Lantadene A-induced apoptosis in human leukemia HL-60 cells

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ABSTRACT

Objectives: Lantadene A (LA, 22β-angeloyloxy-3-oxoolean-12-en-28-oic acid) a pentacyclic, triterpenoid isolated from the leaves of the obnoxious weed, *Lantana camara* L. was evaluated for apoptosis induction in the human leukemia HL-60 cell line.

Materials and Methods: The effect of LA on cell proliferation of HL-60 cancer cells was determined by using the MTT assay. The morphological effects of LA-treated HL-60 cancer cells were observed under a fluorescence microscope. DNA fragmentation was observed using gel electrophoresis. Flow cytometry was carried out to observe changes in the cell cycle distribution of the cells. The expression of Bcl-2 and Bax proteins in HL-60 cells was visualized by means of an immunohistochemical assay and cell viability was determined upon treatment with DEVD-CHO (inhibitor of caspase-3) and LA.

Results: Typical morphological changes including cell shrinkage, chromatin condensation and characteristic DNA ladder formation in agarose gel electrophoresis were observed. LA-induced marked concentration- and time-dependent inhibition of cancer cell proliferation with an IC_{50} value of 19.8 ± 0.10 µg/ml following 48 h incubation. Flow cytometric analysis showed suppressed cell proliferation associated with cell cycle arrest in the G_0/G_1 phase. LA significantly inhibited cell proliferation of HL-60 cells and induced cell apoptosis by downregulating Bcl-2 and upregulating Bax expression. The peptidic caspase-3 inhibitor, DEVD-CHO (NH_2-Asp-Glu-Val-Asp-CHO, 2 µM), increased the viability of HL-60 cells, which had been previously treated with LA.

Conclusions: The results indicated that LA induces efficient cell apoptosis by activating the caspase-3 pathway and through down- and upregulation of Bcl-2 and Bax expression respectively.

**KEY WORDS:** Bcl-2/Bax, caspase-3, DEVD-CHO, pentacyclic triterpenoid

Introduction

Apoptosis or programmed cell death is an essential event that plays an important role in the homeostasis and development of an organism. A tumor is a disease state characterized by uncontrolled proliferation and absence of apoptosis.[1-5] The potential mechanism for a cell to undergo apoptosis exists in a balance between its induction and inhibition factors. Bax, an apoptosis induction factor, Bcl-2 an apoptosis inhibition factor[6] and the caspase family, especially caspase-8, -9 and -3, all play important roles in the apoptotic response. When a cell receives sufficient pro-apoptotic stimuli or lacks anti-apoptotic stimuli, the effector caspase is activated. During apoptosis, the cell experiences a cascade of events that ultimately result in nucleus condensation and DNA fragmentation. Thus, induction of apoptosis is an efficient method of treating cancer.[7]

Triterpenoids represent a diverse class of natural products. Recently, pentacyclic triterpenoids have been described to induce apoptosis in different cell types.[8] *Lantana camara* L. is one of the most noxious weeds in the world, which grows wild in tropical and subtropical parts of the world.[9] Its wild growth provides a huge amount of biomass and currently there is a lot of interest to exploit its natural products in drug research.[10] We have earlier reported the chemopreventive effect of the methanolic extract of *Lantana camara* L. on 7,12-dimethylbenz[a]anthracene (DMBA)-induced squamous cell carcinogenesis in Swiss albino mice.[11] Lantadene A (LA, 22β-angeloyloxy-3-oxo-olean-12-en-28-oic acid) [Figure 1] is the most abundant pentacyclic triterpenoid (0.7% on dry weight basis) in the *Lantana camara* var. *aculeate* (Red).[12] LA has been reported to demonstrate inhibition of Epstein-Barr virus activation in Raje cells induced by 12-*O*- tetradecanoylphorbol-13 acetate (TPA) and possesses tumor inhibitory activity in a two-stage carcinogenesis model in mice.[13,14] However, the molecular mechanism responsible for its tumor inhibitory potential is not well understood. Thus, in this study, we investigate the growth inhibitory effect of LA and the underlying mechanism using HL-60 cells.

Figure 1: Structure of L antadene A
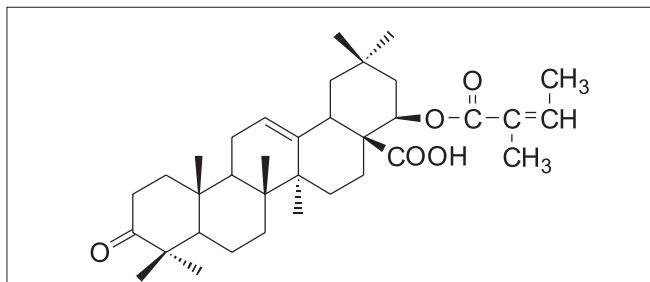
Materials and Methods

Extraction and isolation of LA

The leaves of *Lantana camara* were collected in September from Palampur (HP), India. The leaves were dried in the shade and powdered. A voucher specimen was deposited in the Herbarium in the University Institute of Pharmaceutical Sciences, Panjab University. To 100 g of lantana leaf powder, 500 ml methanol was added and incubated for 24 h with intermittent shaking. The extract was separated by filtration through a muslin cloth and decolorized with 20 g of activated charcoal, which yielded a golden yellow extract. The solvent was removed under reduced pressure, the residue was suspended in a methanol-water (1:7) mixture and extracted with chloroform ($CHCl_3$, 2×15 ml). The organic layer was dried over anhydrous sodium sulphate and the solvent was removed under reduced pressure. The solid residue obtained was recrystallized from methanol to obtain partially purified lantadenes (1.06 g, 1.06%) as a white crystalline product. For isolation of LA, the partially purified lantadene fraction (1.0 g) was chromatographed over a silica gel column (30 g, 60-120 mesh) using chloroform and chloroform-methanol (99.5:0.5) as the eluting solvent. The LA-enriched fractions were pooled, the solvent was removed *in vacuo* and the resulting solid residue was recrystallized twice from methanol to obtain pure LA (0.45 g) as white crystals, melting point 283°C (285–288°C).[15]

IR (KBr): 2960 (aliphatic C-H stretch), 1715 (C=O, 3-keto), 1677 (C=O, C-22 ester).

^{1}H NMR ($CDCl_3$) δ: 5.98 (1 H, dd, $J = 7.1$; 1.2 Hz, 3' H), 5.38 (1 H, t, $J = 3.5$ Hz, C-12 H), 4.97 (1 H, t, $J = 3$Hz, C-22 H), 3.07 (1 H, dd, $J = 14.2$; 4.0 Hz, C-12 H), 1.96 (3 H, dd, $J = 7.1$; 1.6 Hz, 4' CH_3), 1.76 (3 H, d, $J = 1.6$ Hz, 5' CH_3), 1.18, 1.05, 1.04, 1.02, 1.00, 0.90, 0.86 ($CH_3 \times 7$).

^{13}C NMR ($CDCl_3$) δ: 38.41 (C-1), 34.11 (C-2), 217.66 (C-3), 47.42 (C-4), 55.29 (C-5), 19.59 (C-6), 32.17 (C-7), 39.21 (C-8), 47.73 (C-9), 36.76 (C-10), 23.50 (C-11), 122.46 (C-12), 143.10 (C-13), 41.97 (C-14), 27.57 (C-15), 24.18 (C-16), 50.60 (C-17), 38.41 (C-18), 46.87 (C-19), 30.03 (C-20), 37.71 (C-21), 75.88 (C-22), 26.44 (C-23), 21.45 (C-24), 15.09 (C-25), 16.83 (C-26), 25.79 (C-27), 180.10 (C-28), 33.67 (C-29), 26.13 (C-30), 166.26 (C-1'), 127.61 (C-2'), 138.88 (C-3'), 20.56 (C-4'), 15.64 (C-5').

Cells and cell culture

HL-60 cancer cells obtained from the National Center for Cell Science (NCCS, Pune India) were maintained in their logarithmic phase of growth in RPMI 1640 medium (Gibco, Grand Island), supplemented with heat-inactivated 10% fetal bovine serum (Gibco), 100 kU/L benzylpenicillin, 100 mg/L streptomycin and 2 mM L-glutamine (Sigma) in humidified air with 5% CO_2. Stock solutions of LA were made in dimethylsulfoxide (DMSO) (Sigma, USA) and solutions of different concentrations were obtained by diluting with culture medium. The final concentration of DMSO was less than 0.1% in all experiments.

MTT assay and cell viability assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is a common method used to assess cell proliferation and cytotoxicity. The MTT assay was carried out as previously described.[16] Briefly, 1×10^4 exponentially growing cells were seeded per well in 96 well plates and exposed to various concentrations of LA. After incubation with LA for a certain period of time, MTT (Sigma) was added to achieve a final concentration of 0.125 mg/ml and incubated at 37°C for 4 h. The precipitated formazan salt was dissolved in DMSO and the plate samples were read at 570 nm with an ELISA microplate reader (Bio-tek). The 50% inhibitory concentration (IC_{50}) of LA was calculated using the NDST software. Each experiment was preformed in triplicate. The effect of LA on the growth curve of HL-60 cancer cells was determined by means of the Trypan Blue exclusion technique. Briefly, 3×10^4 cells cells were seeded per well in a 24 well plate and then treated with various concentrations of LA. The plate was incubated at 37°C and the number of cultured cells in the different wells was counted using a hemocytometer after staining with 0.4% Trypan Blue every 24 h to calculate the doubling time.

Morphological analysis

Cell morphology of LA-induced apoptosis was investigated by staining the cells with a combination of fluorescent DNA-binding dyes-acridine orange (AO) and ethidium bromide (EB). Briefly, cells were harvested and washed with PBS after being exposed to different concentrations of LA for 24 h. After staining with 100 µg/ml AO/EB for 5 min, the cells were observed under a fluorescence microscope (Olympus).

DNA fragmentation analysis

HL-60 cells were incubated with 0, 5, 10, 15, 20 and 25 µg/ml of LA for 24 h at 37°C. DNA fragmentation was analyzed by electrophoresis as described earlier.[17] Briefly, after exposure to trypsin, the cells (10^7 cells per sample) were washed with Tris-buffered saline (TBS) buffer (pH 7.6) and collected by centrifugation at 1000 *g* for 10 minutes. The pellet was resuspended for 2 h at 50°C in a lysing solution made up of 10 mM Tris-HCl (500 µL; pH 8.0), 150 mM NaCl, 10 mM ethylenediamine tetraacetic acid (EDTA, edetic acid), 0.4% sodium dodecyl sulfate (SDS) and 100 µg/ml proteinase K. The lysate was then extracted with equal volumes of phenol/ $CHCl_3$/ isoamyl alcohol (25:24:01). The DNA was precipitated with ethanol (EtOH), air-dried and dissolved in TE buffer (5 mM Tris-HCl (pH 8.0) and 20 mM edetic acid containing RNase A [0.1 mg/ml; Sigma]). The samples were run in agarose gel containing ethidium bromide (0.5 µg/ml) and were visualized under ultraviolet (UV) light.

Flow cytometric analysis

The HL-60 cancer cells exposed to LA at different concentrations were collected and fixed in 70% EtOH. Quantitative detection of apoptotic cells and analysis of cell-cycle distribution in cultures were performed with FACScan flow

cytometer (Becton Dickinson) using Cell Quest software. The data was finally analyzed with the Modfit 3.0 DNA software. The proliferation index was calculated according to formula:

$$\text{Proliferation index} = \frac{(S + G_2/G)}{G_1 + S + G_2/M} \times 100$$

where S = DNA synthesis phase, G_1 = Gap 1 phase, G_2 = Gap 2 phase, M = Mitosis phase.

Each experiment was performed at least five times.

Immunohistochemical analysis

The expression of Bcl-2 and Bax proteins after 24 h of exposure to different concentrations of LA was visualized in HL-60 cancer cells with the aid of an immunohistochemical assay kit (Zymed Laboratories Inc, San Francisco, CA). The cultured cell sections were fixed with 4% paraformaldehyde and endogenous peroxidase activity was blocked with H_2O_2 and normal goat serum. The sections were incubated with rabbit anti-human Bcl-2 or Bax polyclonal antibodies, biotinylated goat anti-rabbit IgG and avidin-biotin peroxidase complex in that order. The sections were stained with DAB (diaminobenzidine tetrahydrochloride) and observed under light microscope. Quantitative analysis was conducted with the *td*2000 pathology cell image analysis system (Alpha Innotech Corp San Leandro, CA) in five areas of each slide.

Determination of cell viability upon treatment with DEVD-CHO and LA

The viability of HL-60 cells treated with DEVD-CHO (NH_2-Asp-Glu-Val-Asp-CHO) and LA was evaluated using the MTT assay. After incubation with DEVD-CHO (at 0 and 2 μM) for 1 h, cells were treated with various concentrations of LA for 48 hafter which viability was determined.

Statistical analysis

Data was expressed as mean ± standard deviation (SD) and examined for statistical significance of differences with student's *t*-test, *P* values of < 0.05 being considered statistically significant.

Results

Effect of LA on cell proliferation

The effect of LA on the proliferation of HL-60 cancer cells was determined by using the MTT assay. LA significantly inhibits the growth of HL-60 cancer cells in a concentration-dependent manner [Figure 2]. The IC_{50} value of LA was 19.8 ± 0.10 μg/ml after 48 h incubation. The effects of LA on the growth curve of

HL-60 cells were characterized by staining with Trypan Blue. As shown in Table 1, LA induced marked concentration- and time-dependent inhibition of HL-60 cell proliferation and significantly prolonged the doubling time (T_D) in a concentration-dependent manner.

Effect of LA on the morphology of cells

Apoptosis is characterized by distinct morphological features such as cell shrinkage, chromatin condensation, oligonucleosomal DNA fragmentation and finally, breakdown of the cell into smaller units (apoptotic bodies). To determine the morphological effects, LA-treated HL-60 cancer cells were observed under a fluorescence microscope. Uniform green cells with normal morphology were seen in the control group [Figure 3a], whereas orange cells with fragmented chromatin and apoptotic bodies were seen when cells were treated with LA [Figure 3b]. The results suggest that LA was able to induce marked apoptotic morphological changes in HL-60 cells.

Effect of LA on DNA fragmentation

DNA was isolated from the HL-60 cancer cells cultured in the presence of LA at various concentrations for 24 h. The characteristic 'ladder' pattern of apoptosis was observed at 25 μg/ml of LA. A comparison with molecular weight markers indicated that the fragments were multiples consisting of *ca.* 100 base pairs [Figure 4].

Effect of LA on cell cycle

Figure 2: Inhibitory effect of LA on proliferation of HL-60 cells. The growth inhibition was calculated as % of inhibition relative to control using the MTT assay. Data is presented as mean ± SD (n = 4)
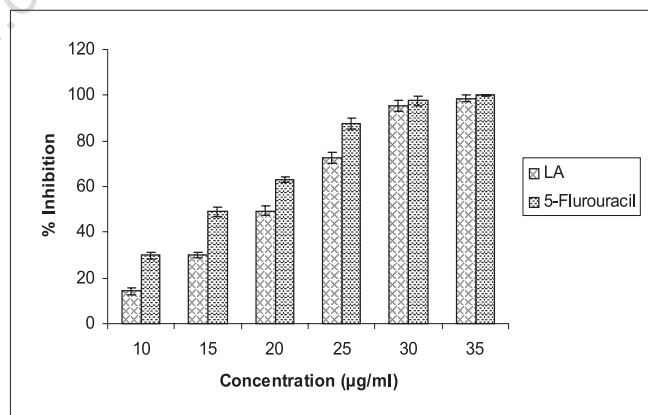

Table 1

Effect of Lantadene A on HL-60 cell growth using the Trypan blue exclusion assay

Concentration LA	Cell number [x 10⁵]			Doubling Time [T_D, h]
(μg/ml)	24h	48h	72h	
Control	1.50 ± 0.27	3.68 ± 0.31	7.50 ± 0.77	29.04 ± 1.30
10	1.68 ± 0.22	3.65 ± 0.33	6.38 ± 0.89[a]	30.87 ± 0.78
15	1.35 ± 0.21	3.07 ± 0.24[b]	5.32 ± 0.57[b]	35.47 ± 1.38[b]
20	1.31 ± 0.19	2.46 ± 0.22[b]	3.13 ± 0.32[b]	51.24 ± 3.87[b]
25	1.12 ± 0.26[c]	1.58 ± 0.22[b]	1.94 ± 0.19[b]	88.37 ± 8.10[b]
30	0.92 ± 0.15[b]	1.30 ± 0.11[b]	1.40 ± 0.14[b]	131.33 ± 13.3[b]

Values in mean ± SD, [a]Significant differences relative to control (< 0.05); *n* = 4, [b]Significant differences relative to control (< 0.001); *n* = 4, [c]Significant difference relative to control was < 0.01; *n* = 4

Figure 3: Fluorescence microscopy of HL-60 cells treated with LA and stained with 100 mg/ml mixture of acridine orange-ethidium bromide for 5 minutes. a) viable cells with green nuclear fluorescence; b) apoptotic cells containing an orange nucleus and exhibiting chromatin condensation. Magnification x200
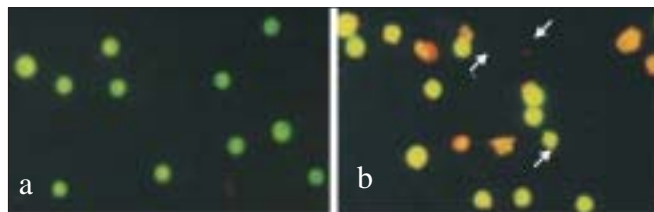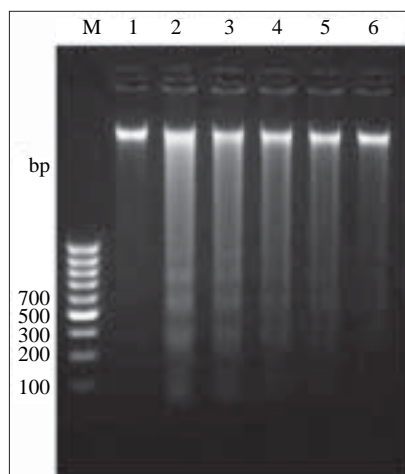

Figure 4: Dose-dependent induction of DNA fragmentation by LA in HL-60 cells visualized by agarose gel electrophoresis. Lane M: DNA marker; Lane 1: Control cells; Lanes 2, 3, 4, 5 and 6: cells treated with 25, 20, 15, 10 and 5 µg/ml of LA respectively.

HL-60 cancer cells were treated with LA at 10, 20 and 30 µg/ml for 24 h and flow cytometric analysis was carried out. There were obvious changes in cell cycle distribution characterized by an increase and decrease in the numbers of cells in the G_0/G_1 as well as S and G_2/M phases respectively in a dose-dependent manner. This indicates that LA suppresses cell proliferation by causing cell cycle arrest in the G_0/G_1 phase [Table 2].

Effect of LA on expression of Bcl-2 and Bax

The proteins Bcl-2 and Bax have been reported to inhibit and enhance apoptosis respectively. To examine the role of Bcl-2 family genes in apoptosis induced by LA, the expression of Bcl-2 and Bax was evaluated in HL-60 cells using the streptavidin-biotin complex (SABC) immunohistochemical method. As shown in Table 3, the expression of Bcl-2 in HL-60 cancer cells treated with LA decreases while the expression of Bax increases. The ratio of Bcl-2 to Bax was found to decrease with increasing concentrations of LA due to the pro-apoptotic effects of LA.

Effect of Caspase-3 inhibitor on the viability of LA-treated cells

Caspase-3 plays a critical role in apoptosis and its activity has been suggested as an index of apoptosis. After incubation with cell-permeable DEVD-CHO (NH_2-Asp-Glu-Val-Asp-CHO) at 2 µM for 1 h, HL-60 cells were exposed to different concentrations of LA. After 48 h, their viability was determined using the MTT assay. As shown in Figure 5, DEVD-CHO significantly increased the viability of cells treated with LA.

Discussion

Lantadene A (LA) is a novel pentacyclic triterpenoid, which was found to inhibit 12-*O*- tetradecanoylphorbol-13 acetate (TPA)-induced Epstein-Barr virus activation in Raje cells and possess tumor inhibitory activity in a two-stage carcinogenesis model in mice. The mechanism of its antitumor activity is still unknown. A reduction in cell growth and induction in cell death are two major ways to inhibit tumor growth.[18] In this study, it was observed that LA induces a marked concentration- and time-dependent inhibition of HL-60 cell proliferation with an IC_{50} value of 19.8 ± 0.10 µg/ml after 48 h incubation. LA induced typical apoptotic characteristics in HL-60 cells such as cell shrinkage, chromatin condensation, plasma membrane blebbing, oligonucleosomal DNA fragmentation and finally breakdown of the cells into smaller units (apoptotic bodies).[19] In addition, apoptotic typical DNA strand-breaks were observed by means of gel electrophoresis. Flow cytometric analysis showed that LA suppressed HL-60 cell proliferation by causing cell cycle arrest in the G_0/G_1 phase.

The proteins Bcl-2 and Bax have been reported to regulate the process of apoptosis. The pro- and anti-apoptotic members of the Bcl-2 family act as rheostats in regulating programmed cell death and are therefore suitable targets for anticancer therapy. *Bcl-2* is an anti-apoptotic gene, regulating the apoptotic pathway and preventing cell death. In contrast, *Bax* is a pro-apoptotic gene, expressed abundantly and selectively during apoptosis-promoted cell death.[20] The Bcl-2 protein encoded by the *Bcl-2* proto-oncogene predominantly localized on the outer mitochondrial membrane, plays a central role in the regulation of cell proliferation and apoptosis. Bax, a related homologue of Bcl-2 is localized in the cytosol and forms heterodimers with Bcl-2, thus effectively antagonizing the Bcl-2 function

Table 2

Effect of Lantadene A on cell cycle and proliferation index in HL-60 cells after 24 h of exposure by flow cytometric analysis

Concentration LA (µg/ml)	Cell cycle %			Proliferation index [%]
	G_0/G_1	S	G_2/M	
0	63.13 ± 1.64	30.46 ± 1.24	6.55 ± 0.67	38.54 ± 1.64
10	73.60 ± 1.04[a]	20.73 ± 1.28[a]	5.70 ± 0.71	26.42 ± 1.03[b]
20	78.05 ± 0.71[a]	7.55 ± 0.35[a]	4.40 ± 0.42[b]	21.95 ± 0.07[b]
30	84.67 ± 1.42[a]	12.77 ± 0.42[a]	0.62 ± 0.78[a]	15.08 ± 1.22[a]

Values in mean ± SD, [a]Significant difference relative to control was < 0.001; *n* = 5, [b]Significant difference relative to control was < 0.01; *n* = 5.

Figure 5: Effect of pretreatment with DEVD-CHO for 1 h on the viability of HL-60 cells treated with LA determined by using the MTT assay. The values are presented as mean ± SD (n =5). Significant differences relative to control were designated as b (< 0.01) and c (< 0.001)
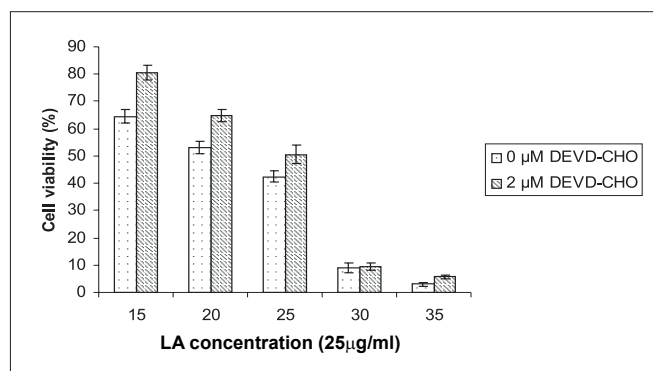

Table 3

Effect of LA on the expression of Bcl-2 and Bax proteins in HL-60 cells after 48 h of exposure using an immunohistochemical assay

Concentration LA (μg/ml)	Bcl-2 [%]	Bax [%]	Bcl-2/Bax ratio
Control	45.7 ± 5.18	21.04 ± 4.16	2.17
10	34.51 ± 4.28[a]	26.22 ± 6.22	1.32
20	22.86 ± 2.54[b]	30.56 ± 4.81[a]	0.75
30	19.63 ± 2.80[b]	44.58 ± 4.25[b]	0.44

Values in mean ± SD, [a]Significant difference relative to control was < 0.05; n = 5, [b]Significant difference relative to control was < 0.001; n = 5.

to promote apoptosis. The Bax/Bcl-2 ratio in a cell acts to regulate the cell's susceptibility to apoptosis. In the present study, it was observed that the expression of Bcl-2 in LA-treated HL-60 cancer cells is decreased while the expression of Bax is increased. The ratio of Bcl-2 to Bax was found to decrease significantly due to the pro-apoptotic effects of LA.

At the execution phase of apoptosis, a series of morphological and biochemical changes appear to result from the actions of cysteine-dependent, aspartate-directed proteases such as caspases. Caspases play an important role in apoptosis triggered by various proapoptotic signals. Caspase-3, in particular, is probably one of the most commonly involved enzymes associated with the execution of apoptosis in various cell types.[21] DEVD-CHO is a highly specific and potent caspase-3 inhibitor. It was observed that pretreatment with DEVD-CHO blocked the LA-induced inhibition of HL-60 cell proliferation, which suggests that the activation of caspase-3 is necessary for apoptosis induced by LA.

p53 is another important factor that affects cell response to drug effects on growth inhibition and apoptosis induction. HL-60 cells lack functional p53 protein. Thus, it appears that in this study, LA triggers apoptosis in a p53-independent manner.

Conclusion

The present study indicates that LA significantly inhibits cell proliferation of HL-60 cancer cells and induces cell apoptosis by downregulating Bcl-2 and upregulating Bax expression and activating the caspase-3 pathway. Therefore, it may be concluded that LA has the potential to be developed as a novel chemotherapeutic agent for cancer. This strategy will also help to utilize the prolific, obnoxious weed *Lantana camara* L. as a resource for drug development.

Acknowledgment

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