Caspase Pathway Activation and Reactive Oxygen Species Generation in Apoptotic Cell Death of Human Leukemic U937 and K562 Cell Line in Response to King Cobra (*Ophiophagus hannah*) Venom

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Abstract: Resistance and decreasing efficacy of current synthetic drug for chemotherapy of leukemic cancer draws attention for development of newer anticancer agent from natural resources. In the present study, king cobra venom (OHV) significantly inhibited leukemic cell growth in dose and time dependent manner. For U937 and K562 cell line, the IC_{50} dose (72 h) was found to be 4.1 µg/ml and 3.9 µg/ml respectively, observed by trypan blue exclusion method and tetrazolium bromide reduction assay. OHV treated morphometry of leukemic cell showed the characteristic features of apoptosis. Both U937 and K562 cells were arrested in the G1 phase of cell cycle with most cells exhibiting the biochemical feature of early and late apoptosis. Mitochondrial membrane potential was lost and reactive oxygen species generated highly in OHV treated leukemic cell line (U937 and K562). Western blot analysis showed OHV increased expression of Bax and decreased expression of Bcl2 in OHV treated cell as compared to untreated control U937 and K562 cell. Upregulation of Cytochrome c, Bid, Bad, Caspase 3/8/9, p21 and NF-κB down regulation of Cyclin D1, CDK4 was also showed by western blot analysis which revealed the possible pathway of OHV in cellular level. The results of this study demonstrated that OHV significantly and selectively induced leukemic cell death through both extrinsic and intrinsic apoptotic pathway.

Keywords: *Ophiophagus hannah*, Venom, Leukemic cell, Apoptosis, Flow cytometry, Cell cycle.

1. INTRODUCTION

Cancer is the major public health concern in all developed and developing countries. In year 2012 a total of 1,638,910 new cancer cases and 577,190 deaths from cancer are estimated [1]. There is urgent need of find better treatment as now cancer becomes the second leading cause of death worldwide. In recent years remarkable progress has been made towards the understanding of molecular pathway of cancer development and hormonal therapy. Various therapies have been used for treating cancer e.g. radiotherapy, immunotherapy, gene therapy and chemotherapy [2]. In these days, predominant option of cancer prevention is the use of chemotherapeutic drug. However, one of the major problems with cancer therapy using chemotherapeutics are that patients often do not respond or eventually resistance is developed after initial treatment, high toxicity and high cost. This has led to the increased use of anticancer agents developed from natural resources. The biodiversity of venoms or toxins of animal, plant, fungal and bacteria made it a unique tool from which new therapeutic agents may be developed. Currently, Snake venom has been shown to possess a wide spectrum of biological activities [3].

From past few decades, research on isolation and characterization of anticancer agents from the snake venom has been undertaken. The antimetastatic activities of crude venom of Indian monocellate cobra (*Naja kaouthia*), Indian cobra (*Naja naja*) Russell's viper (*Vipera russelli*) and Banded krait (*Bungarus fasciatus*) were studied on carcinoma, sarcoma and leukemia models [4-6]. Now we focussed on anticancer activity of Indian king cobra's (*Ophiophagus hannah*) crude venom.

The king cobra of the elapidae family is the world's largest venomous snake [7]. It is known that king cobra snake is extremely poisonous and have strong neurotoxic action [8]. Recently, Lee *et al*. isolated a heat stable form of L-amino acid oxidase from king cobra venom having antibacterial activity [9]. L-amino acid oxidase (LAAO) isolated from King cobra (Ophiophagus hannah) venom, is an extremely potent

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antiproliferative agent against cancer cells. L-amino acid oxidase (OH-LAAO), a heat stable enzyme, has been shown to exhibit very potent anti-proliferative activity against human breast and lung tumorigenic cells but not in their non-tumorigenic counterparts [10, 11]. Previously from our lab, a novel fibrinolytic peptide, Hannahpep was isolated from the Indian king cobra venom [12]. The present investigation reports the antileukemic activity of crude venom of Indian king cobra against two human leukemic cell lines (K562 and U937) and molecular action of this venom to understand its signalling pathway.

2. MATERIALS AND METHODS

2.1. Chemicals

3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), Sodium dodecyl sulphate (SDS), Sodium bi carbonate ($NaHCO_3$), Ethidium bromide, RNase A, Propium Iodide, Acridine Orange, Trypan Blue, Lactate dehydrogenase, De- Methoxy sulphoxide (DMSO) were purchased from Sigma- Aldrich co. (St. Louis, MO, USA). RPMI1640 medium, fetal bovine serum, penicillium-streptomycin was purchased from invitrogen, (USA). Annexin V-FITC, Cell cycle kit, FITC-BrdU kit, Ki-67 anti human antibody kits were purchased from BD-Bioscience, USA. Primary antibody (Bax, Bcl2, Caspase 8, Caspase 9, Cytochrome C, Bid, Bad, Cyclin D, CDK4, p21, NF-κB3 and β-actin) and HRP-conjugated goat anti-rabbit secondary antibody were procured from Santa Cruz, CA, USA All other chemicals were purchased locally and were of analytical grade. Lyophilized *O. hannah* crude venom (OHV) was purchased from the Calcutta snake park, Kolkata, India.

2.2. Cell Culture

U937 (human leukemic lymphoma cell line) and K562 (human myelogenous leukemic cell line) were purchased from National facility for Animal Tissue and cell culture, Pune India. Cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, glucose (1.5g/L), $NaHCO_3$ (1.5g/L), Penicillin (100 units) and streptomycin (10µg/ml). At 95% humidified atmosphere and 5% CO_2 cells were grown to confluence at 37^oC in inside an incubator (Heal Force, China).

2.3. Determination of IC_{50} and Cytotoxicity Studies on Leukemic Cells

The 50% of inhibition concentration (IC_{50}) values of OHV in U937 and K562 cells were determined using different concentration (1, 2, 5 and 10 µg/ml) of the crude venom at 24 h, 48 h and 72 h. The respective viable cells were counted in the phase contrast microscope using Trypan blue [13]. The respective concentration of venom and the percentage of cell death were plotted on the x axis and the y axis of the graph respectively. For cytotoxicty test, MTT assay of treated cells were done to confirm its cytotoxic effect [14]. 40 µl of MTT solution (5 mg/ml) was added 4 h prior to end of 24h, 48 h and 72 h of incubation. The violet colour formazan granules formed by viable cells were dissolved in DMSO and the absorbance at 570 nm was estimated by measuring with the plate reader (BioTek, ELx800).

2.4. Detection of Apoptosis

2.4.1. Observation under Florescence Microscope

1x 10^6 cells of U937 and K562 cell were treated with OHV (IC_{50} dose of respect to two cell lines) for 24 hour and observed using a fluorescence microscope for changes of morphometry. After harvesting, the untreated control cells and the OHV treated cells washed with PBS and then stained with Acridine orange (100 µg/ml) and Ethidium bromide (100 µg/ml) (1:1) [15, 16]. With mounting on slides, the cells were then immediately observed under fluorescence microscope (Motic Image plus) for determination of the cell apoptosis.

2.4.2. Flow Cytometric Analysis

Flow cytometric analysis was done by performing dot plot assay to investigate the type of cell death induced by OHV [17, 18]. The leukemic cells (1 x 10^6) were treated with IC_{50} (for respective cell) dose for 24 h. The cells were pelleted down and washed with Annexin V-FITC binding buffer. After washing, cell pellets were dissolved in Annexin V-FITC binding buffer containing annexin V-FITC and Propidium iodide. With 15 min incubation in dark at room temperature flow cytometric analysis was done. All data were acquired with a Becton-Dickinson FACS Verse double laser cytometer.

2.5. Lactate Dehydrogenase (LDH) Activity Assay

Plasma membrane integrity loss was indicated by release of LDH from the cytoplasm of cultured cells into the medium. Culture medium was collected after OHV treatment and cell debris was removed by centrifugation. LDH activity was measured using a commercial LDH assay kit according to manufacturer's protocol (Sigma –Aldrich, USA) [17]. Briefly, the

samples were read on a spectrophotometer at the absorbance of 440 nm. LDH activity was calculated according to a standard curve and expressed in U/L.

2.6. Study of Cell Cycle by Flow Cytometric Analysis

1x 10^6 leukemic cells were treated with respective IC_{50} dose of OHV for 24 h. Cells were washed with PBS, fixed with ice-cold 70% ethanol. After pelleted down, cells were washed twice with cold PBS and finally dissolved in PBS treated with RNase A for 30 min at 37°C and stained with propium iodide (20 µl from 50 µg/ml) and kept in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector [19, 20, 21].

2.7. Estimation of BrdU Incorporation Using FACS

Exponentially growing U937 and k562 cells (1 x 10^6) were cultured on RPMI medium containing 10µl BrdU (10µg/ml) [22, 23]. After 1 h, cells were treated with respective IC_{50} dose of OHV and kept at CO_2 incubator for 24 h. Cells were fixed with permealization buffer for 20 min. After washing again with PBS, Dnase (5 µg/ml) was added and incubated for 1 h at 37°C. Anti Brdu antibody was added to it and kept at dark for 20 min after PBS washing. Samples were analysed with FACS after incubated with 7-ADD for 15 min at dark condition.

2.8. Reactive Oxygen Species (ROS) Assay

The method was obtained from the report of Condello *et al*. [24]. The H_2DCF-DA is the probe that could quantify the amount of intracellular ROS. For the assay, the leukemic cell lines (U937 and K562) were seeded at 1x 10^6cells/well in the 6 –well plate and cells were treated with IC_{50} dose of OHV for respective cell for 24 h. Cells were incubated with 5 µM H2DCF-DA for 30 min at 37°C, and then cells were washed twice with PBS and resuspended in PBS. After centrifugation at 5000 rpm for 10 min, the fluorescence of the supernatant intensity was analysed at an excitation wavelength of 480 nm and an emission wavelength of 540 nm by a Spectrofluorometer (Bio-Rad, USA) [25].

2.9. Effect of OHV on Caspase 3 Activity

U937 and K562 cells from OHV treated plate were collected after 24 h incubation. Cell lysate were prepared by BD cell lysis buffer (BD Bioscience). Caspase 3 activity was performed according to manufacture protocol by spectrofluorometer (Bio-Rad, USA) [26].

2.10. Western Blot Analysis of Pro and Anti-Apoptotic Protein Expression of Leukemic Cells

OHV treated U937 and K562 cell lysate of were prepared by cell lysis buffer. Equivalent amount of proteins were electrophoresed in 10% SDS-PAGE and transferred to a PVDF membrane which was then blocked with 5% skimmed milk in PBS-T. Incubation with primary antibodies of Bax, Bcl2, caspase 8/9, Cytochrome C, Bid, Bad, Cyclin D1, p21, CDK4, NF-κB3 and β-actin were done followed by subsequent wash with PBS-T (phosphate buffer saline with 0.1% Tween 20). Then membrane was incubated with horse-redox peroxidase conjugated goat antirabbit secondary antibody followed by a detection reagent (Amersham Bioscience) [19, 27].

2.11. Separation and Culture of Normal Lymphocytes

After taking informed consent (Ref. No. IHEC/AG/ HUM/P17/12), blood was collected aseptically from the vein of healthy adults and transferred to heparinised vial. Using Ficoll-histopaque, lymphocytes were collected from whole blood [16]. Then it was cultured in sterile complete RPMI 1640 media. 1x 10^6 cells were treated with sterile PBS, OHV (twice IC_{50} dose of U937 cell) and Imatinib mesylate (standard drug – 100 µg/ml) and cells were grown in a CO_2 incubator at 37° C with 5% CO_2 for 72 h in humidified condition.

2.12. Statistical Analysis

All values are represented as arithmetic mean ± SEM. Statistical analysis was done by student's t test. A probability value of *P≤ 0.05 was chosen as the criterion of statistical significance.

3. RESULTS

3.1. IC_{50} Value of OHV in U937 and K562 Cell Line

After 72 h, IC_{50} value of OHV in U937 cells was 4.2 µg/ml at 95% of fiducial probalities the confidence intervals (Figure **1A**). In K562 cells at 95% of fiducial probabilities the confidence intervals of IC_{50} value of OHV was 3.9 µg/ml after 72 h (Figure **1B**). Viability of cell dereased as incubation time increase from 24 h to 72 h.

3.2. Cytotoxicity Study

OHV at concentrations of 1-10µg/ml (U937 cell line) and 1-10µg/ml (K562 cell line) significantly inhibited the

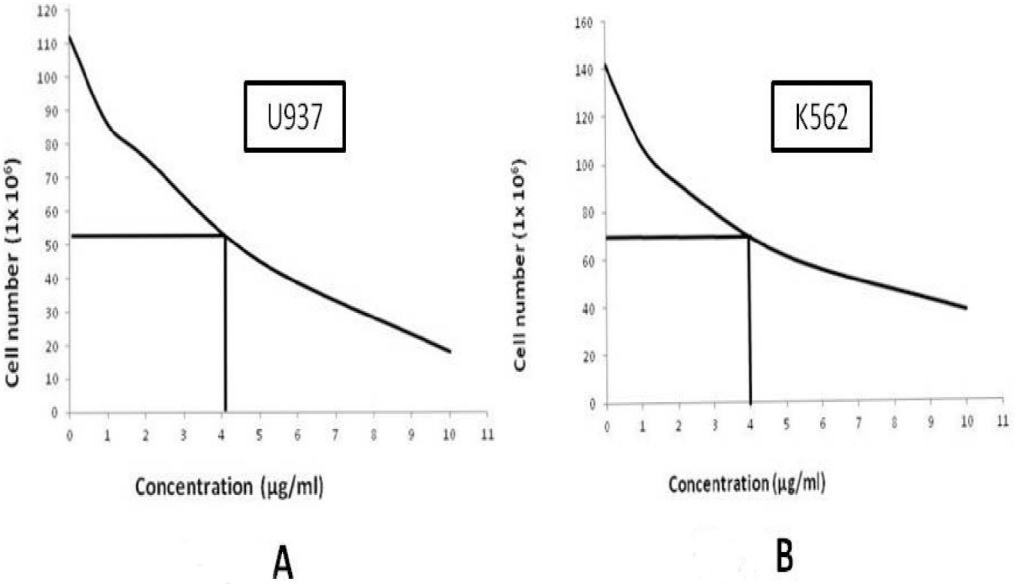

Figure 1: Determination of IC_{50} dose of OHV using trypan blue exclusion test after 72 h. (**A**) IC_{50} dose of U937 was 4.2 µg/ml. (**B**) IC_{50} dose of K562 was 3.9 µg/ml.

growth of the respective leukemic cells compared with the control cell in a time and concentration dependent manner. MTT assay revealed that 1-10 µg/ml of OHV showed 22.9 %- 69.5 % U937 cell decreases and 27% - 76% K562 cell decreases in 72 h as compared to untreated control (Figure **2A** & **B**).

3.3. Detection of Apoptosis (Morphometry)

Morphological observations of treated cells from both the cell lines under fluorescent microscope showed intact nuclei in control cell that gave bright green fluorescence (Figure **3A** & **C**) whereas treated cells showed intense orange-red fluorescence showing signs of apoptosis (Figure **3B** & **D**).

3.4. Lactate Dehydrogenase (LDH) Assay

In order to characterize LDH activity of OHV treated U937 and K562 cells, the amount of LDH released into the culture medium were evaluated. LDH activity of both the cell lines were increased in dose dependent manner.LDH increased to 76.8% for U937 and 68.2% for K562 at IC_{50} dose when compared to untreated cells (Figure **4**).

3.5. Detection of Apoptosis by Flow Cytometric Analysis

Flow cytometric data analysis revealed that after 24 h of treatment of OHV (IC_{50} dose) 21.73 % in LL

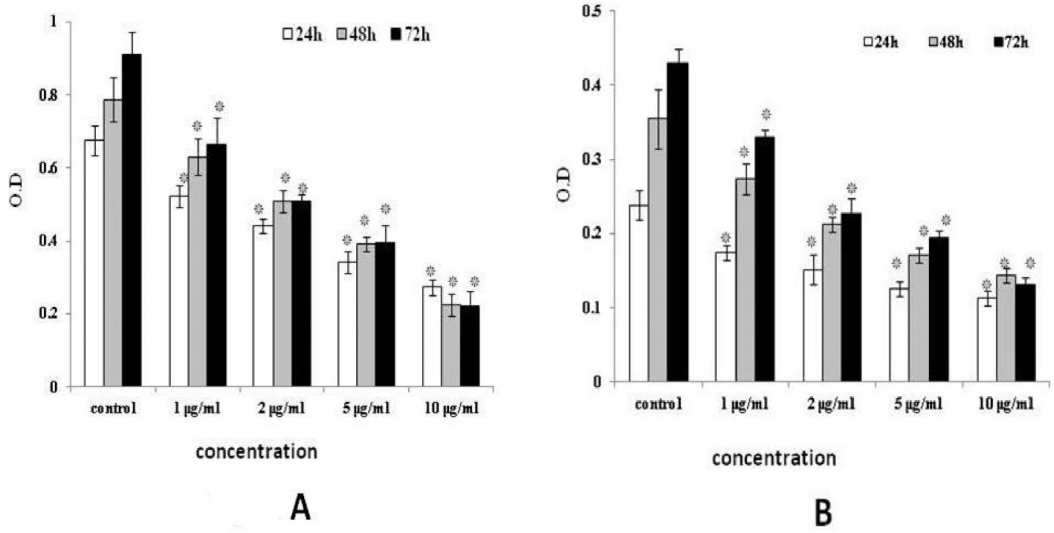

Figure 2: Cytotoxicity effect of OHV showed by MTT assay. Dose and time dependent cell inhibition of U937 cells (**A**) and K562 cells (**B**). The bar displays the mean±SEM of triplicate experiments (P< 0.05 compared to untreated controls).

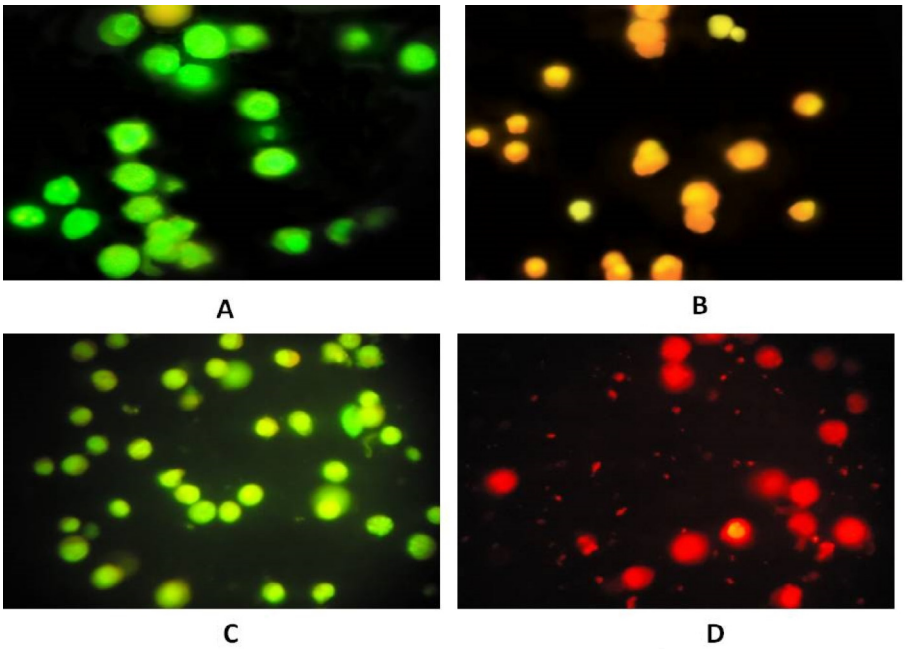

Figure 3: Representative micrographs of untreated control cells and OHV treated cells. Fluorescent microscopic images of control U937 (**A**), K562 (**C**) and OHV treated U937 (**B**), treated K562 (**D**) cell line.

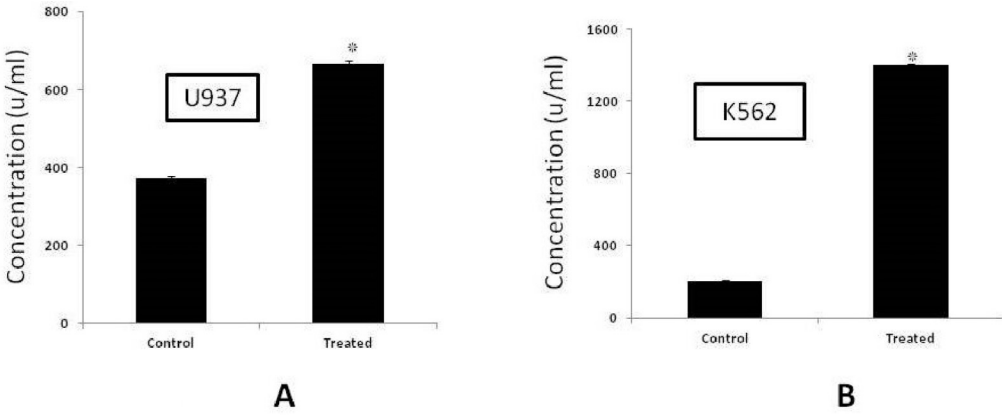

Figure 4: Lactate dehydrogenase (LDH) activity assay. U937 cells (**A**) and K562 cells (**B**) were treated as indicated for 24 h. LDH activity is expressed in U/L according to standard cruve. Results from at least three separate experiments are presented, $p<0.05$ compared to controls.

quadrant, 54.33% in LR quadrant, 21.69 % in UR quadrant and 2.25% in UL quadrant of U937 cells (Figure **5B**) with respect to 89.17 % in LL quadrant, 1.37% in LR quadrant, 0.89% in UR quadrant and 8.57% of control cell (Figure **5A**) and 44.84% in LL quadrant, 42.89% in LR quadrant, 12.24 % in UR quadrant and 0.035% of K562 cells (Figure **5D**) with respect to 98.42 % in LL quadrant, 1.45%in LR quadrant, and 0.06% in UR quadrant and 0.07% of control cell (Figure **5C**).

3.6. Study of Cell Cycle Arrest by Flow Cytometric Analysis

The flow cytometric analysis showed OHV (IC_{50} dose) treatment caused a progressive increase in the number of cells with 2N DNA content, which indicated G1 phase cell cycle arrest of U937 and k562 cells. Where treated U937 cell showed 52.60% G1+G0, 40.56% S and 2.85% G2+M (Figure **6B**), the control cell showed 21.02% G1+G0, 65.03% S and 11.50% G2+M (Figure **6A**) and where treated k562 cells showed 76.92% G1+G0, 23.08% S and 0.00% G2+M (Figure **6D**), the control cell showed 43.02% G1+G0, 44.85% S and 10.07% G2+M (Figure **6C**).

3.7. Estimation of Replication Cycle by BrdU Incorporation Using FACS

BrdU incorporation *via* replication cycle (synthesis phase) was much less in treated cell with respect to

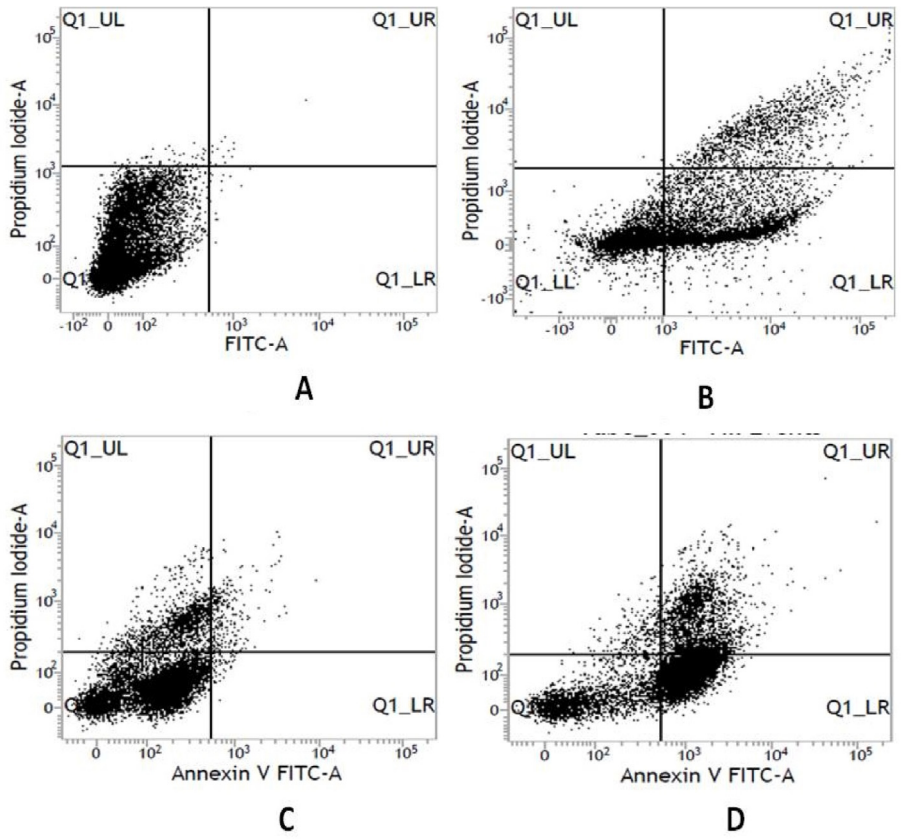

Figure 5: Apoptotic cell death was assayed by flow cytometry. Dot plot showed of U937 cells (**B**) and K562 cells (**D**) after treated with IC_{50} dose of OHV for 24 h. Control untreated U937 cells (**A**) and Control untreated K562 cells (**B**).

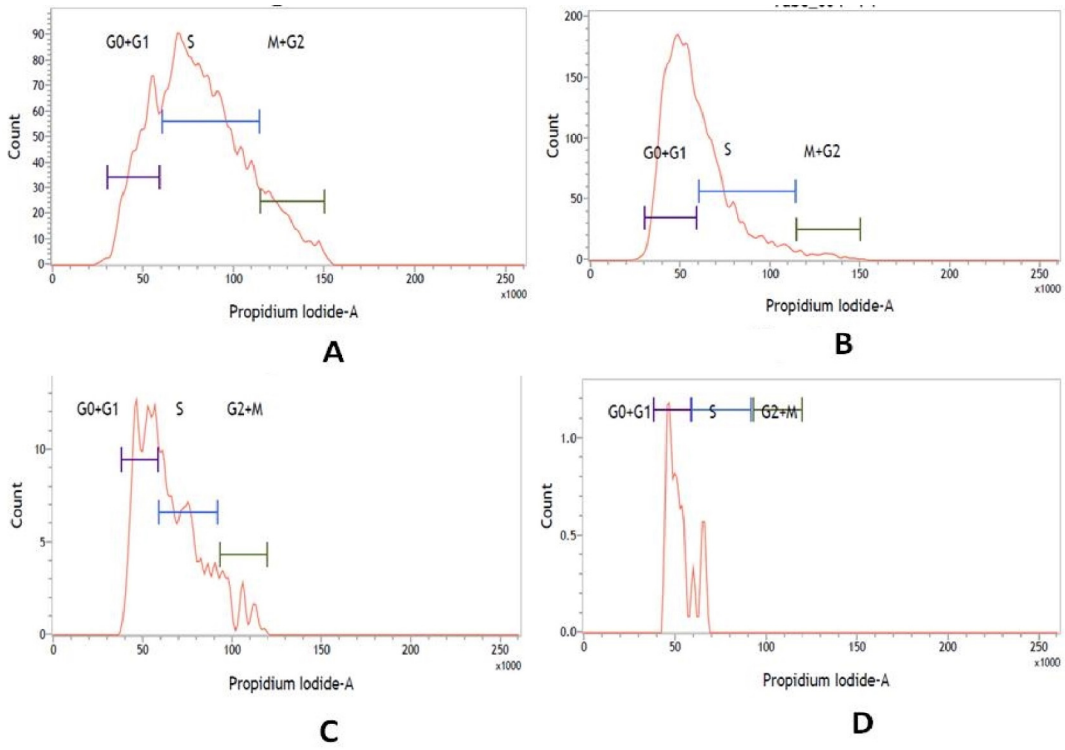

Figure 6: Flow cytometry analysis of cell cycle of untreated control U937 cells (**A**), OHV treated U937 (**B**), control K562 cells (**C**) and treated K562 cells (**D**). Histogram of both the treated cell line showed the arrest of cell cycle in G1 phase. (*x*-axis denotes fluorescence intensity of propium iodide and *y*-axis denotes cell count).

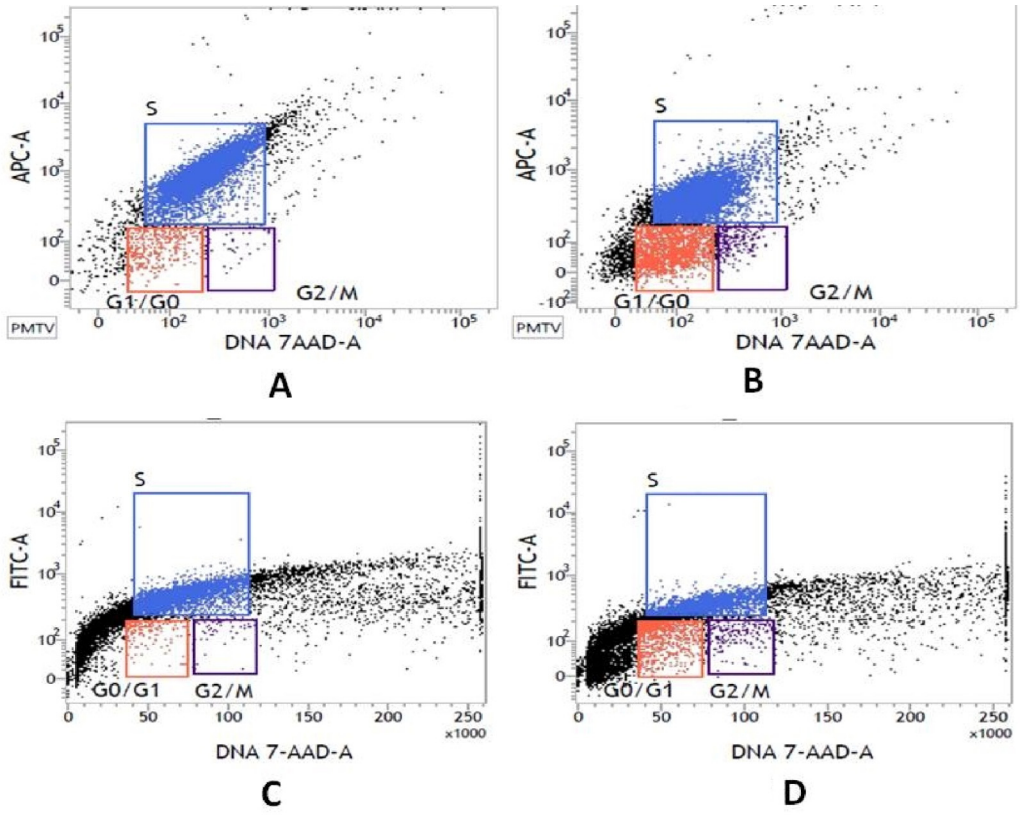

Figure 7: Flow cytometry analysis of replication cycle of control U937 (**A**), OHV treated U937 (**B**), control K562 (**C**) and OHV treated K562 (**D**) cell lines. Control cells showed more Brdu incorporation by indicating more synthesis phase, whereas treated cell line showed less Brdu incorporation indication less synthesis phase and signs of cell cycle arrest.

Control cell. Where control U937 cell showed 89.50% synthesis phase, treated cell showed it just 69.59% (Figure **7A** & **B**). In other experiment, treated K562 cell showed 38.67% Brdu incorporation with respect to 20.64% of Brdu in control K562 cell (Figure **7C** & **D**).

3.8. Analysis of ROS Generation

This study set out to evaluate the effect of OHV in the intracellular ROS production through the evaluation of increase in the fluorescence intensity of the dichlorofluorescein (DCF) by a micro plate reader. U937 and K562 cell lines were incubated with OHV and caused increase in ROS production. At the IC50 dose, OHV induced the ROS levels approximately 2 fold (in U937) and 1.5 fold (in K562) with respect to untreated cells (Figure **8A**).

3.9. Caspase3 Assay

As shown in Figure **8B**, caspase 3 activity was significantly increased in OHV treated cells, with at least 6 fold and 4 fold greater in U937 cell line and K562 cell line respectively than in control cells. Low levels of caspase 3 activity was also detected in control cells, which might be due to small numbers of cells undergoing normal apoptosis in the growing cell population.

3.10. Analysis of Apoptotic Protein and their Regulation

To determine the effect of OHV on the expression of pro and anti apoptotic marker proteins like Bax, Bid, Cytochrome C, and Bcl2 levels were analyzed. OHV upregulated the levels of Bax, Bid, Cytochrome C and p21 whereas downregulated the Bcl2 in both the cell lines. The Cyclin D and CDK4 expression levels were significantly inhibited with same treatment situation indication the cell cycle arresting nature of OHV. Along with caspase 3, expression of caspase 8 and 9 also upregulated in OHV treated cell lines. The expression of NFκB was upregulated that supports the dependency of p53 in cell proliferation control (Figure **9**).

3.11. Effect of OHV on Normal Lymphocyte

The viable human peripheral blood mononuclear cells (PBMNC) were counted after the treatment of OHV (Twice IC50 dose of U937) and Imatinib mesylate (100 µg/ml –standard dose for patient). The count in

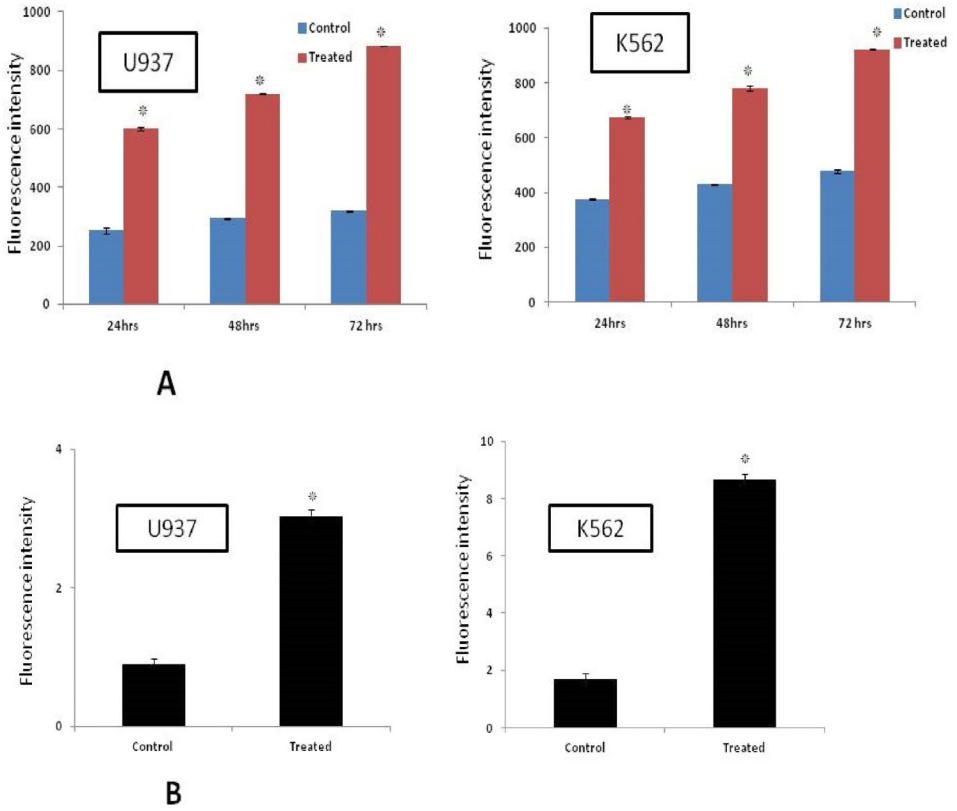

Figure 8: The effect of OHV on the level of reactive oxygen species (ROS) produced from U937 cells and K562 cells (**A**). Cells incubation with OHV caused both dose and time dependent increases in ROS levels quantified by fluorescence plate reader. All results are expressed as mean±SEM (n=4).

(**B**) Caspase 3 activities of OHV treated cells. U937 and K562 cell lines were treated with OHV at IC_{50} dose for 24 h. caspase 3 activities were determined using Caspase-AMC-DEVD -3 kit (n=4). The fold change of the control is considered as 1 and the error bars indicate standard deviations.

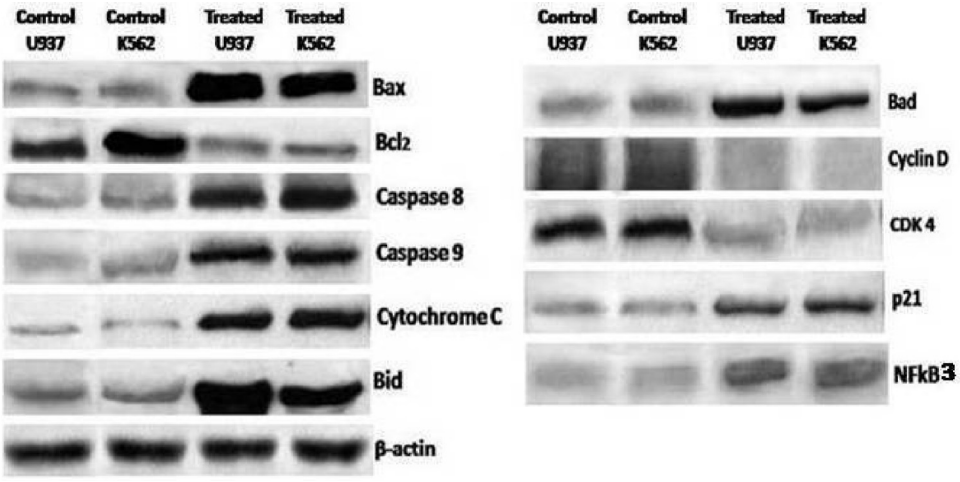

Figure 9: Western blot analysis of control and OHV treated EAC cells. This blot was of twelve independent experiments. Intracellular levels of proapoptotic Bax protein was upregulated along with Bid, Bad, caspase 8/9, p21, Cytochrome c and NFκB3 while antiapoptotic protein Bcl2 was down regulated along with cell cycle regulator protein cyclin D1and CDK4. β-actin was used as the loading control.

OHV and Imatinib mesylate treated cells decreased significantly by 28.30% and 38.7% (*P<0.05) as compared to the PBS treated control (3% loss of cell viability (P<0.05)) which showed normal cell toxicity towards OHV stills less than Imatinib mesylate (used as standard cancer drug in market).

4. DISCUSSION

In current condition, the main goal of cancer therapy is to explore and develop discovery leads that can selectively induce apoptosis in cancer cells [28]. Inhibition of cell proliferation and apoptosis induction through the coordinated cell cycle regulation is to be the fate of the cancer cells after treatment [29]. Debnath *et al.* reported a purified lethal cytotoxic protein from the *Naja kaouthia* venom inhibited cell proliferation in a dose and time dependent manner on human leukemic U937 and K562 cells and which exhibited IC_{50} on U937 and K562 cells were 3.5 µg/ml and 1.1 µg/ml respectively [30]. Das *et al.* identified an antioxidant protein (NN-32) from the Indian spectacled cobra *Naja naja* venom which was cytotoxic on Erlich Ascites Carcinoma cells, increased survival time of inoculated EAC induced mice, reduced solid tumor volume and weight. Whereas Bhattacharya *et al*. reported BF-CT1, a 13 kDa protein isolated from *Bungarus fasciatus* showed cytotoxicity in *in vitro* and *in vivo* experimental models [15, 6]. The present study showed dose dependent reduction of viable cell count and the MTT values due to OHV treatment in the U937 and K562 cells *in vitro*. The IC_{50} dose of OHV on U937 and K562 cell line were 4.1 µg/ml and 3.9 µg/ml respectively after 72 h. Similarly Lee at al. showed OH-LAAO (isolated from King cobra) was a potent cytotoxicity against MCF-7 (human breast adenocarcinoma), A549 (human lung adenocarcinoma) cells and PC-3 (human prostate cacer cell) cells with an IC50 value of 0.04, 0.05µg/mL, 0.05 µg/mL respectively, after 72 h incubation *in vitro* [10, 11]. The reduced value of the optical density has a direct correlation with the growth inhibitory rate and inverse relation with proliferation rate and inverse relation with proliferation rate [31]. The disruption of mitochondrial succinate dehydrogenase system due to the mitochondrial dysfunction or apoptosis might reduce the color formazan production in the MTT assay. This MTT results also reflected in the flow cytometry study of mitochondrial potential assay (▲ψ M). It is important for ATP production and mitochondrial protein transport. Disruption of ▲ψ M is also implicated in various apoptotic phenomena [32]. In order to evaluate the involvement of OHV in mitochondrial potential of leukemic cell lines, membrane potential in cells was determined by a fluorescent dye Rh-123 (JC-1kit). OHV induced the depolarization of proton pump of mitochondria in U937 and K562 cells. The crude venom of jellyfish has also been reported to induce disruption of mitochondrial respiration and membrane permeability in the neuronal like differentiated SH-SY5H cells [33].

Flow cytometric analysis of cell cycle using OHV on U937 and K562 cancer cells revealed that it arrested the cell cycle at Go/G_1 phases indicating its anti proliferating and apoptogenic tendency. Cell cycle of human cell also cancer cell is known to be controlled by anti mitogenic signals with involving p^{21cip1} and Cyclin D1 that bind to and inhibit cyclin-dependent kinases (CDKs) required for initiation of s-phase [34]. OHV downregulated cyclin D1 thus inhibited cyclin dependent kinases in U937 and K562 cells indicated the apoptogenic nature of OHV. OHV induced apoptosis in both leukemic cell lines were detected using Annexin-V-FITC binding by flow cytometry. The main principle of Annexin-V binding is based on the externalization of phosphatidylserine on cell membrane during the early stages of apoptosis. BrdU, analog of the thymidine, is incorporated during the first major round of cell cycle activity. Flow cytometry detected inhibition of replication as treated cell showed less BrdU signal in the synthesis phase. The BrdU incorporation into newly synthesized DNA was analyzed by immunofluorescent staining which showed MDA-MB-231 cells fail to enter s-phase when stimulated by oestradiol [35].Under Fluorescence microscopy, OHV treated U937 and K562 cell exhibited intense red fluorescence and reduced green fluorescence since apoptotic cells could not exclude the dyes and gave a combination of orange red fluorescence. OHV deformed the cancer cell membrane shape and produced membrane blebbing, perforation and nuclear fragmentation. Due to disruption of cytoskeleton protein membrane blebbing and pore formation might occured.

In the present study, however, the anticancer effects of OHV appeared to depend on the activation of alternative cell death pathways. Cancer cell death was associated with an increase in extracellular LDH activity indicating eventual loss of plasma membrane integrity.

The best strategy for cancer therapy and new drug development is to inducing the apoptosis in tumor cells [36].

The present investigation has performed to contribute to the understanding of the mechanism of bioactivity of OHV. The data demonstrated that OHV promoted intracellular ROS formation in dose dependent manner. Cytotoxic drugs might tigger the

intrinsic or mitochondrial mediated apoptosis. Bcl2 family proteins consisting of antiapoptotic as well as proapoptotic proteins play a key role in the intrinsic pathway of apoptosis. Bax, a proapototic protein is considered to be an important marker for the apoptosis pathway. This study demonstrated that OHV induce apoptosis through alternations in Bax: Bcl2 ratio with activation of the caspase 3 cascade in U937 and k562 leukemic cell line. Activation of caspases was occured by nuclear fragmentation of DNA due to apoptogenic trigger. Caspase3 share both caspase 9 (intrinsic) and caspase 8 (extrinsic) mediated pathway of apoptogenic signalling [37]. It was found that OHV diverse proapoptotic signals converged at the mitochondrial level, inducing the translocation of Cytochrome c into the cytosol. Cytochrome c triggered caspase9 activation by initiating a downstream caspase cascade through the complex formation in the cytosol that followed by the activation of the executioner caspase 3 and finally cell death. Similarly, caspase3 mediated apoptosis in A549 cells induced by cinobufocini; a toad skin preparation had been reported [38].

An alternative extrinsic pathway also induced by OHV in U937 and K562 cell line. This study showed protein signalling from plasma membrane receptors to the cytosol, occurred by activation of caspase 8 followed by increased expression of Bid which was induced by upregulation of Bad, hence apoptotic cell death by NFκB signalling. Recently, the inhibition of CDK2 and CDK4 and upregulation of CDKs inhibitor (p21 and p27) was found by the causal of curcumin together with BM-ANF1 [39]. As OHV was crude venom it might contain many cytotoxic fractions which might alone induced cell death by both intrinsic and extrinsic apoptotic pathway.

Interestingly, OHV was less toxic towards normal peripheral mononuclear cells than the standard drug (Imatinib mesylate). The advantage in treatment of cancer with advanced, natural agent based therapies is the inherent leaky vasculature present serving cancerous cells. The defective vascular architecture for serve fast-growing cancers along with poor lymphatic drainage allows them enhanced permeation and retention effect (EPR effect). The ability to target treatment to very specific cancer cells also uses a cancer's own structure in that many cancers overexpress particular antigens, even on their surface. This makes them ideal targets for drug delivery as long as the targets are not expressed in significant quantities anywhere in normal healthy cell [40]. As OHV is crude venom it contains many active subunits. This gives them more cytotoxicity and lesser toxic to normal cell as some of this active subunit might mask the toxic effect of toxic subunit of OHV in normal healthy cell. In other way, Imatinib mesylate is a chemically synthetic drug and its chemical constitute might advrse affect more in healthy normal cell. But the toxicity study was not complete, which requires further study in other normal cell lines in future.

5. CONCLUSION

It may be concluded that, Indian king cobra (*Ophiophagus hannah*) crude venom (OHV) possessed anti proliferative and apoptogenic activity on leukemic (U937 and K562) cell line acting on both extrinsic and extrinsic signalling pathway.

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CONFLICT OF INTEREST

No confliction of interest among authors.

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