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ORIGINAL ARTICLE

Corchorusin-D, a saikosaponin-like compound isolated from *Corchorus acutangulus* Lam., targets mitochondrial apoptotic pathways in leukemic cell lines (HL-60 and U937)

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Abstract
Purpose The presence of triterpene saponins in *Corchorus acutangulus* Lam. has been reported. However, no studies concerning biological activity of the plant extracts have been done so far. In the present study, the anti-leukemic activity of the methanol extract of aerial parts (ME) of *C. acutangulus* has been investigated, and efforts have been made to identify the active ingredient responsible for this activity.
Methods The anti-leukemic activity of ME, its fractions and corchorusin-D (COR-D), the active ingredient, was investigated in leukemic cell lines U937 and HL-60 using cell viability and MTT assays. The molecular pathways leading to the activity of COR-D were examined by confocal microscopy, flow-cytometry, caspase and Western blot assays.
Results ME, its *n*-butanolic fraction and COR-D inhibited cell growth and produced significant cytotoxicity in leukemic cell lines U937 and HL-60. COR-D produced apoptotic cell death via mitochondrial disfunction and was found to pursue the intrinsic pathway by inciting the release of apoptosis-inducing factors (AIFs) from mitochondria. COR-D-induced 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, which resulted in the subsequent accumulation of cells in the sub-G0 phase followed by DNA fragmentation.
Conclusions COR-D possesses significant anti-leukemic activity in U937 and HL-60 cell lines by acting on the mitochondrial apoptotic pathways. Since the 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 view of therapeutic application.

Keywords *Corchorus acutangulus* Lam · Corchorusin-D · U937 · HL-60 · Bax · PARP · Caspase-3 · Caspase-9

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Introduction

Corchorus acutangulus Lam. (*C. aestuans*) is a small annual herb belonging to the family *Tiliaceae* growing throughout the hotter parts of the Indian subcontinent, Indo-China, Australia, Tropical Africa, West Indies and Central America [1]. In Ayurveda, roots and leaves of *C. acutangulus* are said to cure gonorrhea. The seeds are stomachic and are used in the treatment of pneumonia [1].

The aerial parts of *C.acutangulus* contain triterpenoidal glycosides and corchorusins [2]. Corchorusins have structural similarity with saikosaponins [2, 3]. Saikosaponins and saikosaponin-like compounds have been reported to possess potent anti-tumor activity [3–7]. The alcoholic extract of the entire plant of *C. acutangulus* was found to have anti-cancer activity against epidermal carcinoma of nasopharynx in tissue culture [8]. Alcoholic extract and the glycosides of seeds exhibit cardiotonic activity [9]. Digitoxose-containing glycosides are also reported to be present in *Corchorus* sp. [9].

Four corchorusins have been isolated from *C. acutangulus* [2]. However, except a study by a Chinese group [10], not much work has been done to find out the biological activity of the plant extracts. In the present study, the in vitro anti-leukemic activity of the methanolic extract (ME) of aerial parts of *C. acutangulus* has been investigated in human leukemic cell lines U937 and HL-60, and efforts have been made to identify the active ingredient responsible for the anti-leukemic activity. Also, the molecular pathways leading to the activity of the active ingredient were examined by confocal microscopy, flow-cytometry, caspase and Western blot assays.

Materials and methods

Plant material

The aerial parts of *C. acutangulus* were collected and supplied by United Chemicals & Allied Products, Kolkata, India in August, 2007. The plant was authenticated by the Botanical Gardens of Howrah, India where a voucher specimen (SM-001) is deposited.

Chemicals

RPMI 1640 medium, fetal bovine serum (FBS), HEPES, streptomycin, penicillin, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), annexin-V FITC, propidium iodide (PI), cytosine arabinocide (Ara-C) 4′,6-diamidino-2-phenylindole (DAPI) and general reagents were purchased from Sigma (St. Louis, MO, USA). Caspase-3, caspase-8 and caspase-9 colorimetric assay kits were purchased from Biovision (BioVision Research Products, USA). Antibodies against Bcl-2, Bax, PARP, caspase-3, caspase-8 and caspase-9 were purchased from Cell Signaling (USA). All other chemicals and solvents were purchased from local firms and were of high purity grade.

Extraction, fractionation and isolation of Corchorusin D

Air-dried aerial parts of *C. acutangulus* (2 kg) were extracted four times successively with methanol (each time with 5 l for 72 h) and were evaporated to dryness under reduced pressure. The residue (ME, 113 g) was suspended in water and extracted successively with ethyl acetate and *n*-butanol. The solutions were evaporated to dryness in a rotary evaporator to provide ethyl acetate soluble (25 g), *n*-butanol soluble (55 g) and water soluble (30 g) fractions. Each fraction was tested for anti-leukemic activity, and the *n*-butanol fraction was found to be active. A part (20 g) of the *n*-butanol fraction was subjected to silica gel column chromatography eluting with chloroform:methanol (9:1) to yield fraction 1 (5.5 g). Subsequent elution with chloroform:methanol (4:1) afforded fraction 2 (7.3 g) and chloroform:methanol (7:3) yielded fraction 3 (3.5 g) and fraction 4 (2 g). Each fraction was tested for anti-leukemic activity. Fraction 3 showed the activity, and it was then purified by repeated column chromatography over silica gel eluting with chloroform:methanol (4:1) to give a saponin mixture. Preparative HPLC of the mixture (0.45 g) using X terra C18 column [10 μ, 19 × 250 mm; detector UV: λ 210 nm; solvent: methanol: water (7:3 v/v), flow: 20 ml/min] produced 3 chromatographically pure compounds designated as compound A (30 mg), compound B (40 mg) and compound C (80 mg). Compound C, which showed significant activity was identified as Corchorusin D (COR-D; Fig. 1). The identity of COR-D was confirmed by comparing its physical data as well as its infrared (IR), ^{1}H NMR, ^{13}C NMR and ESI Q-TOF mass spectrometry data with those of an authentic sample.

Cell lines

Human leukemic cell lines (U937 and HL-60) were purchased from the National Facility of Animal Tissue and Cell culture, Pune, India. Cells were routinely cultured in RPMI 1640 medium supplemented with 10% of heat inactivated FBS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37°C and 5% CO_2.

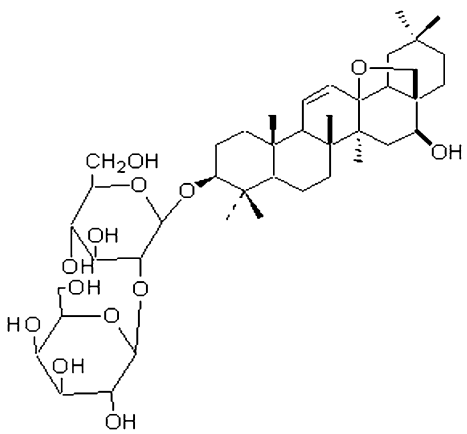

Fig. 1 Structure of Corchorusin-D

The peripheral blood of four volunteers was collected. Mononuclear cells were separated from peripheral blood by the Ficoll–Hypaque technique [11]. The cells were then taken in RPMI 1640 medium with 5% FBS in aseptic condition. In all the experiments, untreated leukemic cells as well as untreated peripheral blood mononuclear cells (PBMC) were used as control.

Cell viability and growth

Cells were grown to 70% confluence and treated with different concentrations of ME (25–350 μg/ml), the *n*-butanol fraction (25–350 μg/ml), the ethyl acetate fraction (25–350 μg/ml), the aqueous fraction (25–350 μg/ml) and COR-D (25 μg to 150 μg/ml). Control cells were then supplemented with complete media. Following treatment, cell number and viability were determined by trypan blue exclusion [12] and MTT [13] assays, respectively.

Nuclear staining

After treatment with COR-D at the IC_{50} concentrations, the cells were harvested, washed with phosphate buffer saline (PBS) and fixed with 3.7% para formaldehyde (Sigma) in phosphate buffer saline (PBS) for 10 min at room temperature. The fixed cells were washed with PBS and stained with 2.5 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) solution for 10 min at room temperature. The cells were washed twice with PBS and analyzed using a confocal laser-scanning microscope (Leica TCS-SP2 system, Leica Microsystem, Heidelberg, Germany) installed with an inverted microscope (Leica DM-7RB). The images of DAPI were acquired by using UV laser whose excitation maxima and emission maxima were 358 nm and 461 nm, respectively.

DNA fragmentation assay

Leukemic cells (U937 and HL-60) were treated with COR-D at different concentrations (75 and 100 μg/ml) in RPMI 1640 and 5% fetal bovine serum (FBS) for 18 h. The cells (1.5×10^6/ml) were washed in ice-cold PBS, and DNA was isolated by following a general phenol–chloroform extraction procedure [14] and dissolved in TE buffer (10 mM Tris–HCl and 1 mM EDTA at pH 8.0). To assess the DNA fragmentation pattern, samples were loaded onto 1.5% agarose gel, and electrophoresis was carried out at 20 V for 12 h.

Detection of apoptosis by flow-cytometry

Apoptotic cell detection was performed using FITC-conjugated annexin-V and propidium iodide (PI). Leukemic cells (U937 and HL-60) were treated with different concentrations of COR-D (50, 75 and 100 μg/ml) for 18 h and pelleted down by centrifugation at 2,000 rpm (4°C) for 10 min. Cells were re-suspended in 0.5 ml of binding buffer (annexin-V binding buffer containing 10 mM HEPES, 150 mM NaCl and 2.5 mM $CaCl_2.2H_2O$: pH = 7.4) and again centrifuged. The pellets were dissolved in 100 μl of same buffer containing 5 μl of 2 μg/ml annexin-V FITC, then 1 μg of PI was added. Cells were incubated in darkness for 15 min and were analyzed by flow-cytometry [15]. All data were acquired with a Becton–Dickinson FACS Caliber (BD) single laser cytometer. Flow-cytometric reading was taken using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). Live statistics were used to align the *X* and *Y* values of the annexin-V FITC or PI stained quadrant populations by compensation. Data analysis was performed with Cell Quest (Macintosh platform) program.

Cell cycle phase distribution analysis by flow-cytometry

Leukemic cells (U937 and HL-60) at a concentration of 1×10^6 were treated with COR-D at different concentrations (50, 75 and 100 μg/ml) for 18 h, harvested, washed and fixed overnight in ethanol. The fixed cells were washed twice in PBS and treated with RNase, 69 μM PI in 38 mM sodium citrate buffer for 1 h in darkness [16]. Cell cycle phase distribution of nuclear DNA was determined on FACS, using a fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using cell Quest software (Becton–Dickinson). A total of 10,000 events were acquired, and analysis of flow-cytometric data was performed using ModFit software. A histogram of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) was displayed.

Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP) of intact cells was measured by flow-cytometry with a cationic dye (mitocapture dye) provided with the Mitocapture Apoptosis Detection Kit (BioVision). This cationic dye fluoresces differently in healthy and apoptotic cells. In healthy cells, mitocapture accumulates and aggregates in the mitochondria giving off a bright red fluorescence. In apoptotic cells, mitocapture cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form, fluorescing green. The cells were analyzed by flow-cytometry using FITC channel for green monomers (*Ex*/*Em* = 488/530 ± 30 nm) and PI channel for red aggregates

($Em = 488/590 \pm 42$ nm). Leukemic cell lines U937 (1×10^6/ml) and HL-60 (1×10^6/ml) were treated with COR-D at different concentrations (50 and 75 μg/ml) for 18 h. The cell pellets were then re-suspended in 1 ml of the diluted mitocapture solution (1 μl of mitocapture was made up to 1 ml with pre-warmed incubation buffer for each assay) and incubated for 20 min at 37°C in 5% CO_2. The cells were centrifuged and re-suspended in 1 ml of the pre-warmed incubation buffer and analyzed by flow-cytometry.

Determination of caspase activity

Colorimetric assays were performed using caspase-3, caspase-8 and caspase-9 activation kits according to the manufacturer's protocol. The kit utilizes synthetic tetra peptides labeled with *p*-nitroanilide.

Leukemic cell lines U937 (1×10^6/ml) and HL-60 (1×10^6/ml) were treated with COR-D at different concentrations (40, 50, 70, 80, 90 and 110 μg/ml) for 18 h, and then the cells were lysed in the supplied lysis buffer. The supernatants were collected, and protein concentration was determined using a protein assay kit (Bangalore Genei). Two hundred micrograms of protein from each sample was incubated with the supplied reaction buffer containing dithiothreitol (DTT) and substrates at 37°C. Caspase activity was determined by measuring changes in absorbance at 405 nm using the microplate reader. Fold increase in activity was determined by comparing the results of the treated samples with the level of uninduced control.

Protein extraction and Western blot analysis

Cells were harvested, washed once with ice-cold PBS and gently lysed for 2 min in 80 μl ice-cold lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris–Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin and 2 μg/ml aprotinin). Supernatants were collected, and the protein concentration was determined using a protein assay kit (Bangalore Genei). Samples were stored at −80°C or immediately used for immunoblotting. Aliquots containing 30 μg of total protein were separated on SDS–polyacrylamide gel and transferred to polyvinyldifluoride (PVDF) membranes for immunoblot analysis using the indicated primary antibodies [17]. Horseradish peroxidase (HRP)-conjugated secondary antibodies were detected using DAB (3,3′-diaminobenzidine).

Data analysis

All results were expressed as mean ± SEM. Statistical analysis was performed by Student's *t* test. *P* value of <0.05 was considered to be statistically significant.

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 1). ME exerted 50% growth inhibition (IC_{50}) of U937 and HL-60 cell lines at concentrations of 235 and 212.5 μg/ml, respectively, while the *n*-butanol fraction showed the IC_{50} at concentrations of 150 and 155 μg/ml in U937 and HL-60, respectively. The IC_{50} value of COR-D in U937 and HL-60 cell lines was 80.64 and 86.99 μg/ml, respectively.

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

COR-D inhibited cell proliferation of U937 and HL-60 cells in a concentration- and time-dependent manner. COR-D at the concentrations of 25, 50, 75, 100, 125 and 150 μg/ml produced a significant inhibition of cell proliferation (Fig. 2a). The same concentrations of COR-D caused statistically significant reduction in OD value in MTT assay (Fig. 2b). COR-D at similar concentrations showed insignificant toxicity in the cellular metabolism of normal PBMC (Fig. 2c). Morphological changes, such as cell shrinkage, membrane blebbing and condensed and fragmented chromatin, associated with apoptotic cell death were observed under confocal microscopy using DAPI in U937 and HL-60 cells treated with COR-D at IC_{50} concentration, but not in control cells (Fig. 3a).

Table 1 IC_{50} of ME, BuOH fraction and Corchorusin-D in two leukemic cell lines

Cell line	Mean ± SEM		
	IC_{50} of ME (μg/ml)	IC_{50} of BuOH Fraction (μg/ml)	IC_{50} of COR-D (μg/ml)
U937	235 ± 6.42	150 ± 3.6	80.64 ± 1.12
HL-60	212.5 ± 2.92	155 ± 4.1	86.99 ± 1.8

$n = 6$

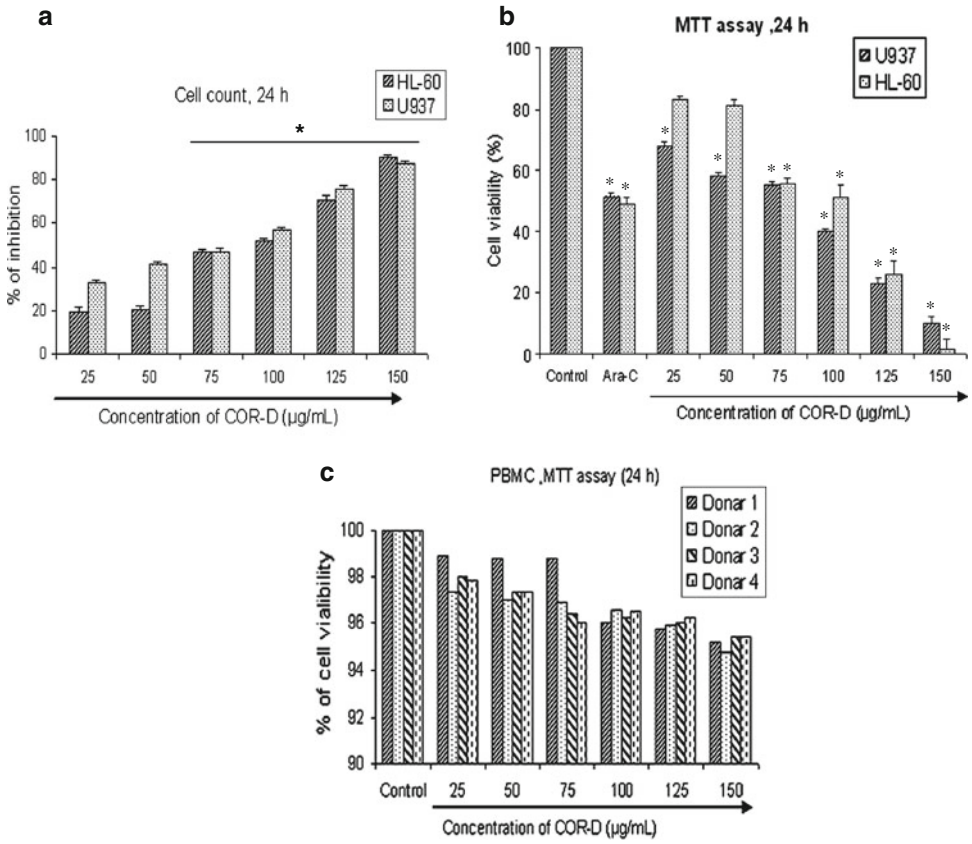

Fig. 2 COR-D reduced cell viability in two leukemic cell lines. HL-60 and U937 cells (1×10^4/well) were incubated with COR-D (25–150 µg/ml) in triplicate for 24 h (**a**). Cell count was taken using trypan blue (**b**). At the end of treatment, 20 µl of MTT (5 mg/ml in PBS) was added to each well and 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 was used as standard reference drug in its IC_{50} value (**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. * $P < 0.05$ versus control

Effect of COR-D on DNA fragmentation

DNA fragmentation is a typical feature of apoptosis [18]. Cellular DNA fragmentation was studied by gel electrophoresis, and a ladder-like pattern, typical of DNA cleavage between nucleosomes, was visible (Fig. 3b).

COR-D induces apoptosis rather than necrosis, as being 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 flow-cytometric data revealed that, in comparison with control untreated cells (U937 and HL-60), COR-D treated cells showed that most of the cells 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 are shown in Fig. 3c.

Flow-cytometric analysis of cell cycle phase distribution of 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 with the concentrations of COR-D (50, 75 and 100 µg/ml) used in the study for 18 h showed a significant increase in percentage of cells in sub-G0 phase, in a concentration-dependent manner. 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 increased with the increasing concentrations of COR-D. With the highest concentration i.e. at 100 µg/ml, 70.14% cells were in the sub-G0 phase (Fig. 4). In HL-60 cells, only 1.83% cells were in the sub-G0 region in control cells and with the highest concentration of COR-D, 82.57% of the cells were in the sub-G0 phase (Fig. 4).

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 by Western blot analysis. A concentration-dependent

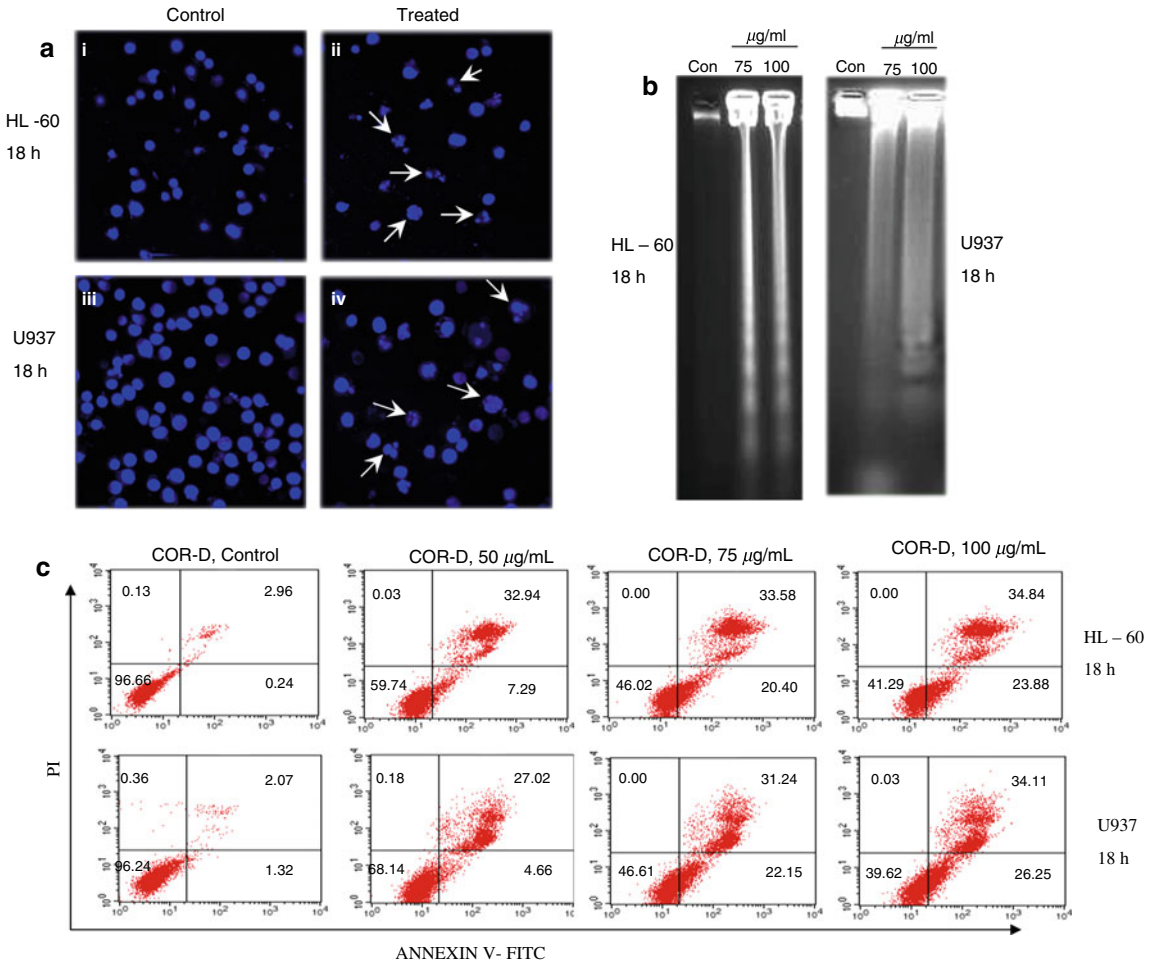

Fig. 3 COR-D induced DNA fragmentation and externalization of phosphatidylserine. **a** Confocal microscopic images showing apoptosis induced by COR-D in HL-60 (*i* and *ii*) and U937 (*iii* and *iv*) cells using nuclear staining dye DAPI (4,6-diamidino-2-phenylindole). *Arrows* indicate the nuclear 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, 75 and 100 μg/ml) 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

decrease in Bcl-2 and increase in Bax were observed in COR-D treated U937 and HL-60 cells (Fig. 5a).

COR-D causes alteration in the mitochondrial membrane potential ($\Delta\psi_m$) in leukemic cell lines

Since Western blot analysis confirmed that COR-D treatment induced a balance between positive and negative regulators of apoptosis shifting toward cell death, an attempt was made to confirm the involvement of mitochondria through observing whether COR-D alters membrane potential triggering further 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 as detected by a shift in fluorescence from red to green during depolarization of the membrane ($\Delta\psi_m$). Exposure of the leukemic cells to COR-D showed a decrease in J-aggregates in mitochondria and increase in monomers in the cytosol after 18 h of treatment (Fig. 5b).

Activation of caspases in COR-D treated leukemic cells

After investigating the decrease in MMP as well as the increase in Bax, it was necessary to find the role of mitochondria in apoptosis induced by COR-D 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 performed in order to determine whether caspase-3 and caspase-9 are really cleaved during COR-D-induced apoptosis in U937 and HL-60 cell lines on exposure to the COR-D at different concentrations (40, 50, 70, 80, 90 and 110 μg/ml) for 18 h

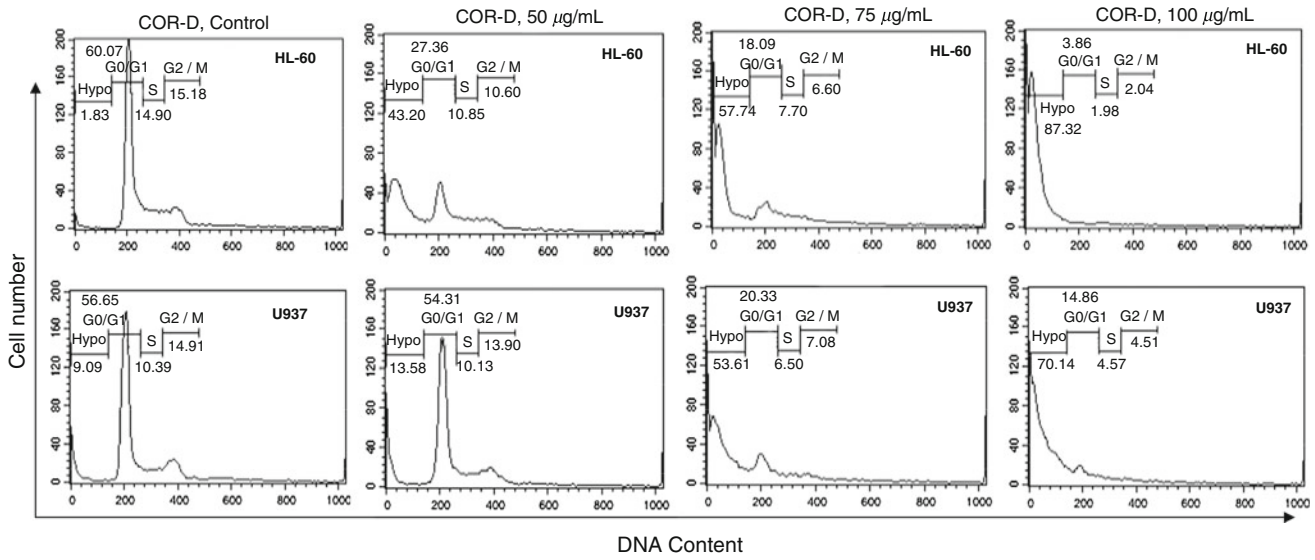

Fig. 4 Cell cycle phase distribution as analyzed by flow-cytometry after COR-D treatment. HL-60 and U937 cells were incubated with different concentrations of COR-D (50–100 μg/ml) for 18 h for DNA cell cycle analysis. The cells were analyzed through FACS after propidium iodide staining. Gates were set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

(Fig. 6b). COR-D exposure resulted in the processing and cleavage of both caspase-3 and caspase-9. The active form of caspase-9 increased and the procaspase-9 decreased with the increase in concentration of COR-D. The activation of caspases was further corroborated by colorimetric measurement (Fig. 6a), and it was found that in U937 cell line caspase-9 showed maximum (eightfold increase) activity at a concentration of 70 μg/ml of COR-D, and in case of caspase-3 the activity was found to be maximum (3.615 fold increase) at 80 μg/ml of COR-D. In HL-60 cell line, caspase-9 activities were maximum at a concentration of 50 μg/ml of COR-D, and caspase-3 showed a maximum activity at a concentration of 70 μg/ml of COR-D. At the same time, COR-D treatment in U937 and HL-60 showed enhanced caspase-8 activity as revealed by its increased expression with increase in concentration of COR-D.

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 [19–21]. 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 with COR-D (40, 50, 70, 80, 90 and 110 μg/ml) in U937 and HL-60 cell lines resulted in the cleavage of PARP to yield an 89-kDa cleaved fragment in the cell lysates (Fig. 6b).

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 [22]. The present study reports, for the first time, the anti-leukemic activity of ME of aerial parts of *C. acutangulus,* and 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 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* [3]. Though both the molecules are terpenoidal glycosides, they vary in the positions of some organic functionality (C-16 and C-23). Because of their steroid-like structure (terpenoid lipid type), saikosaponins have some common steroid-like pharmacological activities [3] and possess therapeutic effects in hyperlipidaemia, hepatic injury, chronic hepatitis and inflammation [23]. Hsu et al. [3] have reported the apoptotic effect of saikosaponin D in human CEM lymphocytes. Studies have also reported that saikosaponins inhibit the proliferation of human leukemia cells [3–7, 24–26] and that saikosaponin D inhibits the cell proliferation of human leukemia cells by up regulating the expression of GR mRNA [27, 28]. The pharmacological activity of COR-D has not been investigated so

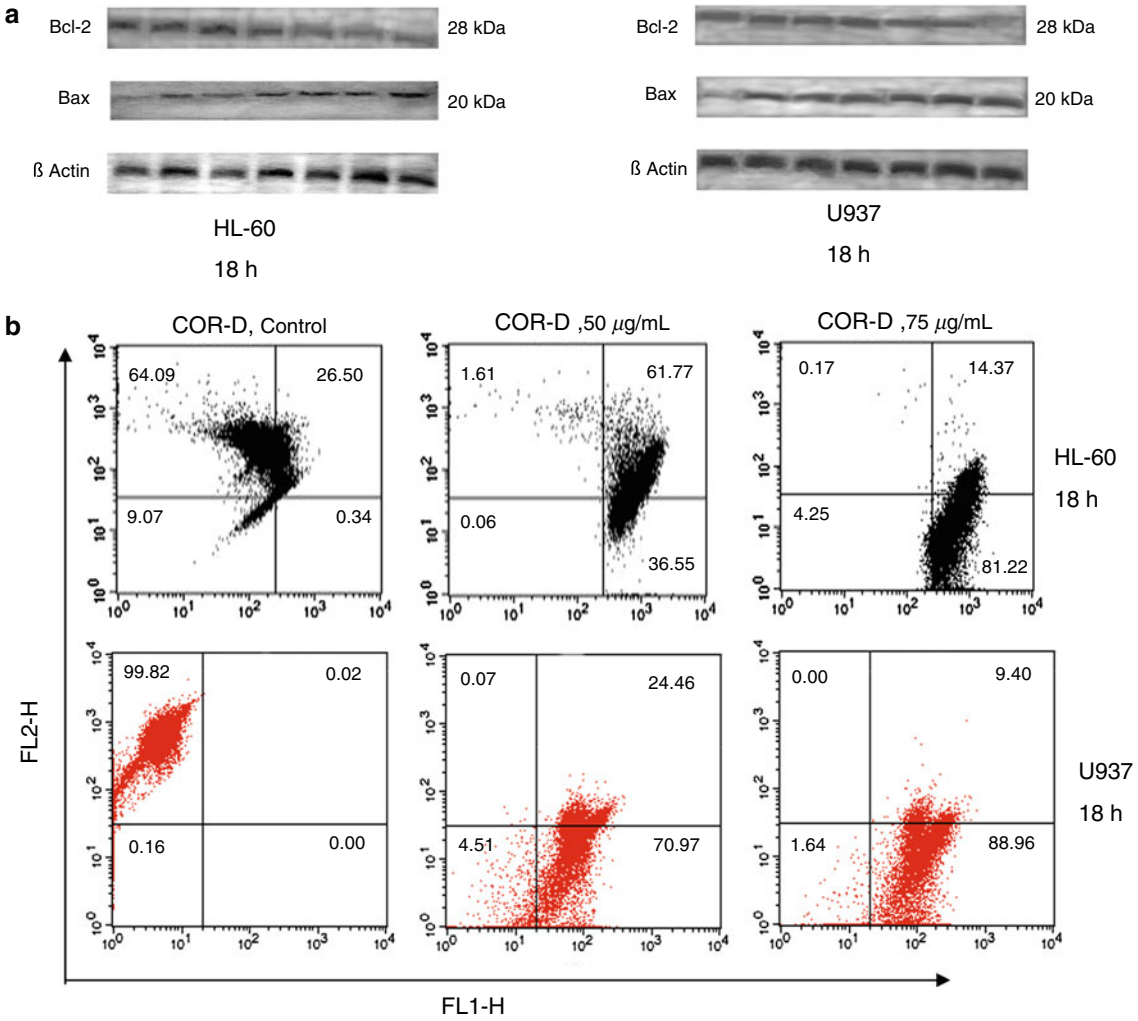

Fig. 5 COR-D induced Bax-dependent mitochondrial death in HL-60 and U937 cells. (**a**) A concentration-dependent increase in Bax band intensity and decrease in Bcl-2 band intensity in the cell lysates of COR-D treated HL-60 and U937 cells were determined by Western blot analysis. (**b**) COR-D-induced 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 ($\Delta\ \psi_m$), was analyzed immediately by flow-cytometry

far. The present study reports the apoptotic effect of COR-D in leukemic cell lines (HL-60 and U937), which may be due to its structural similarity with saikosaponins.

The results demonstrated that COR-D inhibited cell proliferation of U937 and HL-60 cells in a concentration-dependent manner. Further analysis demonstrated that cultured U937 and HL-60 cells treated with COR-D exhibited morphological features of apoptosis, such as membrane shrinkage, nuclear fragmentation and chromosomal condensation. Degradation of higher order chromatin structure of DNA into fragments is a landmark of cellular self-destruction by apoptosis [18]. The COR-D treated cells showed DNA fragments, resulting in a ladder-like pattern when analyzed by agarose gel electrophoresis. The 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 [15, 29]. In apoptotic cells, membrane changes leading to PS exposure occur rapidly and the cells loose membrane integrity later in apoptotic process and 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 [15]. Hence, flow-cytometry by 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 [30]. Flow-cytometric analysis showed that COR-D treatment caused apoptosis in U937 and

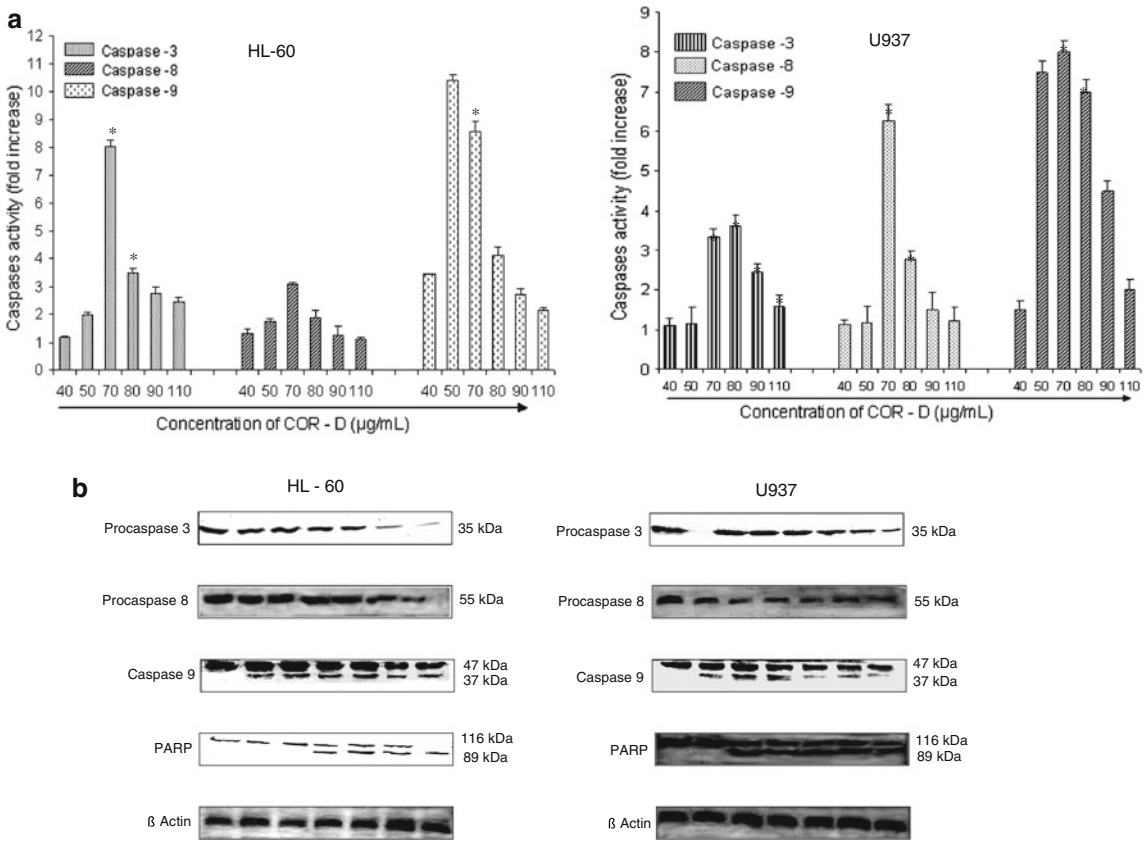

Fig. 6 COR-D triggered cell death in two leukemic cell lines, which involves cleavage of caspases. **a** In order to determine the activation of caspase-3, caspase-8 and caspase-9 in HL-60 and U937 cells on exposure to COR-D for 18 h, colorimetric assay was done using caspase colorimetric assay kits (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

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 in cell viability assay is due to apoptosis rather than necrosis. Based on this finding, it could be postulated that the occurrence of inflammation due to the 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 [31]. In the present study, it was found that COR-D caused the increase in cells in sub-G0 phase in a concentration-dependent manner, with the decrease in cells in G1, G2 and S phases, which supports the contention that cell death after COR-D treatment occurs 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 [32]. In the present study, a concentration-dependent decrease in Bcl-2 and increase in Bax were observed in COR-D treated U937 and HL-60 cells, which reveals that COR-D altered the Bax/Bcl-2 ratio and triggered the mitochondrial pathway of apoptosis.

The activation of the caspase cascade by COR-D 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 caspase-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 [33] and activation of caspase-3 play the central role in the initiation of apoptosis [34, 35]. In the present study, caspase-8 activity was enhanced by COR-D treatment in U937 and HL-60 cell lines, which indicates the

involvement of COR-D in the activation of death receptor and mediation of apoptosis.

The lower intensity in the fluorescence of mitocapture dye aggregates confirmed the loss of mitochondrial membrane potential in COR-D treated U937 and HL-60 cells resulting in the release of different mitochondrial AIFs for sequestering further downstream pathways. Finally, treatment with COR-D in the two cell lines resulted in the cleavage of PARP 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 disfunction is responsible for the induction of apoptotic cell death. COR-D 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 fact that COR-D increased the activation of caspase-8 also suggests the involvement of extrinsic pathway in apoptosis. It would be interesting to examine whether COR-D-induced apoptosis and cell cycle perturbation can play any role in anti-leukemic activity in vivo.

Conflict of interest statement None.

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