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Research Paper

Cytotoxic evaluation and induction of mitochondria-mediated apoptosis in human leukaemia HL-60 cells by *Carissa spinarum* stem isolate

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Abstract

Objective To evaluate *Carissa spinarum* stem isolate for its anti-cancer therapeutic potential.

Methods The n-butanol fraction of aqueous extract from *Carissa spinarum* stem was assessed for its cytotoxic and pro-apoptotic activity.

Key findings We report for the first time the anti-cancer potential of *C. spinarum* stem aqueous extract (CSE) and its n-butanol fraction (CSF). Both inhibited cell proliferation of various human cancer cell lines in which leukaemia HL-60 cells treated with CSF showed maximum growth inhibition having an inhibitory concentration (IC_{50}) value of 34.58 ± 0.91 μg/ml. In addition, CSF induced concentration-dependent apoptosis in HL-60 cells as measured by various end-points (e.g. Annexin V binding, DNA laddering, apoptotic body formation and an increase in hypodiploid subG0 DNA content). Moreover, persistent levels of reactive oxygen species caused translocation of Bax to mitochondria and Bcl-2 degradation, which led to loss of mitochondrial membrane potential and release of cytochrome c to the cytosol. These events were associated with significant activation of caspase-3, caspase-6 and caspase-9 leading to poly (ADP-ribose) polymerase cleavage.

Conclusion All the above parameters revealed that CSF induced apoptosis through the mitochondrial dependent pathway in HL-60 cells.

Keywords anti-cancer; apocynaceae; apoptosis; *Carissa spinarum*; HL-60 cells

Introduction

Cancer has a reputation of being a deadly disease next to cardiovascular diseases due to unavailability of specific drugs. Cancer cells evade self-demise through apoptosis because of the accumulation of several genetic and epigenetic changes within the cells.[1] It is well documented that most cytotoxic anti-cancer agents induce apoptosis, thereby providing a conceptual framework to link cancer genetics with cancer therapy. More than 75% of anti-cancer drugs are directly or indirectly derived from medicinal plants, which continue to be an essential source of novel discovery leads.[2,3] Current clinically useful cytotoxic agents primarily act by inducing apoptosis in cancer cells.[4–6] Apoptosis is a highly regulated process that involves activation of a series of molecular events. It is characterized by cell shrinkage, blebbing of the plasma membrane and chromatin condensation, which are consistent with DNA cleavage. Cell proliferation and apoptosis play a major role in maintaining homoeostasis and as such any disruption within these processes can lead to disease states.[7] Since deregulation of apoptosis is the hallmark of all cancer cells, thus agents that activate programmed cell death in cancer cells could be valuable anti-cancer therapeutics.

Carissa spinarum L. (Apocynaceae), an evergreen and perennial glabrous shrub, is of great medicinal value and is widely distributed in the Indian subcontinent.[8] Previous phytochemical investigations revealed the presence of sesquiterpenes and cardiac glycosides isolated from *Carissa* species. Recently, a germacrane derivative, carenone, was isolated from the stems of *C. spinarum*.[9] The plant exhibited antibacterial[10] and antioxidant properties[11] but no anti-cancer property has been reported so far. Keeping this in mind, *C. spinarum* has been selected to exploit its possible anti-cancer potential by the induction of

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apoptosis in cancer cells for therapeutic benefit. In this study, the aqueous extract of *C. spinarum* stem (CSE), its n-butanol fraction (CSF) and reference compounds (naringenin and ursolic acid) were evaluated for their in-vitro cytotoxic potential against different human cancer cell lines of various tissues. The cytotoxic potential of CSE and CSF against several cancer cell lines is reported for the first time. In addition, *C. spinarum* stem isolate, CSF, containing naringenin and ursolic acid as its active constituents, exhibited promising cytotoxicity with biological implications. Therefore, it was further studied for its possible mode of action and it was observed that CSF was involved in the induction of apoptosis in human leukaemia HL-60 cells. The observed apoptotic activity of CSF is mitochondria-dependent, associated with the generation of reactive oxygen intermediates, release of cytochrome c, degradation of anti-apoptotic protein, translocation of pro-apoptotic protein, activation of caspase cascade and PARP cleavage, which together account for apoptotic cell death.

Materials and Methods

Chemicals and antibodies

RPMI-1640 medium, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine-123 (Rh-123), propidium iodide (PI), sulphorhodamine B (SRB), 3-(4,5,dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), DNase-free RNase, proteinase K, phenylmethanesulfonyl fluoride (PMSF), eukaryotic protease inhibitor cocktail, Triton X-100, penicillin, streptomycin, L-glutamine, pyruvic acid, camptothecin, adriamycin, fetal calf serum and Annexin V-FITC apoptosis detection kit were purchased from Sigma Aldrich (St Louis, MO, USA). Tris-Base, EDTA and phosphate-buffered saline (PBS) were purchased from HiMedia Laboratories Pvt. Ltd (Worli, Mumbai, India) and trichloroacetic acid was from Merck Specialties Pvt. Ltd (Worli, Mumbai, India). Caspase-3, caspase-6 and caspase-9 assay kits were from Bio-Vision, Inc. (Linda Vista Avenue, CA, USA). FITC mouse anti-cleaved PARP (ASP214) antibodies and mouse anti-human antibodies to cytochrome c were from BD Biosciences Pharmingen, (San Diego, CA, USA). Mouse anti-human antibodies to Bcl-2, Bax and β-Actin, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrophoresis reagents, protein estimation kit and protein markers were from Bio-Rad Laboratories (Benicia, CA, USA). Hyper film and ECL Plus Western blotting detection kit were from Amersham Biosciences (Little Chalfont, UK).

Collection and extraction of plant material

The stem of *C. spinarum* L., commonly known as karanda, was collected from the Jammu region of J&K state, India in the months of July–September after identification and authentication by Dr S.N. Sharma, taxonomist of the Indian Institute of Integrative Medicine (CSIR), Jammu. A specimen was also deposited in the herbarium of the Institute (Accession no. 15901).

Dried and powdered stem (500 g) was placed in a 10-l beaker and heated with 4 l distilled water on water bath at 80°C for 2 h. The water-soluble part was removed and the extraction process was repeated three times using 4 l water each time. The aqueous extract was filtered through a bed of celite and freeze dried. The total yield of the extract, CSE, was 5.25%. The suspension of dried aqueous extract was taken in a separating funnel and extracted with n-butanol (4×100 ml). The n-butanol fraction was evaporated to dryness under reduced pressure below 50°C. The total yield of the n-butanol fraction, CSF, was 13.5%.

Sample preparation and standardization

The presence of the flavonoid, naringenin and triterpene acid, ursolic acid, reported earlier from this plant,[12] was observed in CSF by TLC and HPLC comparison with authentic samples. Since ursolic acid is known to possess growth inhibitory potential against various human cancer cell lines and naringenin is a known antioxidant, CSF was quantified for these two compounds by HPLC using authentic compounds as markers. The accurately weighed quantity of the CSF was dissolved in a known volume of HPLC-grade water. The samples were filtered through a millipore micro filter (0.45 µm) and then injected into the HPLC system. Stock solutions of the reference compounds were prepared in HPLC-grade solvents. From the stock solutions, working solutions for each reference compound were prepared by dilution with HPLC-grade solvent for further analysis.

The compounds exhibited linear responses in the calibration curves, which were prepared by using the multipoint calibration curve method. Working solutions of the reference compounds were injected in different amounts (5–25 µl for ursolic acid) and (1–5 µl for naringenin). Excellent calibration curves were obtained for naringenin ($r^2 = 0.997766$) and ursolic acid ($r^2 = 0.999774$). Calibration curves were determined on the basis of five amounts (5–25 µl) for ursolic acid and (1–5 µl) for naringenin solutions.

HPLC analysis

Naringenin and ursolic acid were separated and quantified by using a Waters HPLC system consisting of two pumps, Waters 515 HPLC with Waters pump control module, an automatic sampling unit (Waters 717 plus auto sampler, a column oven, a photodiode array detector Waters 2996 and temperature control module II). Waters empower software was used for data analysis and data processing. The samples were analysed at 30°C on a Merck LiChrospher RP-18 Column (5 µm, 250×4.00 mm) with 0.05% trifluoroacetic acid (TFA) in acetonitrile: 0.05% TFA in water (85 : 15) as mobile phase, flow rate of 1.0 ml/min, UV detection at 206 nm for ursolic acid; and 0.05% TFA in acetonitrile: 0.05% TFA in water (gradient), flow rate 1.0 ml/min, UV detection at 215 nm for naringenin. From the HPLC results the CSF was found to contain 0.32% naringenin and 0.88% ursolic acid (Figure 1).

Cell culture and treatment

The human cancer cell lines were obtained from National Cancer Institute (Frederick, MD, USA). Colon (Colo-205, SW-620), ovary (IGR-OV-1, OVCAR-5), prostate (DU-145, PC-3), lung (A-549, Hop-62), CNS (SK-N-SH, SF-295), acute lymphoblastic leukaemia (MOLT-4) and acute promyelocytic leukaemia (HL-60) cell lines were grown and

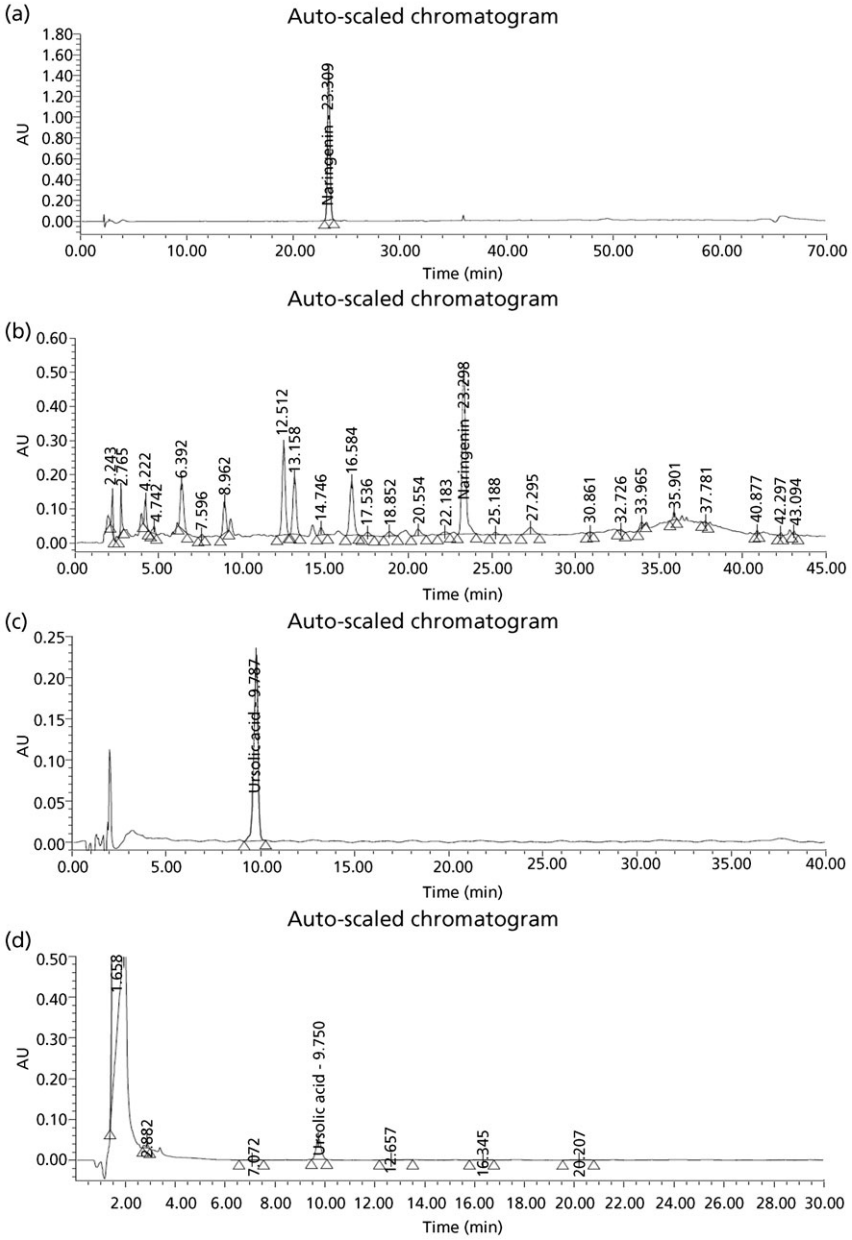


Figure 1 HPLC chromatograms of marker compounds and n-butanol fraction (CSF) of *Carissa spinarum* stem. (a) naringenin (b) naringenin identified in CSF (c) ursolic acid and (d) ursolic acid identified in CSF.

maintained in RPMI-1640 medium, pH 7.4. The media was supplemented with fetal calf serum (FCS) (10%), penicillin (100 U/ml) and streptomycin (100 µg/ml), being referred to as complete medium. The cells were grown in CO_2 incubator (Hera Cell, Heraeus, Germany) at 37°C with 90% humidity and 5% CO_2. Human peripheral blood mononuclear cells (PBMC) were prepared from the blood of normal human by using Sigma's Histopaque-1077 and 1119 solution as described previously.[13,14] Briefly, a double gradient was formed in a 15-ml centrifuge tube by layering an equal volume of Histopaque-1077 over Histopaque-1119 (2 ml : 2 ml). Anticoagulated venous blood (4 ml) was carefully layered onto the upper Histopaque-1077 layer and centrifuged at 700*g* for 30 min at room temperature. After centrifugation, the band at the interface between the Histopaque-1077 and the plasma, containing mononuclear cells, was aspirated carefully and transferred into a clean conical centrifuge tube. Cells were suspended in 10 ml cell culture medium (RPMI 1640 supplemented with fetal calf serum) and centrifuged at 200*g* for 10 min. Supernatant was discarded and pellet was gently resuspended in 10 ml cell culture medium and the washing process was repeated twice to obtain mononuclear cell suspension. Stock solutions (20 mg/ml) of CSE and CSF were prepared in water and serially diluted with complete growth medium containing 50 µg/ml of gentamicin to obtain the desired concentrations.

Cytotoxicity assay

The in-vitro cytotoxicity against human adherent cancer cell lines was determined by SRB assay as described previously.[15,16] The samples were further screened in leukaemia cell lines, namely MOLT-4, HL-60, and normal human PBMC using MTT assay as per standard procedure.[17,18]

Annexin V/propidium iodide flow cytometric analysis

Phosphatidylserine exposed on the outside of the apoptotic cells was determined by an Annexin V-FITC Apoptosis Kit (Sigma, St Louis, MO, USA). Briefly, following treatment with CSF for 24 h, HL-60 cells (5×10^5/ml) were harvested by low-speed centrifugation, washed twice with ice-cold PBS, pelleted and resuspended in 400 µl of 1 X Annexin V-FITC binding buffer, 4 µl of Annexin V-FITC conjugate and 8 µl of PI buffer. The cells were then incubated at room temperature for 15 min in the dark and analysed using a FACS Calibur (Becton Dickinson, San Jose, CA, USA).[19]

DNA agarose gel electrophoresis

Apoptosis was assessed by electrophoresis of extracted genomic DNA from HL-60 cells as described previously.[20]

DNA content and cell cycle phase distribution

HL-60 cells (5×10^5/ml) were treated with 10, 30 and 100 µg/ml of CSF for 24 h, fixed in cold 70% alcohol in PBS, washed, digested with DNase free RNase (10 µg/ml) at 37°C for 1 h and stained with PI (5 µg/ml) for 3 h at 4°C in the dark. Cells were analysed immediately on FACS Calibur (Becton Dickinson). The fluorescence intensity of sub-G0 cell fraction represents the apoptotic cell population.[21]

Measurement of intracellular peroxides (ROS) in HL-60 cells

The level of intracellular peroxides was determined by using DCFH-DA as demonstrated previously.[22,23] HL-60 cells (5×10^5/ml) were incubated with indicated concentrations of CSF in the presence and absence of antioxidant in 6-well plates for 24 h at 37°C. DCFH-DA (1 µM) was added 30 min before termination of the experiment. Cells were washed in PBS and analysed in FL-1 channel (excitation λ488 nm; emission λ535 nm) on FACS Calibur (Becton Dickinson).

Measurement of mitochondrial membrane potential

The change in mitochondrial transmembrane potential ($\Delta\Psi_{mt}$) as a result of mitochondrial perturbation due to ROS generation induced by CSF was measured after staining with rhodamine-123.[24] HL-60 cells (5×10^5/ml) were incubated with CSF in the presence and absence of ROS inhibitor in 6-well plate for 24 h at 37°C. Rhodamine-123 (5 µg/ml; stock, 1 mg/ml PBS) was added 1 h before termination of the experiment. Cells were washed in PBS and centrifuged at 100*g* for 5 min and suspended in PBS. Immediately before analysis, PI (5 µg/ml; stock 1 mg/ml PBS) was added to the samples. The intensity of fluorescence from 10 000 events was analysed in FL-1 channel on FACS Calibur (Becton Dickinson).

Caspase assays

HL-60 cells (2×10^6/3 ml/well, 6-well plate) were incubated with CSF for the indicated time period. At the end of treatment, cells were washed in PBS and the pellet was lysed in cell lysis buffer. The activity of caspase-3, caspase-6 and caspase-9 in the cell lysates was determined colorimetrically using BioVision colorimetric caspase assay kits. Caspase-3, caspase-6 and caspase-9 employed chromophore conjugated peptides DEVD-pNA, VEID-pNA and LEHD-pNA as substrates, respectively. Release of *p*-nitroanilide (pNA) was assayed as per the supplier's instructions.

PARP cleavage

HL-60 (2×10^6/3 ml/well, 6-well plate) cells were treated with CSF at various concentrations for 24 h. Cells were harvested by low-speed centrifugation, washed twice with ice-cold PBS, pelleted and resuspended in 1 ml of 2% (w/v) paraformaldehyde and incubated for 20 min on ice for fixation. Fixed cells were then permeabilized in 0.1% Triton X-100 for 30 min, washed and resuspended in 100 µl of PBS. Further, the cells were incubated with 20 µl of FITC mouse anti-cleaved PARP (ASP214) antibody (BD Biosciences) at room temperature for another 30 min in the dark. Finally, the samples were washed twice and resuspended in 500 µl PBS and analysed for cleaved PARP using a FACS Calibur (Becton Dickinson).

Preparation of cytosolic and mitochondrial lysates

Cytosolic and mitochondrial fractions for analysis of cytochrome c, Bcl-2 and Bax immunoblotting were obtained by selective plasma membrane permeabilization with digitonin.[25] Briefly, 2×10^6 cells/2 ml were lysed for 1–2 min in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, 350 µg/ml digitonin and 1% (v/v) eukaryotic protease inhibitor cocktail). The lysates were centrifuged at 12 000*g* for 1 min, and the supernatant collected as cytosolic fraction was stored at −70°C. Residual pellet was lysed with whole-cell lysis buffer after incubating on ice for 30 min. After centrifugation at 12 000*g* for 10 min at 4°C, cell lysates were transferred to fresh tubes and stored at −70°C as mitochondrial fraction.

Western blot analysis

The protein lysates prepared as described previously[14] were subjected to discontinuous SDS-PAGE analysis. Proteins (50 µg) were resolved on SDS-PAGE and then electrotransferred to PVDF membrane overnight at 4°C at 30 V. Non-specific binding was blocked by incubation with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were probed with respective primary antibodies for 2 h and washed three times with TBST. The blots were then incubated with horseradish peroxidase-conjugated mouse or rabbit secondary antibodies for 1 h, washed again three times with TBST and signals detected using ECL plus chemiluminescence's kit on X-ray film.

Statistical analysis

Statistical significance was assessed by the Kruskal–Wallis test followed by post-hoc (Dunn's test) analysis;

$P \leq 0.05$ was considered statistically significant. Inhibitory concentration (IC_{50}) values were analysed by Tukey–Kramer multiple comparison test ($P \leq 0.05$).

Results

Assessment of in-vitro cytotoxicity

In-vitro cytotoxicity studies of CSE, CSF and reference compounds (naringenin and ursolic acid) showed a dose-dependent effect against several human cancer cell lines of different tissues such as colon, ovary, prostate, lung, CNS and leukaemia (data not shown). IC_{50} values of CSE, CSF, naringenin and ursolic acid for various cell lines of different tissues are shown in Table 1. Treatment with CSF and ursolic acid revealed maximum cytotoxicity against the leukaemia, colon and ovary cancer cell lines. Ursolic acid exhibited maximum cytotoxicity ($P \leq 0.01$) with lowest IC_{50} values (25.15 ± 2.54, 29.25 ± 3.64 and 29.61 ± 3.64 μg/ml) against HL-60, Colo-205 and IGR-OV-1 cell lines (respectively). Significant cytotoxic potential was exhibited by CSF with IC_{50} values of 34.58 ± 0.91, 45.48 ± 3.37, 49.63 ± 2.84 and 49.90 ± 2.15 μg/ml against HL-60, SW-620, Colo-205 and IGR-OV-1 cell lines, respectively, whereas CSE also showed substantial cytotoxicity against all the cell lines. However, naringenin was the least active as its observed IC_{50} values were significantly higher than CSE, CSF and ursolic acid. Moreover, all treatments were almost non-toxic to PBMCs as their IC_{50} values were much higher (≥200 μg/ml for CSE and naringenin; 197.86 ± 8.89 and 183.33 ± 8.81 μg/ml for CSF and ursolic acid, respectively) under similar conditions.

Analysis of apoptosis and necrosis by Annexin V/propidium iodide binding

Early events of apoptosis were analysed using the Apoptosis Detection Kit (APOAF; Sigma Aldrich). To differentiate between apoptosis and necrosis, HL-60 cells (5×10^5/ml) were exposed to CSF (10, 30 and 100 μg/ml) and incubated for 24 h. All preparations were stained with annexin V-FITC+/PI+ for flow cytometric analysis. Cells were acquired for FSC and SSC and major gated cell population was analysed for apoptosis and necrosis. The CSF at 10, 30 and 100 μg/ml and camptothecin (4 μM) increased the percentage of annexin V positive cells by 7.03, 21.50, 25.95 and 25.62%, respectively, whereas annexin V-/PI+ cells for the same were 3.65, 10.15, 12.50 and 4.23%, respectively (Figure 2). The values for the control were 3.32 and 4.51%, respectively, for annexin V + / PI– and annexin V-PI+ cells. Apoptosis thus appeared to be the primary mode of cell death induced by CSF.

DNA fragmentation by agarose gel electrophoresis

Analysis of DNA from apoptotic cells by agarose gel electrophoresis is known to produce a characteristic DNA ladder that is widely regarded as a biochemical hallmark of apoptosis. A distinct DNA laddering pattern was observed in HL-60 cells treated with CSF at 10, 30 and 100 μg/ml concentration but precisely more prominent at the higher concentration after 24 h incubation. Camptothecin (4 μM) used as a reference compound also showed a clear DNA ladder while no such ladder was observed in untreated HL-60 cells and treated human PBMC (Figure 3).

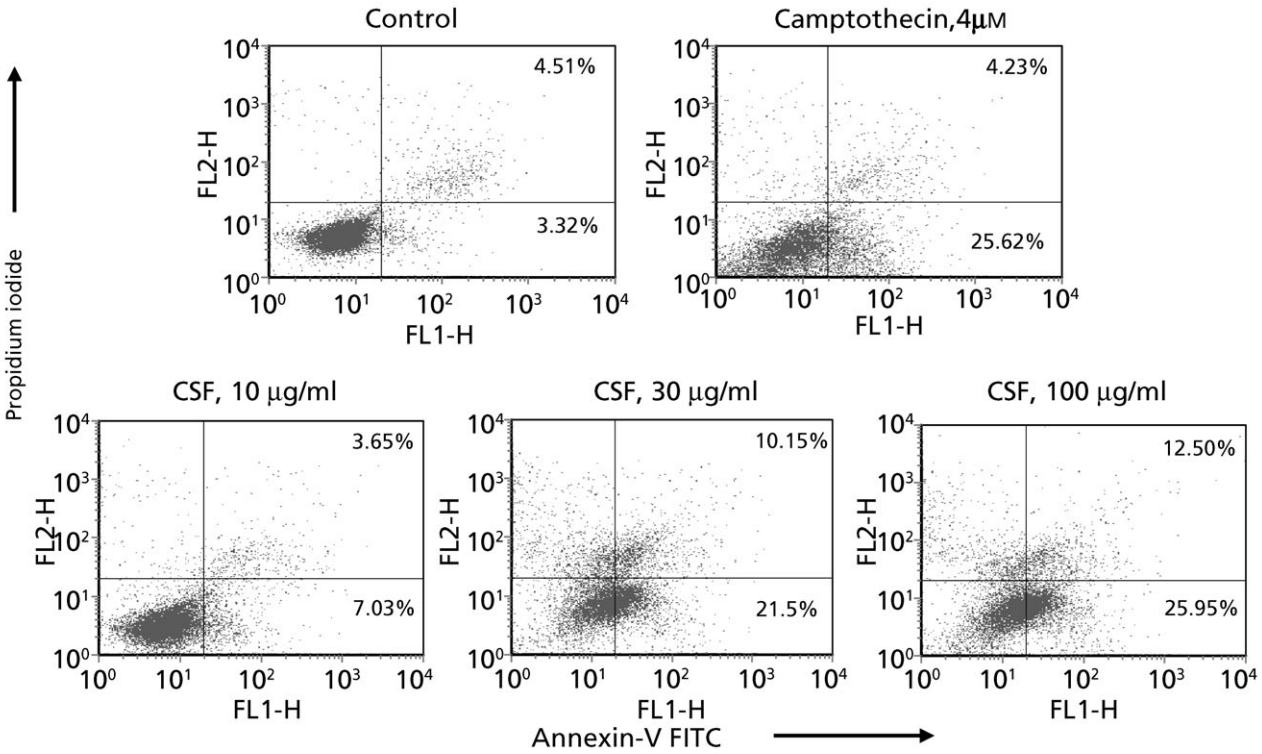
C. spinarum stem n-butanol extract increased sub-G0 fraction of cell cycle measured by flow cytometry

HL-60 cells treated with CSF at 10, 30 and 100 μg/ml up to 24 h clustered in the hypodiploid (sub G0) phase of the cell cycle if compared with the control by 7.35, 63.77 to 80.72% versus 4.28%, respectively. Moreover, a concentration-dependent increase in sub-G0 fraction was observed, which may comprise both apoptotic and debris fraction (Figure 4).

Table 1 Inhibitory concentration (IC_{50}) values of aqueous (CSE) and n-butanol fraction of *Carissa spinarum* stem (CSF) and reference compounds naringenin and ursolic acid against various cell lines

Tissue	Cell line	IC_{50} value (μg/ml)			
		CSE	CSF	Naringenin	Ursolic acid
Colon	Colo-205	76.21 ± 3.17	49.63 ± 2.84^{a}	$122.00 \pm 7.57^{b\dagger,d\dagger}$	$29.25 \pm 3.64^{c\dagger,f\dagger}$
	SW-620	67.59 ± 1.47	45.48 ± 3.37^{a}	$114.53 \pm 5.79^{b,d\dagger}$	$31.36 \pm 2.48^{c\dagger,f\dagger}$
Ovary	IGR-OV-1	76.48 ± 2.18	$49.90 \pm 2.15^{a\dagger}$	$102.50 \pm 6.61^{b\dagger,d\dagger}$	$29.61 \pm 3.14^{c\dagger,e,f\dagger}$
	OVCAR-5	81.58 ± 2.11	$53.50 \pm 2.70^{a\dagger}$	$95.41 \pm 5.02^{d\dagger}$	$35.46 \pm 2.77^{c\dagger,e,f\dagger}$
Prostate	PC-3	66.18 ± 2.03	62.22 ± 1.61	$102.33 \pm 3.75^{b\dagger,d\dagger}$	$37.93 \pm 2.51^{c\dagger,e\dagger,f\dagger}$
	DU-145	64.66 ± 1.36	63.77 ± 1.47	$111.66 \pm 4.80^{b\dagger,d\dagger}$	$40.98 \pm 4.21^{c\dagger,e\dagger,f\dagger}$
Lung	A-549	82.94 ± 3.74	61.87 ± 2.18^{a}	$104.00 \pm 5.50^{b,d\dagger}$	$38.76 \pm 3.52^{c\dagger,e,f\dagger}$
	Hop-62	64.37 ± 1.52	54.25 ± 4.08	$107.25 \pm 5.19^{b\dagger,d\dagger}$	$34.81 \pm 2.86^{c\dagger,e,f\dagger}$
CNS	SK-N-SH	58.78 ± 3.22	51.84 ± 2.70^{a}	$100.20 \pm 4.45^{b\dagger,d\dagger}$	$42.16 \pm 2.14^{c\dagger,f\dagger}$
	SF-295	77.53 ± 2.40	56.67 ± 2.35^{a}	$104.66 \pm 6.17^{b,d\dagger}$	$37.48 \pm 4.07^{c\dagger,f\dagger}$
Leukemia	MOLT-4	83.35 ± 0.62	60.76 ± 1.22^{a}	$99.33 \pm 7.12^{d\dagger}$	$32.37 \pm 4.52^{c\dagger,e\dagger,f\dagger}$
	HL-60	48.72 ± 1.28	34.58 ± 0.91^{a}	$93.66 \pm 2.96^{b\dagger,d\dagger}$	$25.15 \pm 2.54^{c\dagger,f\dagger}$
Blood	PBMC	>200	197.86 ± 8.89	>200	183.33 ± 8.81

IC_{50} values of CSE and CSF of *Carissa spinarum* stem and reference compounds (naringenin and ursolic acid) against human cancer cell lines. Data are expressed as Mean ± SEM of three similar experiments. IC_{50} values of each group are compared. $^{a}P < 0.05$ CSE vs CSF; $^{b}P < 0.05$ CSE vs naringenin; $^{c}P < 0.05$ CSE vs ursolic acid; $^{d}P < 0.05$ CSF vs naringenin; $^{e}P < 0.05$ CSF vs ursolic acid; $^{f}P < 0.05$ naringenin vs ursolic acid; †$P < 0.01$.



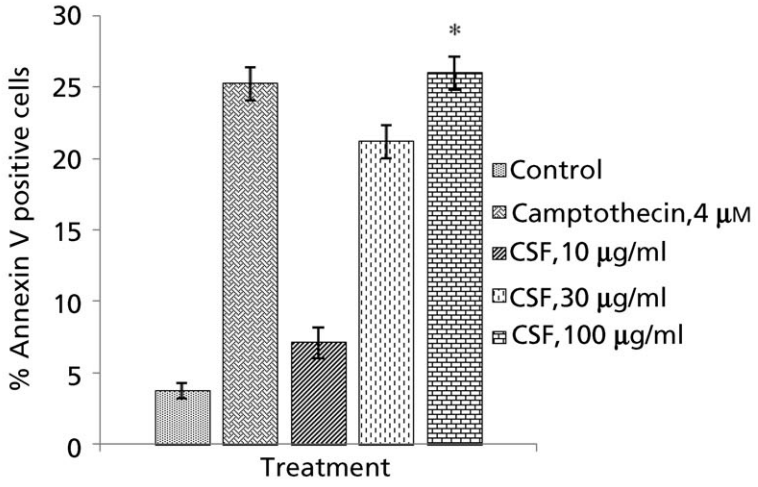


Figure 2 Flow cytometric analysis of n-butanol fraction of *Carissa spinarum* stem (CSF)-induced apoptosis and necrosis in HL-60 cells using Annexin V-FITC and propidium iodide double staining. Cells (5×10^5/ml) were incubated with indicated concentrations of CSF for 24 h and stained with Annexin V-FITC/PI to analyse apoptotic and necrotic cell populations. Data are representative of one of three similar experiments.

Intracellular generation of peroxides mediated by *C. spinarum* stem n-butanol extract in HL-60 cells

HL-60 cells were incubated with CSF at different concentrations and analysed by flow cytometry after staining with DCFH-DA. Cells treated with CSF at 10, 30 and 100 μg/ml in the absence of antioxidant showed a concentration-dependent increase in DCF-positive cell population by 12.44, 19.02 and 23.90%, respectively. The level of ROS generation was found to be selectively more in CSF-treated cells at the higher concentration. It was also observed that the presence of 5 mM ascorbate inhibited ROS generation induced by 100 μg/ml of CSF treatment as only 11.85% cell population was DCF positive. However, there was very low DCF fluorescence (1.61%) in the untreated HL-60 cells (Figure 5).

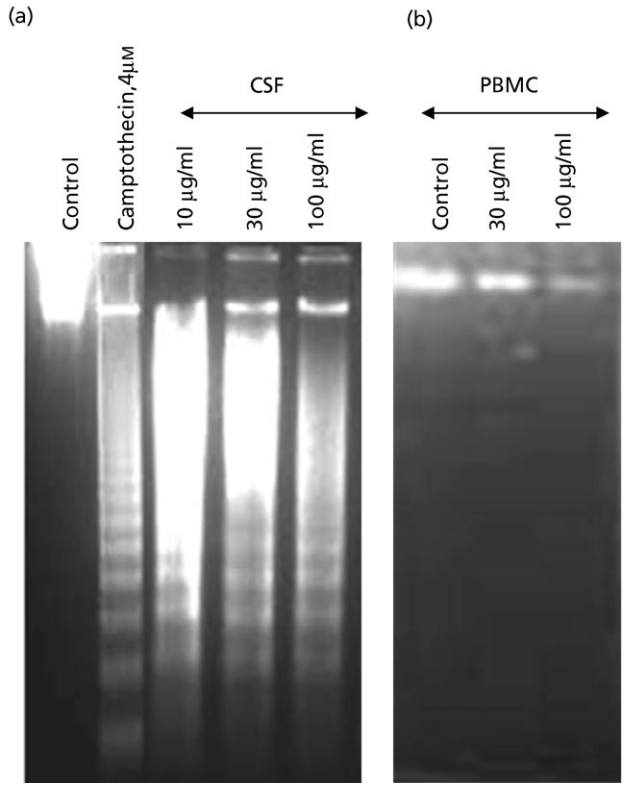


Figure 3 Agarose gel electrophoresis demonstrating apoptotic DNA fragmentation in HL-60 cells. Cells (2×10^6/2 ml) were treated with indicated concentration of n-butanol fraction of *Carissa spinarum* stem (CSF) for 24 h. Genomic DNA of HL-60 cells (a) and human PBMC (b) was isolated and electrophoresed as described in Material and Methods.

Effect on Rhodamine-123 uptake by HL-60 cells

Mitochondria play an essential role in the propagation of apoptosis. Since the disruption of mitochondrial membrane potential is a critical step occurring in cells undergoing apoptosis, the rhodamine fluorescence was used to determine the mitochondrial membrane potential in HL-60 cells following treatment with CSF at different concentrations. As shown in Figure 6, the fluoriscan data demonstrated significant concentration-dependent mitochondrial damage and hence the decrease in mitochondrial membrane potential was observed in the absence of ROS inhibitor. The flow cytometric analysis revealed that 17.45, 42.50 and 79.22% of the HL-60 cell population exhibited loss of mitochondrial membrane potential after 24 h exposure with treatment by CSF at 10, 30 and 100 μg/ml, respectively. However, presence of ROS inhibitor (5 mM ascorbate) protected the cells' mitochondria from undergoing mitochondrial membrane depolarization induced by ROS generation in CSF-treated cells. In untreated HL-60 cells, approximately 90% of cells were functionally active with high ^{123}Rh signals.

Up-regulation of caspase activity

HL-60 cells treated with CSF for 24 h produced a concentration-dependent increase in caspase activity. The CSF displayed higher than 2.5-fold caspase-3 activity at 100 μg/ml compared with control. Similarly, the activity of caspase-6 and caspase-9 displayed an increase of ~2 fold at the same concentration. Caspase-9 activation by CSF revealed that it induces apoptosis through the intrinsic or mitochondria-dependent pathway (Table 2).

Table 2 Induction of caspases by n-Butanol fraction of *Carissa spinarum* stem

Treatment	Fold change in caspase activity		
	Caspase-3	Caspase-6	Caspase-9
Control	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.05
CSF 10 μg/ml	1.26 ± 0.03	1.11 ± 0.02	1.10 ± 0.02
CSF 30 μg/ml	2.54 ± 0.05	1.43 ± 0.05	1.55 ± 0.02
CSF 100 μg/ml	2.74 ± 0.07	1.85 ± 0.02	2.05 ± 0.05
Camptothecin-4 μM	3.00 ± 0.05*	2.91 ± 0.02*	2.8 ± 0.05*

Induction of caspase activity was determined colorimetrically by using BioVision colorimetric caspase assay kits. Data are mean ± SEM from three similar experiments. *$P < 0.05$ vs control.

PARP cleavage induced by *C. spinarum* stem n-butanol extract

An additional evidence of caspase-3 activation was the cleavage of PARP as investigated by flow cytometric analysis using a mouse monoclonal antibody. Incubation of HL-60 cells with CSF resulted in the formation of cleaved protein in a concentration-dependent manner. The percentage of cleaved PARP by CSF was 8.34, 28.24 and 38.84% at 10, 30 and 100 μg/ml, respectively, as opposed to 5.76% in the untreated HL-60 cells (Figure 7).

Mitochondrial dysfunction induced by *C. spinarum* stem n-butanol extract associates with cytochrome c release, Bcl-2 degradation and Bax translocation

Translocation of Bax from cytosol promotes the permeabilization of outer mitochondrial membrane, resulting in the loss of membrane potential accompanied by the subsequent release of cytochrome c from mitochondria to cytosol. Here, HL-60 cells were treated with CSF at 10, 30 and 100 μg/ml for 24 h and evaluated for apoptotic key enzyme/proteins by immunoblot analysis. It was observed that treatment with CSF increased the release of cytochrome c into the cytosol with simultaneous translocation of Bax to mitochondria due to degradation of anti-apoptotic protein Bcl-2 in a concentration-dependent manner (Figure 8), thereby confirming that CSF induces apoptosis through the intrinsic pathway.

Discussion

Much of the contemporary research in the development of anti-cancer therapeutics from plants has been focused on investigating the molecular mechanism by which an agent induces cytotoxicity and apoptosis in cancer cells.[26] Anti-cancer drugs inducing apoptosis, having low incidence of side effects and targeting specific cytotoxicity to cancer cells are the drugs of choice. In this regard, the cytotoxic potential

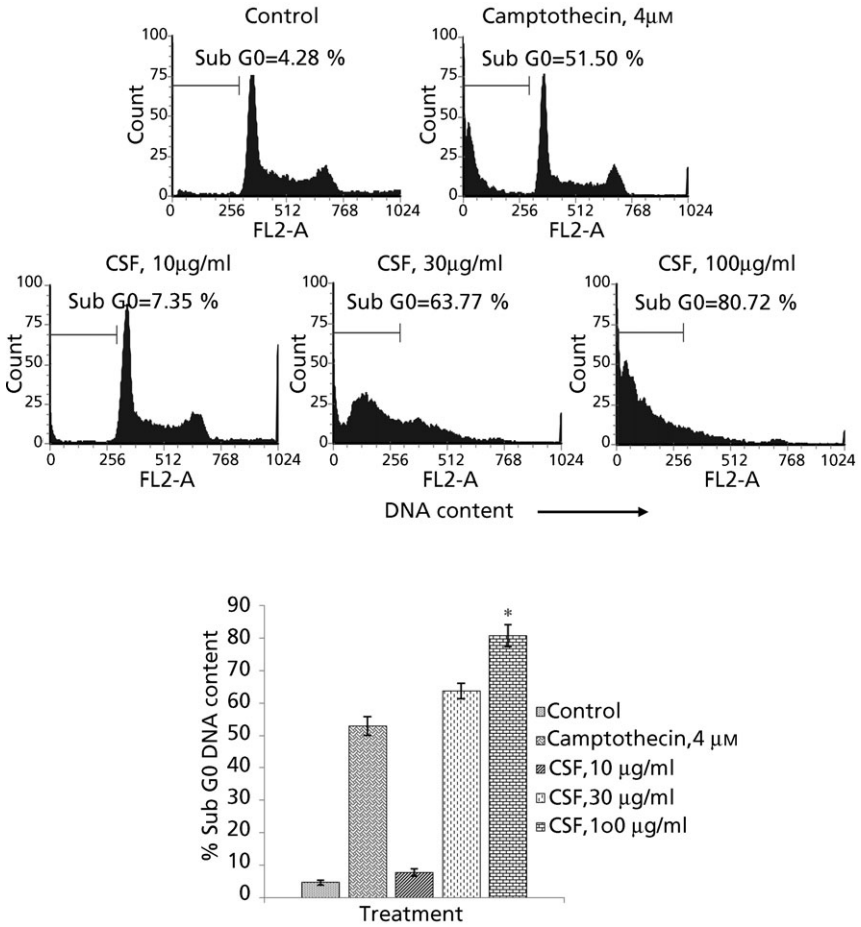


Figure 4 DNA cell cycle analyses in HL-60 cells exposed to n-butanol fraction of *Carissa spinarum* stem (CSF). HL-60 cells (5×10^5/ml) in culture were treated with CSF at indicated concentrations for 24 h. Cells were stained with propidium iodide to determine DNA fluorescence and cell cycle phase distribution as described in Material and Methods. Fraction of cells for DNA content of hypo-diploid (sub-G0, <2n DNA) population was analysed from FL2 A vs cell counts. Data are representative of one of three similar experiments.

of aqueous extract (CSE) and n-butanol fraction (CSF) of *C. spinarum* stem and the reference compounds (naringenin and ursolic acid) was investigated in several human cancer cell lines. The results of this study for the first time describe the cytotoxicity and pro-apoptotic activity of CSF on human leukaemia HL-60 cells. Both CSE and CSF were able to inhibit proliferation of human cancer cell lines of different tissue origin. Ursolic acid was the most cytotoxic compound, followed by CSF and CSE, whereas naringenin was almost ineffective against all cell lines. However, both, CSE and CSF did not exert any cytotoxic effect on the normal cells. The varying degree of cytotoxicity observed against different cancer and normal cells may be due to difference in their molecular characteristics.[27] Since CSF possesses the potential for development as an anti-cancer agent, we further sought to understand the mechanism of apoptotic cell death. Apoptotic cell death is the consequence of a series of precisely regulated events, which are frequently altered in tumour cells.[28] This study provides valuable insight into the pro-apoptotic nature of CSF as evidenced from measurement of several biological end-points of apoptosis, such as Annexin V binding, appearance of apoptotic bodies, DNA fragmentation and increase in sub-G0 DNA fraction in HL-60 cells within a specified time period.

Fluorescence microscopy of CSF-treated HL-60 cells illustrated typical morphological changes such as cell shrinkage, chromatin condensation, plasma membrane blebbing and formation of apoptotic bodies (data not shown) which was confirmed by a concentration-dependent increase in apoptotic cell population with externalized phosphatidylserine analysed by Annexin V/PI staining assay. Since differential sensitivity to apoptosis is linked to distinct phases of the cell cycle,[29,30] likewise the CSF caused a concentration-dependent increase in the apoptotic sub G1 fraction. Sometimes, the DNA damage causes ATP depletion or circumvents activation of the caspase cascade, impairing cell death by apoptosis and, consequently, the cell progresses towards necrosis. The process of necrosis is related to the cytoplasmic content, extravasation and local inflammatory reaction. For this reason, there is a great interest in new drugs that can induce tumour cells to apoptotic cell death.[31] The results of DNA agarose gel electrophoresis showed that CSF activated the necessary endonucleases, such that endonucleolytic cleavage of DNA at internucleosomal linker sites resulted in fragmentation of

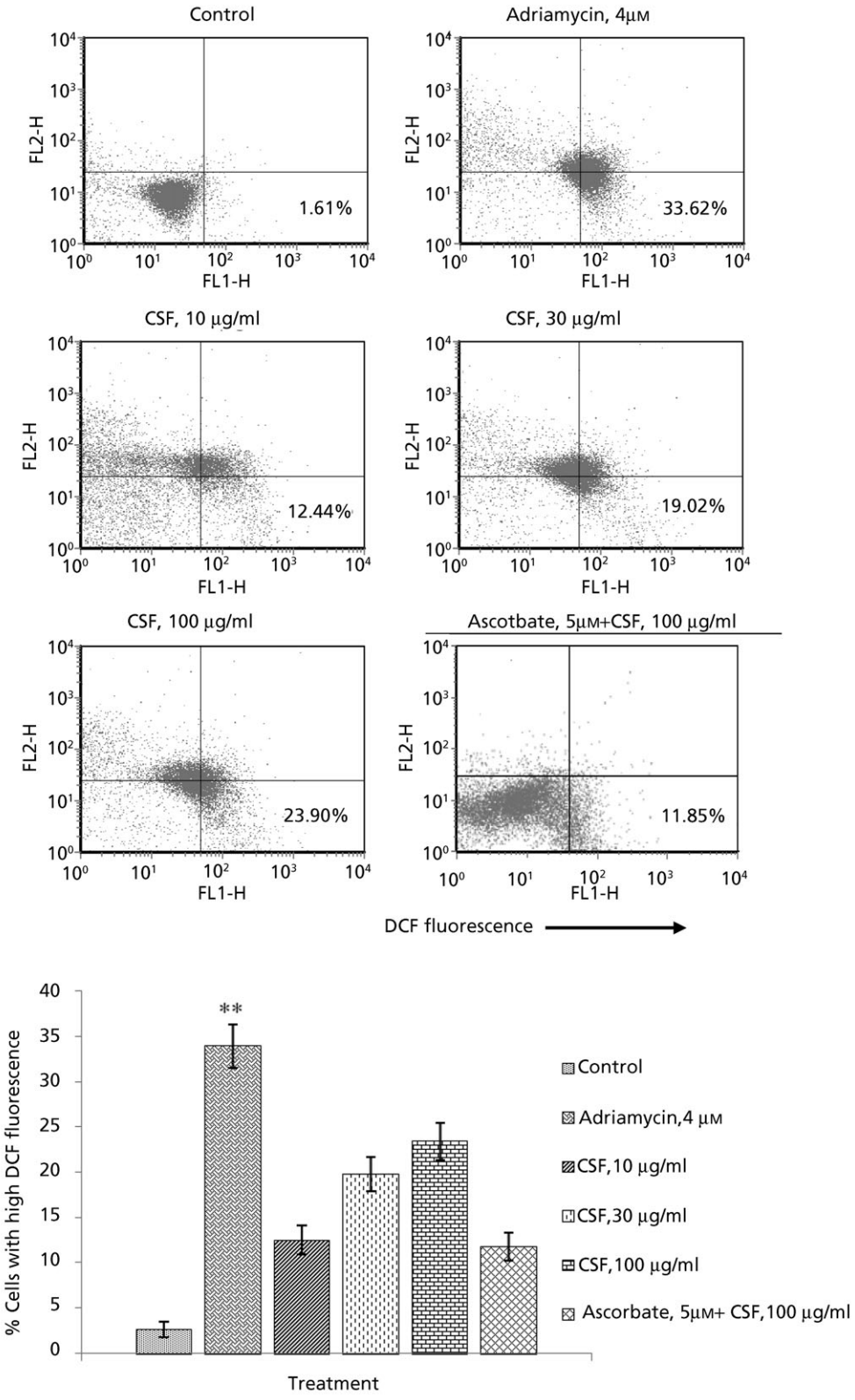


Figure 5 Effect of n-butanol fraction of *Carissa spinarum* stem (CSF) on the generation of reactive oxygen species in HL-60 cells. HL-60 cells (5×10^5/ml) were incubated with CSF at various concentrations for 24 h in the presence and absence of antioxidant. Cells were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 1 μM), incubated for 30 min, 10 000 events acquired and gated population analysed using FACS Calibur flow cytometer. Data are representative of one of three similar experiments.

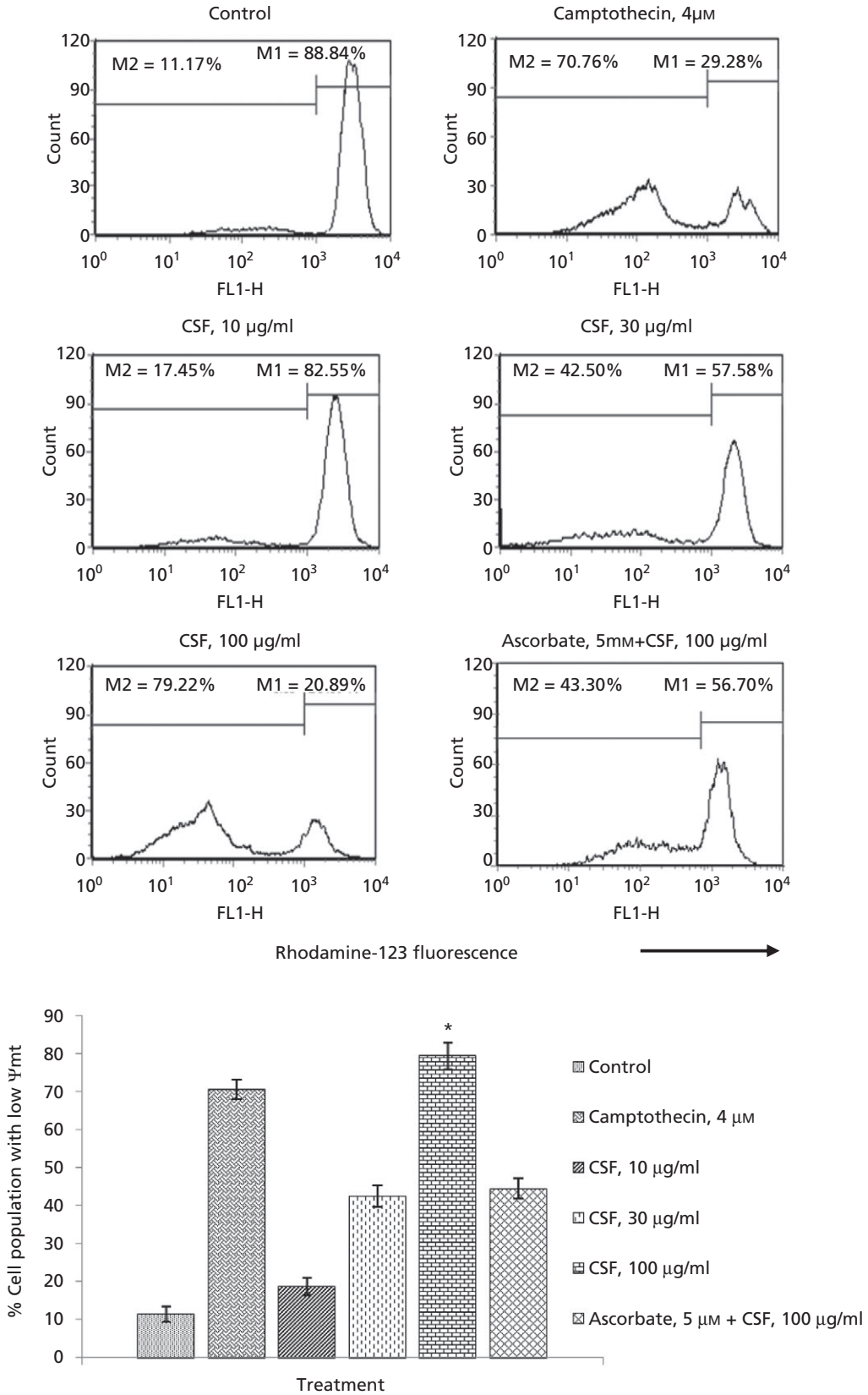


Figure 6 n-Butanol fraction of *Carissa spinarum* stem (CSF) induced concentration-dependent loss of mitochondrial membrane potential (Ψmt) in HL-60 cells. Cells were stained with rhodamine-123 dye and flow cytometric analyses of $\Delta\Psi_{mt}$ in HL-60 cells treated with CSF at indicated concentrations for 24 h in the presence and absence of antioxidant was done using FACS Calibur. Data are representative of one of three similar experiments.

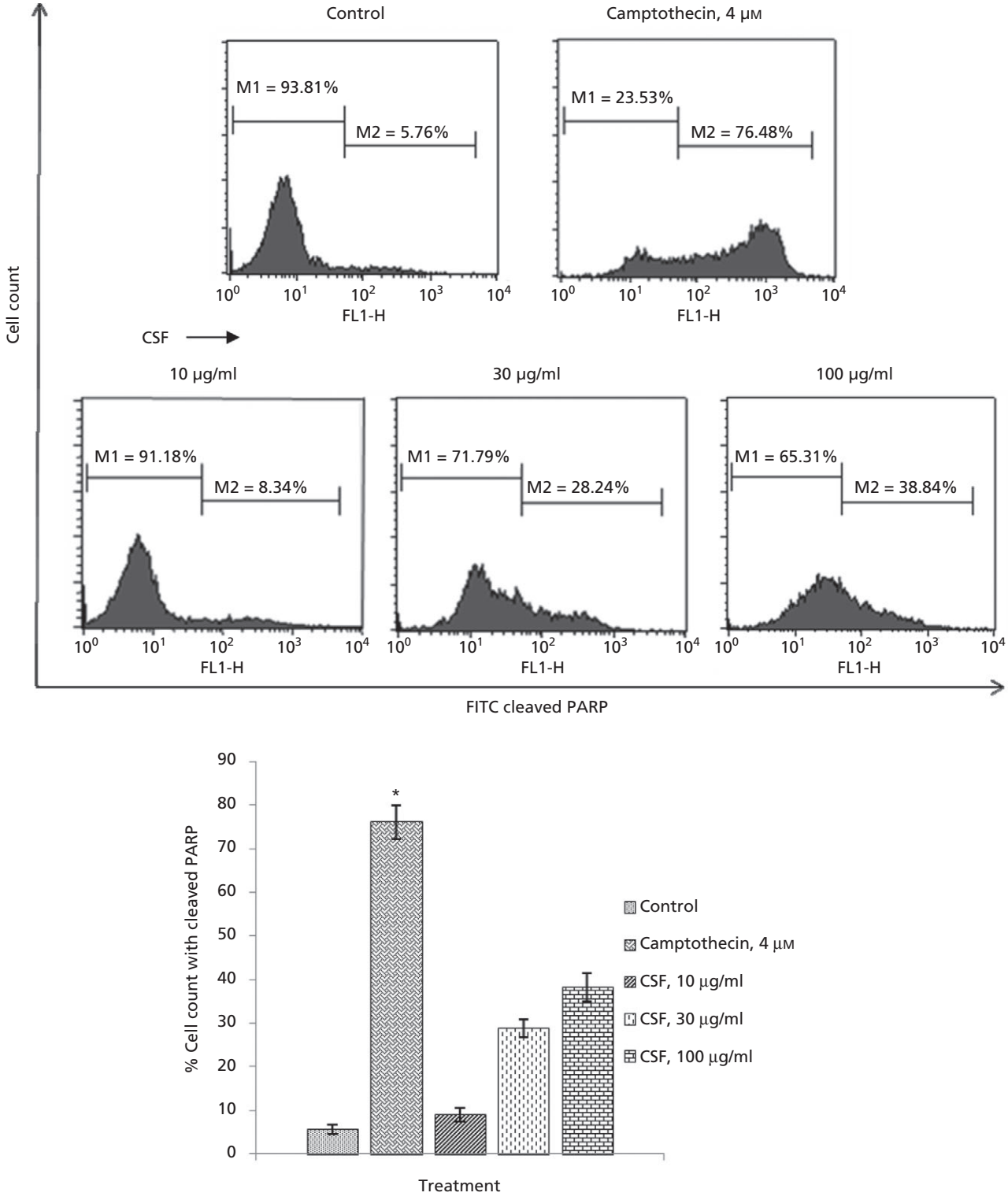


Figure 7 n-Butanol fraction of *Carissa spinarum* stem (CSF) induces concentration-dependent cleavage of PARP in HL-60 cells. HL-60 cells (2×10^6/3 ml/well) exposed to CSF at indicated concentrations for 24 h were incubated with FITC mouse anti-cleaved PARP antibody (20 μl, 30 min) in the dark as described in Material and Methods. Analysis of cleaved PARP was done using a FACS Calibur. Data are representative of one of three similar experiments.

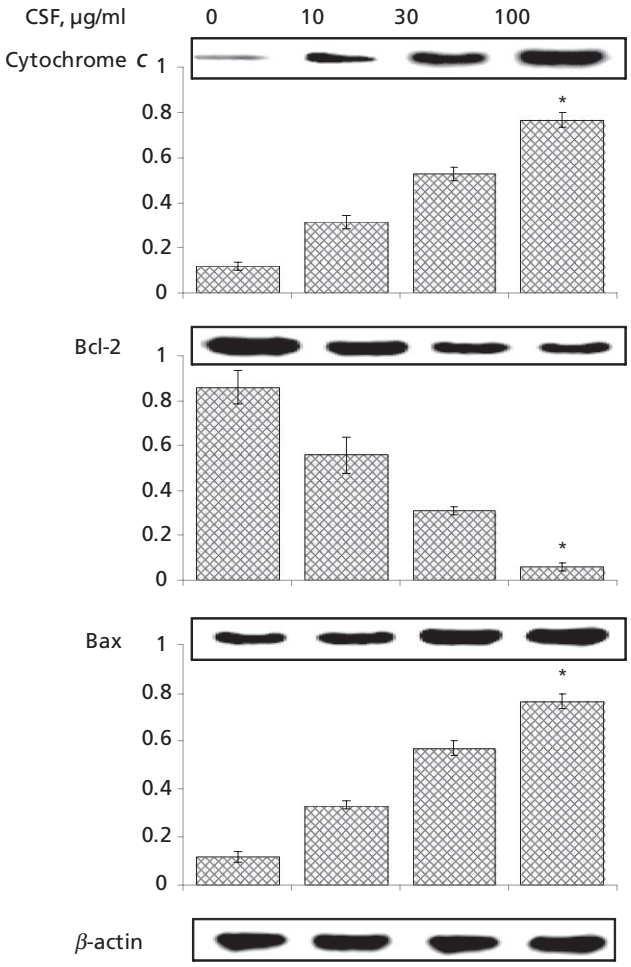


Figure 8 Influence of n-butanol fraction of *Carissa spinarum* stem (CSF) on the expression of important proteins involved in the initiation of apoptosis. HL-60 cells (2×10^6/2 ml/well) were treated with indicated concentrations of CSF for 24 h and cytosolic and mitochondrial fractions were prepared and subjected to Western blot. β-Actin was used as internal control to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of cytochrome c, Bcl-2 and Bax. Relative density of each band for cytochrome c, Bcl-2 and Bax indicates arbitrary units of expression analysed by Quantity One software of Bio-Rad gel documentation system. Data are mean ± SEM from three similar experiments compared with CSF untreated group, i.e. control. $^*P < 0.05$.

DNA, which is one of the later irreversible events during apoptosis.[32] Furthermore, it was speculative to find out the primary targets affected by CSF.

It is quite well-known that mitochondria play a decisive role in the intrinsic apoptotic pathway.[33] Specifically, different pro-apoptotic proteins, such as cytochrome c, which are normally present in the intermembrane space of these organelles, are released during the early stages of apoptosis.[34,35] Permeabilization of the outer mitochondrial membrane and oxidative stress induced by free radicals allow the release of proapoptotic molecules, which results in the activation of downstream caspases, caspase-3 and in particular, caspase-9.[36] Caspases act either as initiator or executioner within the apoptotic process. Executioner caspases are activated from their pro-enzymatic form by the action of other caspases within a cascade reaction.[37] The elevation in the activity of caspase-3, caspase-6 and caspase-9 as observed in this study suggests that apoptosis of cancer cells induced by CSF is mediated through activation of these proteases. In addition, the elevated level of caspase-3 could utilize PARP, a DNA repair enzyme, as its substrate leading to its degradation. As such, PARP cleavage induced by CSF was observed in our study, which directed DNA-damaged cells to undergo apoptosis. Furthermore, the activated caspases might target the permeabilized mitochondria resulting in the corresponding loss of mitochondrial membrane potential ($\Delta\psi_{mt}$) concomitant with increased production of ROS and eventual disruption of membrane integrity. In this study, CSF-treated HL-60 cells exhibited a shift in mitochondrial membrane potential across the membrane possibly induced by generation of observed reactive oxygen intermediates. In particular, the loss of mitochondrial membrane potential is facilitated through opening of mitochondrial permeability transition pores (PTP) largely due to Bax translocation, which in turn conduct the leakage of cytochrome c and pro-apoptotic proteins from mitochondria to the cytosol.[14] Interestingly, the release of caspase-activating proteins during early apoptosis is regulated primarily by the Bcl-2 family of proteins. Among these, degradation of anti-apoptotic protein Bcl-2 and overexpression of pro-apoptotic protein Bax observed in this study induce the release of cytochrome c from the mitochondria, which is an important event during apoptosis mediated by the mitochondrial pathway. Overall, it was observed in this study that apoptosis in HL-60 cells treated with CSF was executed by the release of cytochrome c from the mitochondria, activation of caspases, reduction of mitochondrial membrane potential induced by ROS generation, degradation of Bcl-2 and enhancement of Bax expression. The results of this study strongly support further research and development of the bioactive constituents from *C. spinarum* as potential anticancer agents with possible therapeutic implication. Since *C. spinarum* produces different sesquiterpenes, cardiac glycosides, flavonoids like naringenin and triterpene acids such as ursolic acid, a detailed phytochemical investigation is going on to identify the active principle of *C. spinarum* for further development.

Conclusion

This study lead us to conclude that the *C. spinarum* aqueous extract (CSE) and its n-butanol fraction (CSF) exhibit a potential cytotoxic effect on a wide range of human cancer cell lines, with apoptotic activity of CSF in human leukaemia HL-60 cells. CSF was able to induce loss of mitochondrial membrane potential due to generation of ROS, which causes release of cytochrome c from the mitochondria induced by Bcl-2 degradation and translocation of Bax, which further activate procaspase-9 to caspase-9 and the executioner caspase-3 that leads to PARP cleavage and finally DNA fragmentation. So we can say that CSF induced apoptosis through the intrinsic or mitochondrial-dependent apoptotic pathway in HL-60 cells and proved a promising candidate for developing into a potential anti-cancer therapeutic.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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