control untreated cells (U937 and HL-60), were bound to annexin-V FITC but not to PI,
and few with both, products indicating that the mode of cell death was apoptosis. Percentage of apoptotic cells increased in a concentration-dependent manner as shown in [Fig 4.2C]. From this it can be concluded that COR-D shifts the cells towards apoptosis rather than necrosis.

**Flow-cytometric analysis of cell cycle phase distribution in U937 and HL-60**

To find out the mechanism of neoplastic cell death by COR-D, the cell cycle phase distribution was analyzed by FACS. Treatment of the cells for 18 h with 50 µg/ml (65 µM), 75 µg/ml (97.5 µM) and 100 µg/ml (130 µM) of COR-D caused a significant increase in percentage of cells in sub-G0 phase in a concentration-dependent manner.

![Cell cycle phase distribution as analyzed by flow-cytometry after COR-D treatment](image)

Fig 4.3: Cell cycle phase distribution as analyzed by flow-cytometry after COR-D treatment. HL-60 and U937 cells were incubated with different concentrations of COR-D [50 µg/ml (65 µM), 75 µg/ml (97.5 µM) and 100 µg/ml (130 µM)] for 18 h for DNA cell cycle analysis. The cells were analyzed through FACS after propidium iodide staining. Gates were set to assess % of cells in sub- G0 (<2n DNA, M1), G0/G1 (2n DNA, M2), S (>2n DNA, M3) and G2/M (4n DNA, M4). Bars denote the boundaries of cell cycle phases.

In U937 cells, there were only 9.09% cells in the sub-G0 region in the control cells whereas the percentage of cells in the sub-G0 region in treated cells increased with the increasing concentrations of COR-D. With the highest concentration, i.e. at 100 µg/ml
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(130 μM), 70.14% cells were in the sub-G0 phase [Fig 4.3]. In HL-60 cells, only 1.83% cells were in the sub-G0 region in control cells but 82.57% were in the sub-G0 phase in cells treated with the highest concentration of COR-D, which is also an indication of apoptosis [Fig 4.3].

**COR-D induces enhanced expression of Bax and suppresses the expression of Bcl-2**

Bax, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic protein, are involved in cell death and cell survival, respectively. While the oligomerized Bax may form a pore big enough for the apoptogenic proteins to pass through or destabilize the mitochondrial outer membrane, Bcl-2 can neutralize the activities of Bax. Therefore, to unveil the mechanism of apoptosis by COR-D further, the expression level of these two proteins was studied in U937 and HL-60 cells by Western blot analysis. A concentration-dependent decrease in Bcl-2 and increase in Bax was observed in treated cells [Fig 4.4A], suggesting that COR-D altered the Bcl-2/Bax ratio.

**COR-D causes alteration in the mitochondrial membrane potential (ΔΨm) in leukemic cell lines**

Since Western blot analysis confirmed that COR-D treatment induced a balance between positive and negative regulators of apoptosis shifting towards cell death, an attempt was made to confirm the involvement of mitochondria by studying whether COR-D alters membrane potential, triggering further the downstream pathway of apoptosis. Accordingly, COR-D treated U937 and HL-60 cells were characterized by FACS analysis using mitocapture dye, which undergoes a transition from molecular aggregation to molecular monomer formation, the process being detected by a shift in fluorescence from red to green during depolarization of the membrane (ΔΨm). Exposure of the leukemic cells to the compound showed a decrease in J-aggregates in mitochondria and increase in monomers in the cytosol after 18 h of treatment, confirming the involvement of mitochondria in COR-D induced apoptosis in these cells [Fig 4.4B].
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A concentration-dependent increase in Bax band intensity and decrease in Bcl-2 band intensity in the cell lysates of COR-D treated HL-60 and U937 cells determined by Western blot analysis. (B) COR-D-induced apoptosis in leukemic cells was mitochondria dependent. Mitochondrial involvement in COR-D treated HL-60 and U937 cells was characterized using a mitocapture dye (Bio Vision). The ratio of emission of red (585 nm) and green (530 nm) fluorescence from mitochondria and cytosol, i.e. polarization of (∆Ψm), was analyzed immediately by flow-cytometry.

Activation of caspases in COR-D treated leukemic cells

The observed decrease in MMP and increase in Bax in COR-D treated cells suggested studies on the role of mitochondria in apoptosis induced by the compound. This was done by comparing the levels of different caspases in treated and untreated cells, as caspase-dependent apoptotic cell death features caspase activation. Western blot analysis was
Fig 4.5: COR-D triggered cell death in two leukemic cell lines, which involves cleavage of caspases. (A) In order to determine the activation of caspase 3, caspase 8 and caspase 9 in HL-60 and U937 cells on exposure to COR-D for 18h, colorimetric assay was done using caspase colorimetric assay kits (Bio Vision). The enzyme activity was expressed in terms of fold increase compared to untreated cells. The data shown are from a representative experiment performed three times with comparable results. The values reflect the mean ± SE of triplicate determination. (B) In order to determine whether caspase 3, caspase 8 and caspase 9 are really cleaved during COR-D-induced apoptosis in HL-60 and U937 cells on exposure to COR-D, Western blot analysis was performed with total protein extracts. The cleavage of PARP in COR-D treated HL-60 and U937 cells for 18 h was determined by Western blot analysis using PARP antibodies (1:100, over-night) as described in “Materials and methods”. * P < 0.05 versus control.

performed in order to determine whether caspases 3 and 9 are really cleaved during COR-D induced apoptosis in U937 and HL-60 cell lines. We found that exposure to the compound at different concentrations- 40 µg/ml (52 µM), 50 µg/ml (65 µM), 70 µg/ml (91 µM), 80 µg/ml (104 µM), 90 µg/ml (117 µM) and 110 µg/ml (143 µM)- for 18h [Fig 4.5B] caused cleavage of both the caspases. The active form of caspase 9 increased and
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procaspase 9 decreased with increase in concentration of COR-D. The activation of caspases was further corroborated by colorimetric measurement [Fig 4.5A]. In U937 cell line, caspase 9 showed maximum activity (eightfold increase) at a concentration of 70 µg/ml (91 µM) of COR-D, while in case of caspase 3 the activity was found to be maximum (3.615 fold increase) at 80 µg/ml (104 µM) of the compound. In HL-60 cell line, maximum activity was observed at a concentration of 50 µg/ml (65 µM) of COR-D for caspase 9, but at a concentration of 70 µg/ml (91 µM) for caspase 3. At the same time, the treatment showed enhanced caspase 8 activity in U937 and HL-60 cells as revealed by its increased expression with increase in concentration of the test compound.

Effect of COR-D on the cleavage of PARP

Poly ADP-ribose polymerase (PARP, 116 kDa) is a key signaling enzyme involved in triggering the repair of single-strand DNA damage (Haeijmakers et al., 2001; Caldecott et al., 2005; Farmer et al., 2005). The activity of PARP to repair DNA is disrupted by its cleavage through the activation of caspase 3, resulting in DNA damage and apoptosis. As PARP is one of the substrates of caspase 3, cleavage of PARP is the indicator of apoptosis. Treatment of U937 and HL-60 cell lines with COR-D at 40 µg/ml (52 µM), 50 µg/ml (65 µM), 70 µg/ml (91 µM), 80 µg/ml (104 µM), 90 µg/ml (117 µM) and 110 µg/ml (143 µM) resulted in the cleavage of PARP to yield an 89-kDa cleaved fragment in the cell lysates, confirming that the cells underwent apoptosis.
DISCUSSION

One of the goals of cancer chemotherapy is to explore and develop new molecules and/or therapies, which can selectively induce apoptosis in cancer cells (Denicourt et al., 2004). The present study reports, for the first time, the anti-leukemic activity of methanolic extract of aerial parts of C. acutangulus. Through bioactivity guided fractionation COR-D has been identified as the active ingredient responsible for this activity. The study also provides a novel insight into the mechanisms involved in COR-D induced early events leading to the activation of signaling cascades and culminating in apoptotic cell death in human myelomonocytic (U937) and promyelocytic (HL-60) leukemic cell lines.

COR-D has structural similarity with saikosaponin D, a well-known saikosaponin which was isolated from Bupleurum falactum (Hsu et al., 2000). Though both the molecules are terpenoidal glycosides, they vary in the positions of some organic functionalities (C-16 and C-23). Because of their steroid-like structure (terpenoid lipid type), saikosaponins have some common steroid-like pharmacological activities (Hsu et al., 2000) and possess therapeutic effects in hyperlipidaemia, hepatic injury, chronic hepatitis and inflammation (Ohuchi et al., 1985). Hsu et al. (2000) have reported the apoptotic effect of saikosaponin D in human CEM lymphocytes. Saikosaponins have also been reported to inhibit the proliferation of human leukemia cells (Hsu et al., 2000; Chen et al., 2003; Qian et al., 2003; Wen-Sheng 2003; Hsu et al., 2004a, 2004b; Bachran et al., 2008; Sun et al., 2009). Saikosaponin D inhibits the cell proliferation of human leukemia cells by up regulating the expression of GR mRNA (Guinea et al., 1994; Kato et al., 1995).

The pharmacological activity of COR-D has not been investigated so far. The present study reports its apoptotic effect in leukemic cell lines (HL-60 and U937), which may be due to its structural similarity with saikosaponins. The results demonstrated that it inhibited proliferation of U937 and HL-60 cells in a concentration-dependent manner. Further analysis demonstrated that cultured U937 and HL-60 cells treated with the compound exhibited morphological features of apoptosis, such as membrane shrinkage, nuclear fragmentation and chromosomal condensation. Degradation of higher order
Discussion

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Chromatin structure of DNA into fragments is a hallmark of cellular self-destruction by apoptosis (Cohen et al., 1994). The COR-D treated cells showed DNA fragmentation, resulting in a ladder-like pattern when analyzed by agarose gel electrophoresis. DNA fragmentation was also confirmed by means of confocal microscopy using DAPI staining.

In the first stage of apoptosis, the changes in the membrane composition lead to extra cellular exposure of phosphatidyl serine (PS) residues, which bind annexin-V in a calcium-dependent manner (Martin et al., 1995; Konoshima et al., 1999). In apoptotic cells, membrane changes leading to PS exposure occur rapidly and the cells loose membrane integrity later in the apoptotic process to expose DNA. Hence using PI, a DNA-binding dye, together with fluorochrome-conjugated annexin-V, apoptotic cells and necrotic cells can be discriminated by flow-cytometry (Martin et al., 1995). This double staining provides clear detection of viable, apoptotic and necrotic cells. The live cells do not take any stain, early apoptotic cells bind to annexin-V only, late apoptotic cells bind with both annexin-V FITC and PI, and the necrotic cells bind to PI only (Darzynkiewicz et al., 1992). Flow-cytometric analysis showed that COR-D treatment caused apoptosis in U937 and HL-60 cell lines in a concentration-dependent manner. However, only 0.03% of treated cells showed PI positivity indicating that the formation of necrotic bodies was less after COR-D treatment. Thus, cell death produced by COR-D in U937 and HL-60 cell lines is due to apoptosis rather than necrosis. Based on this finding, it could be postulated that the inflammation due to necrotic bodies in in vivo system may be quite reduced.

Cell proliferation inhibition is highly correlated with the activation of a variety of intracellular signaling pathways to arrest the cell cycle in the sub-G0, G0/G1, S or G2/M phase (Roy et al., 2004). In the present study, COR-D was found to cause increase in cells in sub-G0 phase in a concentration-dependent manner along with decrease in cells in G1, G2 and S phases, which supports the contention that cell death occurred via apoptotic signaling pathway. In the complex signaling events of apoptosis, the ratio of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins in mitochondria changes, causing
loss of membrane potential, release of AIF and activation of caspases, leading to apoptosis (Yang et al., 1997). In the present study, a concentration-dependent decrease in Bcl-2 and increase in Bax were observed in treated U937 and HL-60 cells, which reveals that COR-D altered the Bax/Bcl-2 ratio and triggered the mitochondrial pathway of apoptosis. Activation of the caspase cascade by the molecule indicated that the promotion of apoptosis in response to death inducing signals originated from cell surface receptors, mitochondria or endoplasmic reticulum. The present study revealed that COR-D induced elevation of some caspases, including caspases 3, 8 and 9. Activation of initiator caspases such as caspase-8 or caspase-9 in response to the pro-apoptotic signals activates caspase-3, the major effector caspase (Patel et al., 1996), and this plays the central role in the initiation of apoptosis (Nicholson et al., 1995; Salvesen et al., 1999). As caspase 8 activity was enhanced by COR-D treatment in U937 and HL-60 cell lines, the involvement of the compound in the activation of death receptor and mediation of apoptosis is indicated.

The lower intensity in the fluorescence of mitocapture dye aggregates confirmed the loss of mitochondrial membrane potential in treated U937 and HL-60 cells resulting in the release of different mitochondrial AIFs for sequestering further downstream pathways. This resulted in the cleavage of PARP in the two cell lines to yield an 89-kDa cleaved fragment in the cell lysates, which ultimately confirms triggering of apoptosis through the intrinsic pathway. In conclusion, this study reveals that COR-D-induced mitochondrial dysfunction is responsible for the induction of apoptotic cell death. The compound pursued the mitochondrial intrinsic pathway by release of AIF from mitochondria and translocation of Bax from cytosol to mitochondria, facilitating caspase 9 activation and up-regulation of downstream pathways leading to caspase 3 activation and PARP cleavage. The involvement of extrinsic pathway in apoptosis by the increase in activation of caspase 8 is also suggested.
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