Original Article

Cytotoxic and Antitumor Activity of the Extract of *Clerodendron infortunatum*: A Mechanistic Study

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ABSTRACT

Objective: To investigate the molecular mechanism of anti tumor activity of *Clerodendron infortunatum* extract (CIE) under *in vitro* condition.
Materials and Methods: The cytotoxic activity of CIE extract was tested on murine tumor cells (Dalton's lymphoma ascites cells - DLA) and normal mouse cells (splenocytes) by trypan blue dye exclusion assay. The ability of the extract to induce apoptosis was measured via acridine orange and propidium iodide dual staining method. Apoptosis induction was confirmed by the analysis of level of expression of various apoptotic genes like *bax*, *bcl-2*, *caspase 8* and *caspase 9*.
Results: The extract showed a dose dependent anti-proliferative activity on DLA cells whereas mouse splenocytes were found to be not affected. Treatment of DLA cells with CIE increased the number of apoptotic events in DLA cells. Increase in the *bax*/*bcl-2* ratio along with an escalation in the expression of *caspase 9* by almost 60 fold confirmed the induction of intrinsic pathway of apoptosis.
Conclusions: CIE showed effective anti-proliferative activity on DLA cells by the induction of intrinsic pathway of apoptosis.

Keywords: *Clerodendron infortunatum,* Dalton's lymphoma ascites cells, Apoptosis, *bax*/*bcl-2* ratio.

INTRODUCTION

Cancer is the second largest common disease and a major Global health burden[1,2]. It was estimated that, worldwide there were 10.9 million new cancer cases, 6.7 million deaths due to cancer, and 24.6 million persons living with cancer in 2012[3-5]. Development of cancer is a multistage process due to the accumulation of mutagenic alterations occurring over a span of years resulting in uncontrolled cell growth and division. These genetic

alterations are sometime carried in the germline[6].

Plants have been used as medication for cancer[7]. Over 60% of the currently used anti-cancer agents are derived from natural sources[8] - plants, marine organisms and micro-organisms[9]. Many of the commonly used anti neoplastic agents such as taxol, vinblastine, vincristine and camptothecin are derived from plants. Though the discovery of these natural compounds and their derivatives have facilitated in our combat against various types of cancers, there is still a vast repertoire of phytochemicals useful for treating cancer, are yet to be identified and characterized. Hence initial research focuses on screening the ability of various plant extracts for their potential cytotoxic and anti-proliferative property[10]. One of the major advantage of plant derived drugs is the absence of side effects, which is often found associated with synthetic drugs in the market[11].

Clerodendron, is a very large and diverse genus, widely distributed in Asia, Australia, Africa and America[10]. The plants of this genus have been used by tribal people in colic, scorpion string, snake bite, tumor and certain skin diseases, also used in Indian folk medicine for the treatment of bronchitis, asthma, fever, diseases of the blood, inflammation, burning sensation and epilepsy[12]. The roots of the plant has laxative, diuretic, analgesic, anti-inflammatory, anti-tumor and antibacterial activities[13,14].

Quercetin is a polyphenol which has recently gained considerable attention due to its prophylacytic and therapeutic properties in the prevention of cardiovascular diseases, cancer, cataract etc[15]. Various parts of *C. infortunatum* contain quercetin, having the highest concentration in the flowers, followed by leaves and then roots[16]. The present work aims to explore the antitumor activity of hydro-alcoholic extract of *C. infortunatum* on the murine tumor cells. The extract induced mortality and apoptosis in murine tumor, Dalton's Lymphoma Ascites (DLA), cells, while causing minimal cytotoxicity and no apoptosis induction in mouse splenocytes. The levels of expression of genes involved in apoptotic pathways were studied by quantitative real time PCR in the tumor cells to understand the mechanism.

MATERIALS AND METHODS

Cells

DLA cell line was obtained from Amala Cancer Research Centre (Thrissur, India) and was propagated as transplantable ascites tumors in male Swiss albino mice. Cells were withdrawn freshly from mouse peritoneum, washed with PBS and counted using hemocytometer. Six million cells per milliliter of DMEM (dulbeco's modified Eagle's Medium) with 10% FBS were used for further studies.

Preparation of hydro-alcoholic extract of Clerodendron infortunatum

Dried roots of *Clerodendron infortunatum* were finely powdered. The powder was weighed and subjected to soxhlet extraction at 70^{o}C with 50% ethyl alcohol. The extract was evaporated in a rotary evaporator at 50^{o}C under vaccum and finally, the extract was subjected for lyophilization to get a dry crude extract with a yield of 12% of the powder. This hydro-alcoholic extract thus obtained was termed as CIE and stored at 4^{o}C.

HPLC

The extract was subjected for HPLC analysis using Agilent - Model No. 1260 System. An aqueous solution of CIE, 10 mg/ml, was prepared and filtered through a 0.2 µm filter and 20 µL was injected into HPLC equipped with PAD (Pixel Array Detector) detector and SunFire- C 18,

5 µm column. 1 mg/ml standard quercetin was prepared and 20 µl was injected. The solvents used for gradient elution were (A) Acetonitrile and (B) HPLC grade water. Detection Wavelength was 280 nm. The percentage of quercetin in the CIE was also calculated.

Cytotoxicity of Clerodendron infortunatum on DLA and mouse spleenolytes

Suspensions of DLA cells and mouse splenocytes, in DMEM with 10% fetal calf serum, at (6×10^6 cells/ml) were treated with five different concentrations of CIE. The cells were incubated for 20 hours after addition of the extract at 5% CO_2 and 37°C. CIE induced cytotoxicity was checked up to 20 hours using trypan blue dye exclusion assay. Briefly, 80 µl of cell suspension was mixed with 80 µl of 0.2% trypan blue, kept for 2-3 minutes and loaded on a haemocytometer. The no. of stained and unstained cells was counted separately. More than 100 cells of each type were counted. The percentage survival of cells were calculated and plotted against concentration of drug.

% of survival = (Live cell count/total cell count) ×100.

Induction of apoptosis on DLA by Clerodendron infortunatum

Suspensions of DLA cells (6×10^6 cells/ml) in DMEM with 10% fetal calf serum, were treated with five different concentrations of CIE (0 – 500 µg/ml). The cells were incubated for 16 hours after addition of the extract with 5% CO_2 and at 37°C. CIE induced apoptosis was checked on 16 hours using Acridine Orange (AO) and Propodium Iodide (PI) Double Staining, described in detail elsewhere[17].

The cells were pelleted by centrifugation (200 x g; 10 mm) and 80 µl of cell-free supernatant was removed. One microliter of a mixture of 100 µg/ml acridine orange and 100 µg/ml ethidium bromide (both prepared in isotonic saline) was added to the cell pellet, the cells were resuspended, and 10 µl of the cell suspension was placed on a clean, ordinary microscope slide and covered with a 22-mm square cover slip. The number of live and dead cells with normal vs. apoptotic nuclei was determined by UV microscopy using a 40 X dry objective and a filter combination suitable for observing fluorescence. Viable and apoptotic cells were quantified in a population of 200 cells. The results were expressed as a proportion of the total number of the cells examined. Percentage of apoptosis was calculated with the formula given below.

% of apoptosis = (number of apoptotic cells/total number of cells) × 100.

Expression of various apoptotic genes in DLA cells treated with CIE

RNA extraction

Total RNA from the cells treated with different concentrations of CIE for 16 hours were isolated by the method developed by Chomczynski and Sacchi[18] which is a modification of Acid Guanidium Thiocyanate-Phenol-Chloroform Extraction. The isolated total RNA was converted to cDNA.

Preparation of complementary DNA

Reverse transcription of isolated RNA and PCR were done using Applied Biosystems Geneamp Thermal Cycler 2720. cDNA was synthesized as per the manufacturers protocol. Briefly, mRNA isolated from DLA was mixed with random hexamer for priming at 65°C for 5 minutes. This was mixed with the master mix containing dNTPs, DTT, reverse transcriptase and RNase inhibitor. The condition for cDNA synthesis was 50°C for 1 hour followed by inactivation at 95°C for 5 minutes.

Analysis of levels of expression of *bax* and *bcl-2*

The levels of *bax* and *bcl-2* is used as a marker for identify the induction of apoptosis in cells. The cDNA was subjected to PCR to find out whether there was any change in the expression of *bax* and *bcl-2* while gapdh was used as the house keeping control. The forward and reverse primers for PCR were 5'- AAG GGC TCA TGA CCA CAG TC-3' and 5'-TGT GAG GGA GAT GCT CAG TG-3' for *gapdh*, 5'-CTC GTC GCT ACC GTC GTG ACT TCG-3'and 5'-CAG ATG CCG GTT CAG GTA CTC AGT C-3' for *bcl-2* and 5'-AAG CTG AGC GAG TGT CTC CGG CG-3' and 5'-GCC ACA AAG ATG GTC ACT GTC TGC C-3' for *bax* respectively. The cycling conditions of *bcl-2* and *bax* is as follows,

94ºC for 1minute (denaturation)
64ºC for 1 minute (annealing)
72°C for1 minute (extension)
} 35cycles

For the housekeeping gene gapdh the cycling condition is as given below,

95ºC for 10 minutes (denaturation)
56ºC for 30 seconds (annealing)
72ºC for 59 seconds (extension)
} 35cycles

The final amplicons were electrophoresed in a 2% agarose gel containing 2 mg of ethidium bromide and the gel was visualized under a gel documentation system. With the software provided with the gel documentation system, the thickness of the bands were analyzed and expressed as a change in expression with respect to the untreated control.

Quantitative polymerase chain reaction (qPCR)

The cDNA prepared were subjected to qPCR for checking the levels of expression of various genes like *bax, bcl-2, caspase-8* and *caspase-9*. *gapdh* was used as the house keeping control. The levels of expression of various genes were expressed as relative fold change in expression in comparison with the control group.

Real-time PCR was done on an AB Applied Biosystem Step One Plus Real-time PCR System. The forward and reverse primer pairs for detecting the expression of *bax* were 5'-TGC TAC AGG GTT TCA TCC AG-3'and 5'-CAC GTC AGC AAT CAT CCT CT-3'. PCR primers for *caspase 8* were forward 5'- GAT GTT GGA GGA AGG CAA TC-3' and reverse 5'-ATT CCA ACT CGC TCA CTT CT-3'. The primer pairs for gene *caspase 9* is forward 5'-TGA CAT CCT TGT GTC CTA CTC-3' and reverse 5'- CCA GGA ATC TGC TTG TAA GTC-3'

The forward and reverse primers for the gene *bcl-2* were 5'-AGG ATT GTG GCC TTC TTT GA-3' and 5'ATG CTG GGG CCA TAT AGT TC-3'and for *gapdh* were forward 5'-GCG AGA CCC CAC TAA CAT C-3' and reverse 5'- GAG TTG TCA TAT TTC TCG TGG T-3'. The cycling conditions for all these genes were as provided below.

95ºC for 15 seconds (denaturation)
53ºC for 1 minute (annealing)
72°C for 30 seconds (extension)
} 45cycles

The quantitative real-time data is presented as comparative C_T method also known as the $2^{-\Delta\Delta CT}$ method[19]. The comparative C_T method makes several assumptions, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene[20]. The advantage of this method of presentation is that data can be represented as fold change.

Fold change = $2^{-\Delta\Delta CT}$

$2^{-\Delta\Delta CT}$ = [(C_T gene of interest - C_T internal control) Sample A – (C_T gene of interest - C_T internal control) Sample B].

The data obtained were presented as a histogram denoting the fold change of expression of the genes of interest compared with the house keeping gene.

RESULTS

High pressure liquid chromatography analysis of CIE

The HPLC Chromatogram of Hydro-alcoholic CIE and the reference standard Quercetin are presented in figure **1a** and **figure 1b**.

The HPLC chromatogram of reference standard Quercetin showed a peak of 44.507 minutes with an area of 16424464 while Hydro-alcoholic CIE showed a corresponding peak of 44.827with an area of 975343. 0.59% of quercetin was present in CIE extract.

Cytotoxicity of CIE on DLA cells

The viable cells were counted in a hemocytometer. Upon treatment with various concentrations of CIE (50 - 500µg/ml) the number viable DLA cells was found to decrease in a concentration dependent as well as time dependent manner. Almost all the cells in the treatment group with the highest extract concentration were found to be dead at 19th hour (**Supplementary figure 1**). CIE did not exhibit much toxicity on splenocytes. At 20 hours of incubation, splenocytes treated with the highest concentration of CIE showed 94.9% survival. This shows that, CIE at this concentration did not exert much toxicity to normal cells. The comparative data of survival percentage of DLA cells and splenocytes at 20 hr of CIE treatment is presented in **figure 2**.

Induction of Apoptosis by CIE

The percentage of apoptosis was acquired on 16th hour of incubation on DLA cells with various concentration of CIE and the data is presented as apoptotic index in **figure 3**. Apoptotic index was observed to increase with the increase in concentration of CIE treatment, with the lowest concentration of CIE (50 µg/ml) showing an apoptotic index of 17% while the highest concentration, 500µg/ml induced 58% of the DLA cell to undergo apoptosis.

Levels of expressions of *bax* and *bcl-2*

The expression levels of the anti-apoptotic gene *bcl-2* and pro-apototic gene *bax* in DLA cells treated with various concentration of CIE were assayed to confirm the induction of apoptosis. In many systems, members of the *bcl-2* family modulate apoptosis. In the present experiment, treatment of DLA cells with various concentrations of CIE shifted the ratio favoring the induction of apoptotic pathway as can be evident from the data presented in **figure 4**. The expression levels of *bcl-2, bax* and the house keeping gene *gapdh* upon 16 hour of application of the extract have been shown in **supplementary figure 2**.

Relative expression levels of various apoptotic genes on DLA cells treated with various concentrations of CIE

Upon treating DLA cells with various concentrations of CIE (50-500µg/ml), a relative change in expression of the anti-apoptotic gene *bcl-2* and pro-apototic gene bax were observed. *Bax* was found to be increased in concentration dependent manner (**figure 5a**). This confirmed the induction of apoptosis.

The pathway with which apoptosis progress can be discerned by analyzing the expression of Caspase-8 and Caspase-9 which corresponds to extrinsic and intrinsic pathways. From **figure 5b**, it can be observed that expression of caspase 9 increased dramatically corresponding to the activation of intrinsic pathway. *Gapdh* served as the house keeping control in both the experiments.

DISCUSSION

Apoptosis and necrosis are the two pathways through which anticancer drugs induce cell death[21]. Anticancer agents at

lower concentration cause apoptosis while higher cause necrosis[22]. The anticancer agents inflict injuries and damages to cancer cells resulting in the activation of the pro-apoptotic factors to induce apoptosis. The cells escape apoptosis by developing resistance to anticancer agents[23].

Apoptosis or programmed cell death is an orchestrated mechanism of cell death which ensures neat and ordered elimination of unwanted cells and cells with damage from normal tissue[24]. There are 2 commonly described initiation pathways of apoptosis – the extrinsic pathway and the intrinsic pathway. Intrinsic pathway is triggered by irreparable genetic damage, hypoxia, higher concentration of cytosolic Ca^{2+}, severe oxidative stress etc[25]. As a result of these death signals, pro apoptotic multi-domain Bcl-2 family proteins such as Bax and Bak form oligomer in mitochondrial outer membrane which confers the release of cytochrome C and other pro apoptotic molecules into cytoplasm[26]. Cytochrome C triggers the activation of initiation Caspase 9 which in turn activates downstream executioner caspases such as effector caspases - caspase-3, 6 and 7[27]. These effector caspases cleave nuclear lammins, cytoskeletal proteins and inhibitor of DNase leading to cell death. This culminates in the formation of apoptotic bodies which are engulfed and eliminated by nearby and phagocytic cells[28]. The extrinsic pathway of apoptosis is activated by tumor necrosis factor (TNF) which triggers a downstream process culminating in the activation of pro-caspase 8, which activates downstream effector and executioner caspases 3 and 7[29]. Apoptosis induction in cells can be confirmed by an increase in the ratio of Bax to Bcl-2[30,31].

Several studies established that some phytochemicals present in medicinal plants exert anti-tumorigenic activity by inducing apoptosis[32]. Treatment of DLA cells with CIE induced apoptosis. This was primarily established visually by the typical morphological changes a cell undergoes during apoptosis, including chromatin condensation and membrane blebbing[33]. The apoptosis induction was confirmed by the increased *bax/bcl2* ratio[34].

To identify the pathway by which apoptosis occured, the expression levels of caspase 8 and 9 were studied. Caspase 8 activation occurs during extrinsic pathway, when the death signal comes from outside the cell. Intrinsic pathway is characterized by the activation of caspase 9. In the present study, DLA cells treated with CIE showed a significant increase in the expression of caspase 9 confirming the activation of intrinsic pathway.

The trypan blue dye exclusion assay is used for evaluating the viability of cells. The cellular damage is due to membrane lipid peroxidation and protein denaturation by extracellular or intracellular insult mediated through toxic substances[35]. CIE showed specific toxicity towards DLA cells clearly indicating its specificity on rapidly dividing cells suggestive of its anti-proliferative activity.

Quercitin has been shown to possess anti-proliferative and cytotoxic activity on various cell lines[36]. The mechanism involved for these activities have not yet been properly elucidated. The hydro-alcoholic extract also showed presence of various other compounds which may also be involved in exerting cytotoxicity to DLA cells. The present study showed that apoptosis induction is a major pathway by which CIE induced cytotoxicity in DLA cells. The presence of such phytochemicals in the extract could be the reason for such an activity. It is also interesting to note that toxicity was confined only to rapidly dividing cancer cells. The induction of toxicity can be due to the effect of a single compound or a group of compounds in the extract. Isolation, identification, and purification of these

compounds and elucidation of their biological activities would be a more rewarding and fruitful endeavor.

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REFERENCES

1. Kainsa S, Kumar P, Rani P. Medicinal Plants of Asian Origin Having Anticancer Potential: Short Review. *Asian J Biomed and Pharma Sci*. 2012; 2: 1-7.
2. Ma X, Yu H. Global Burden of Cancer. *Yale J Biol Med*. 2006; 79: 85-94.
3. Parkin MD, Bray F, Frelay J, Pisani P. Global Cancer Statistics, 2002. *CA-Cancer J. Clin*. 2005; 55: 74-108.
4. Dervan PA. Understanding Cancer. Jefferson, NC: McFarland, 1999.
5. Hartwell JL. Plants used against cancer: a survey. Lawrence, MA. Quarterman Publications, 1982, 438-439.
6. Devitta V, Hellman S, Roseberg SA (1993) In: Cancer: Principle and Practice of Oncology. J.B. lippincot Company, Philadelphia, USA.
7. Koshland D, Kent JC, Hartwell LH. Genetic analysis of the mitotic transmission of minichromosomes. *Cell*. 1985; 40: 393-402.
8. Privitera G, Napoli E, Luca T, Ruberto G, Castorina S. *In vitro* Anti-Proliferative Effect of Salvia officinalis Essential Oil and its Three Main Components on Human Lung Cancer Cells. *AJPCT*. 2014; 2: 1159-1168.
9. Ramesh KS. A Review: Biomedical Compounds and Anticancer drugs from Marine and Tropical Herbal medicine. *International Journal of Chemistry and Pharmaceutical Sciences*. 2014; 2: 997-1006.
10. Valiyari S,Baradaran B, Delazar A, Pasdaran A, Zare F. Dichloromethane and methanol extracts of scrophularia oxysepala induces apoptosis in MCF-7 human breast cancer cells. *Drugs*. 2012; 15: 223-231.
11. Namian P, Talebi T, Germi K G, Shabani F. Screening of Biological Activities (Antioxidant, Antibacterial and Antitumor) of Nerium oleander Leaf and Flower Extracts. *AJPCT*. 2013; 1: 378-384.
12. Shrivastava N, Patel T. Clerodendrum and Heathcare: An Overview. *Medicinal Aromatic Plant Sci. Biotech*. 2007; 1: 142-150.
13. Khatry N, Kundu J, Bachar SC, Uddin MS and Kundu JK. Studies on Antinociceptive, Antiinflammatory and Diuretic Activities of Methanol Extract of the Aerial Parts of Clerodendron viscosum Vent. *Dhaka Univ. J. Pharm. Sci*. 2005; 5: 63-66.
14. Das S, Haldar PK, Pramanik G, Bala A, Kar B. Anticancer Activity of Clerodendron infortunatum Linn. *Extract in Swiss Albino Mice Asian Journal of Chemistry*. 2010; 22: 6387-6392.
15. Kutty NAA, Mathews SM, Leena PM.Physical, Phytochemical Screening and Antibacterial Activities of Clerodendron infortunatum Linn Root. *Int J Pharm Bio Sci*. 2011; 2: 182-187.
16. Gupta S, Gupta R. Detection and quantification of quercetin in roots, leaves and flowers of Clerodendrum infortunatum L. *Asian Pac J Trop Dis*. 2012; 2 (2), 940-943.
17. Duke, R. C., and Cohen, J. J. (1992) Morphological and biochemical assays of apotosis. In Current Protoco in Immunology (Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, eds) pp. 3.17.1-3.17.16, John Wiley and Sons, New York.
18. Chomczynski P, Sacchi N.The single-step method of RNA isolation by acidguanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. *Nat Protoc*. 2006; 1: 581–585.
19. Schmittgen T D, Livak K J. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*. 2008; 3: 1101–1108
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. *Methods*. 2001; 25: 402–408.
21. Sandeep D, Ramasamy S, Krishnan R, Karunagaran D, Alsuhaibani E, Nair C K K. Evaluation of Cytotoxic and Anti-Tumor

Activities of Coscinium fenestratum Extract. *AJPCT*. 2014; 2: 1346-1360.
22. Zong WX, Thompson CB. Necrotic death as a cell fate. Genes Dev. 2006; 20: 1-15.
23. Inoue S, Salah-Eldin AE, Omoteyama K. Apoptosis and anticancer drug resistance. *Hum Cell*. 2001; 14: 211-221.
24. Vassiliki Nikoletopoulou, Maria Markaki, Konstantinos Palikaras, Nektarios Tavernarakis. Crosstalk between apoptosis, necrosis and autophagy. *Biochimica et Biophysica Acta*. 2013; 1833: 3448–3459
25. Karper G. Cell and molecular biology. 6th ed. United States of America: Wiley & Sons; 2006. 1-1819.
26. Cooper GM, Hausman ER. The Cell- A Molecular Approach. 2nd ed. Boston: Sinuaer Associates. 2013; 1-832.
27. Flink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect Immun*. 2005; 73: 1907-1916.
28. Erwig LP, Henson PM. Clearance of apoptotic cells by phagocytes. *Cell Death Differ*. 2008; 15: 243-250.
29. Joaquín H. Patarroyo S. and Marlene I. Vargas V (2013). Apoptosis and Activation-Induced Cell Death, Apoptosis, Dr. Justine Rudner (Ed.), ISBN: 978-953-51-1133-7, InTech, DOI: 10.5772/52805.
30. Oltvai Z, Milliman C and Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conversed homolog, Bax, that accelerates programmed cell death. *Cell*. 1993; 74: 609-619.
31. Korsmeyer SJ. Bcl-2 gene family and the regulation of programmed cell death. *Cancer Res*. 1999; 59: S1693-S1700.
32. Peng, Lei , Liu, An , Shen, Yue , Xu, Hua-Zi , Yang, Shi-Zhou , Ying, Xiao-Zhou , Liao, Wei , Liu, Hai-Xiao , Lin, Zhong-Qin , Chen, Qing-Yu , Cheng, Shao-Wen , Shen, Wei-Dong "Antitumor and anti-angiogenesis effects of thymoquinone on osteosarcoma through the NF-κB pathway". Oncology Reports 29.2 (2013): 571-578.
33. Georg Häcker. The morphology of apoptosis. *Cell Tissue Res*. 2000; 301: 5-17.
34. Yi X, Yin XM, Dong Z. Inhibition of Bid-induced apoptosis by Bcl-2. tBid insertion, bax translocation, and bax/bak oligomerization suppressed. *Journal of Biological Chemistry*. 2003; 278: 16992-16999.
35. Stadtman ER. Protein Oxidation in Aging and Age-Related Diseases. *Ann. N. Y. Acad. Sci*. 2001; 928: 22–38.
36. Durgo K, Halec I, Sola I, Franekić J. Cytotoxic and genotoxic effects of the quercetin/lanthanum complex on human cervical carcinoma cells *in vitro*. *Arh Hig Rada Toksikol*. 2011; 62: 221-227.

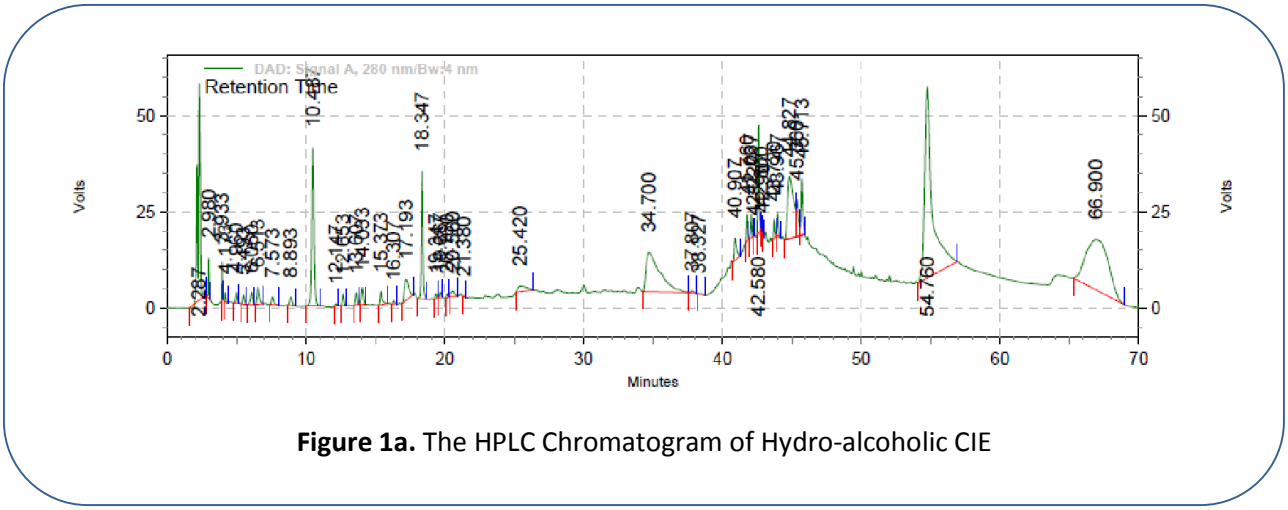

Figure 1a. The HPLC Chromatogram of Hydro-alcoholic CIE

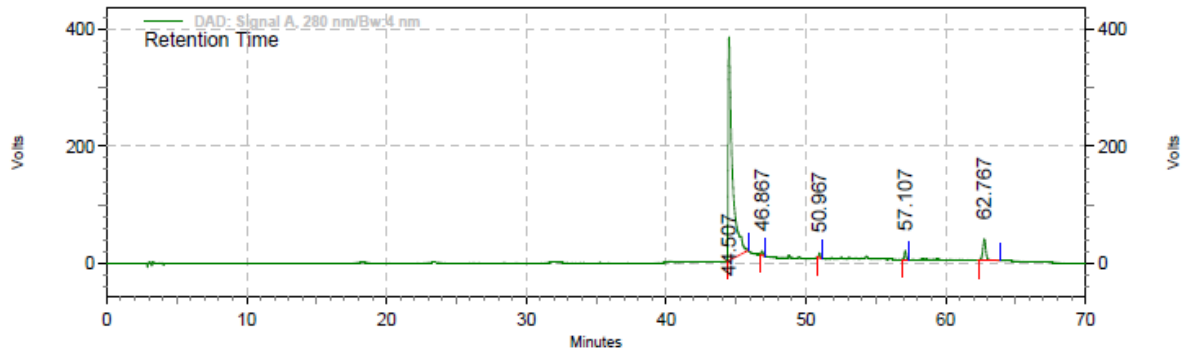

Figure 1b. The HPLC Chromatogram of reference standard Quercetin

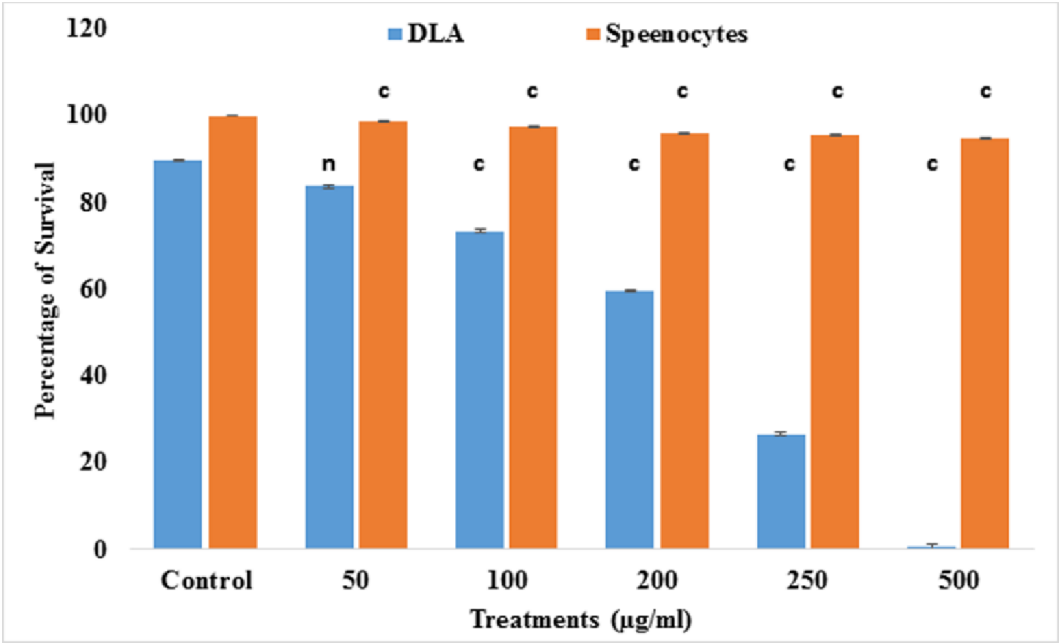

Figure 2. Percentage survival of DLA cells and mouse splenocytes treated with CIE for 20 hours

Note: 'a' indicates $p<0.05$, 'b' indicates $p>0.001$, 'c' indicates $p<0.001$ when compared with respective control.

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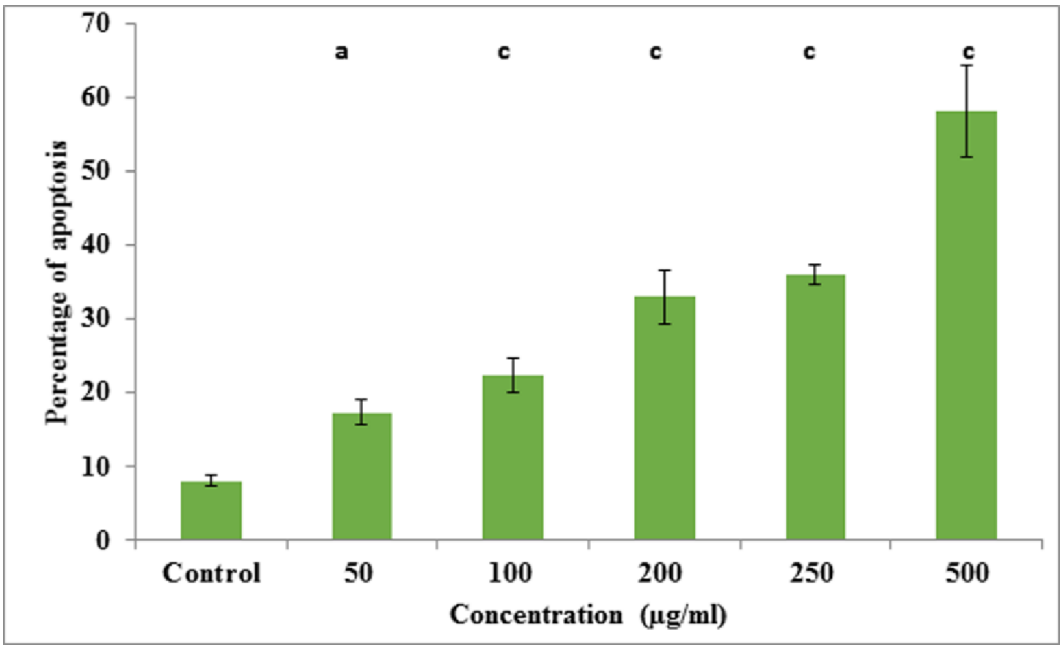

Figure 3. Effect of different concentration of CIE on induction of apoptosis on DLA cells presented as percentage of apoptotic index

Note: 'a' indicates $p<0.05$, 'c' indicates $p<0.001$ when compared with respective control.

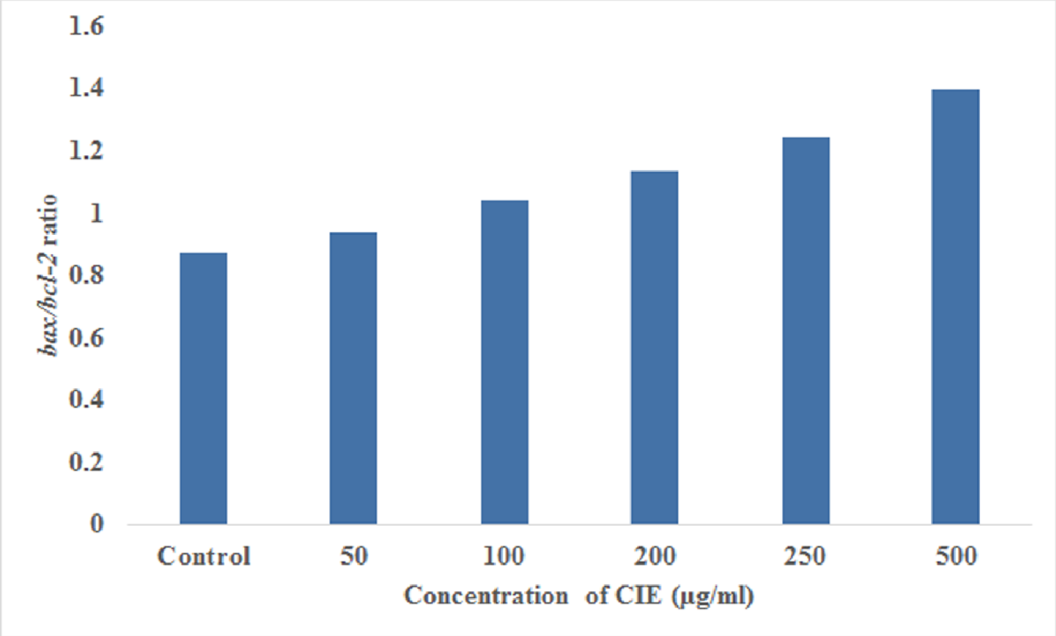

Figure 4. *bax/bcl-2* ratio of DLA cells treated with various doses of CIE (0-500 µg/ml). The ratios were calculated densitometric scans of the PCR amplicons of the respective genes

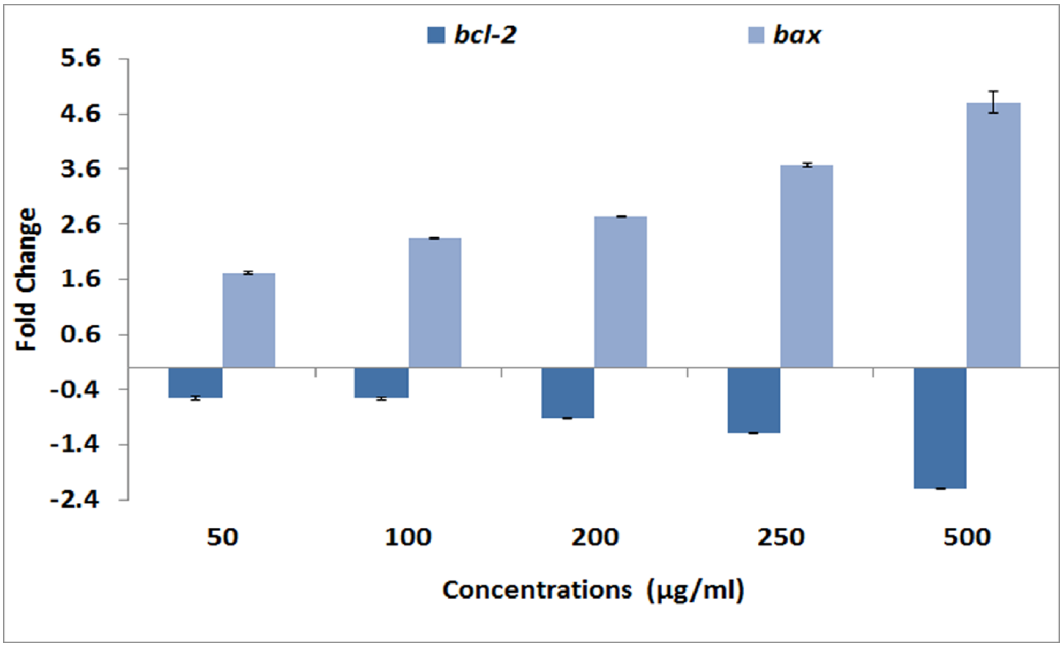

Figure 5a. The relative fold change of various apoptotic genes (*bax and bcl-2*) in DLA cells treated with various concentration of CIE (50- 500 µg/ml)

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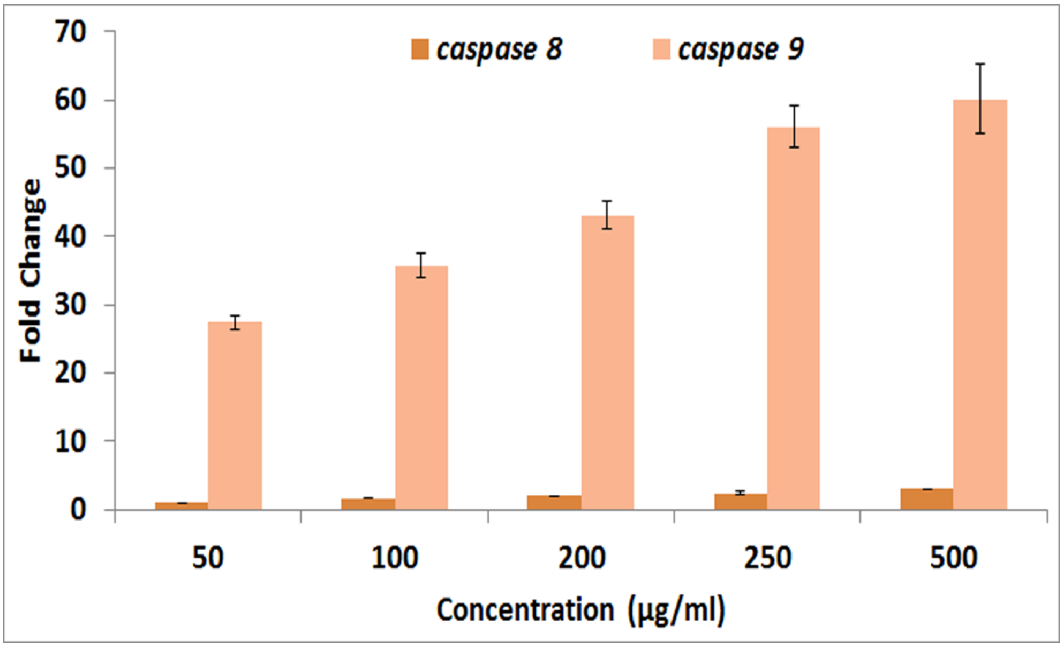

Figure 5b. The relative fold change of various apoptotic genes (*Caspase-8 and Caspase-9*) in DLA cells treated with various concentration of CIE (50-500 µg/ml)

SUPPLEMENTARY FIGURES:

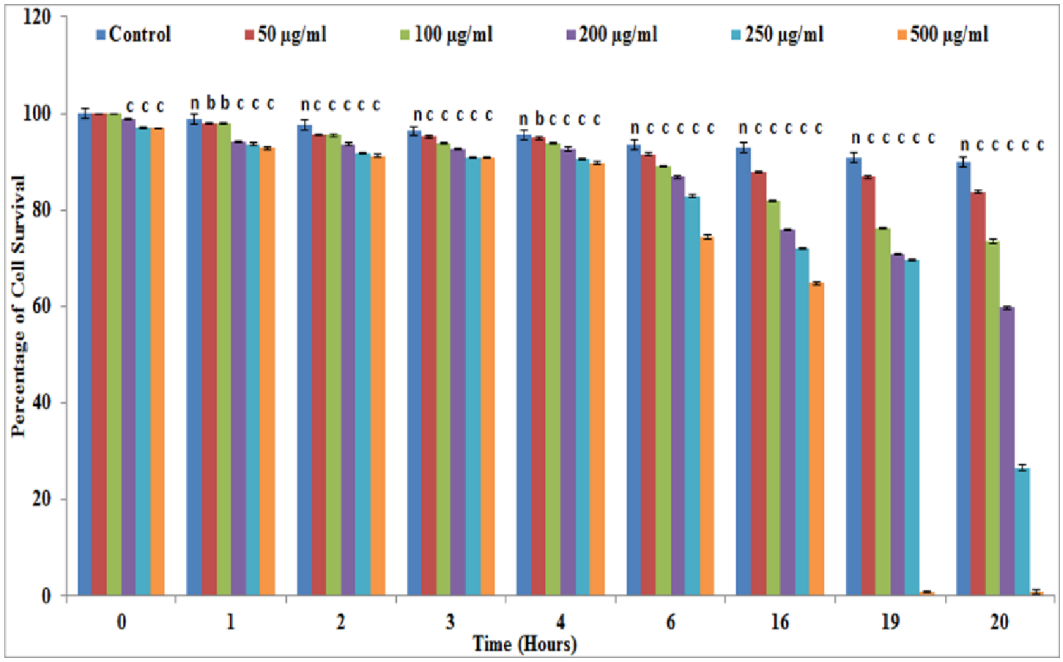

Figure 1. Percentage survival of DLA cells treated with *Clerodendron infortunatum* extract

Note: 'a' indicates $p<0.05$, 'b' indicates $p>0.001$, 'c' indicates $p<0.001$ when compared with respective control.

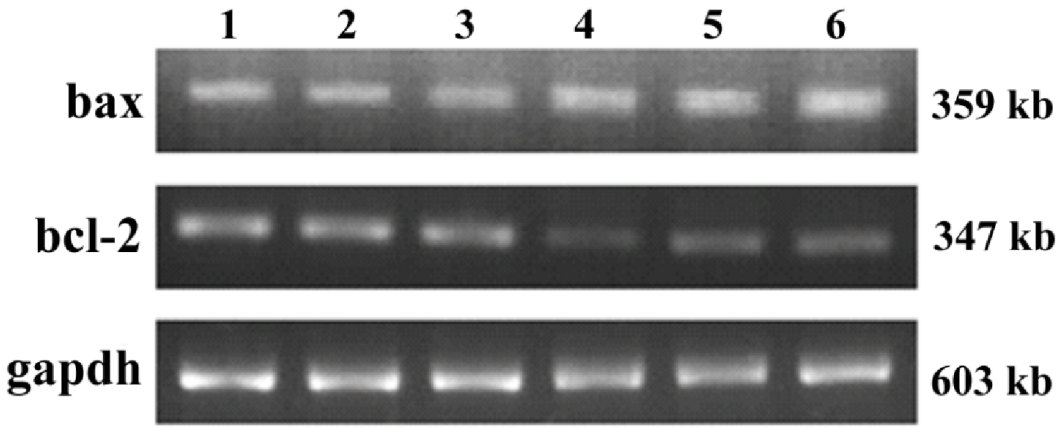

Figure 2. Electrophoretic separation of RT–PCR samples. Lane 1: PCR product from control DLA cells for *bcl2, bax* and *gapdh.* Lane 2. 3, 4, 5 and 6 for 50, 100, 200, 250, and 500 µg/ml *Clerodendron infortunatum* treated cells respectively