

Research Article



Apoptogenic Activity of Secretion Extract of *Bellamya Bengalensis* f. *Annandalei* via Mitochondrial Mediated Caspase Cascade on Human Leukemic Cell Lines

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ABSTRACT

Secretion extract of *Bellamya bengalensis* f. *annandalei* have been traditionally used for many ailments. The anti-leukemic activity of secretion extract of *Bellamya bengalensis* f. *annandalei* (SEBB) has been investigated against three human leukemic cell lines U937, K562 and HL-60 where IC₅₀ values are calculated to be 22.21µg/ml, 10.3µg/ml & 12.79µg/ml respectively. Morphologically, externalization of phosphatidyleserine has been found on all the treated cells but not in control cells. Cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies are characteristic features of apoptosis. Gel electrophoresis study shows fragmented DNA in the form of ladder and Flow cytometric analysis showed appreciable number of cells in early & late apoptotic stages. The cells are getting arrested in the sub-G₁ & G₁ phases of cell cycle. The apoptosis is mediated through activation of caspase-9 & Caspase-3.

Keywords: Apoptosis, *Bellamya bengalensis*, Cytotoxic, Leukaemia, Secretion.

INTRODUCTION

Cancer is a complex multifactorial disease of the cell. Cancer development and progression are dependent on cellular accumulation of various genetic and epigenetic events.¹ Radiotherapy, chemotherapy and surgical measure are the key tools for cancer treatment. In the haematopoietic malignancies, chemotherapeutic approaches are widely applied in practice. Drug discovery against cancer is ventured throughout the world especially from the natural products.² Animals are therapeutic arsenals that have been playing significant roles in the healing processes, magic rituals, and religious practices of peoples from the five continents. In the folk-traditional medicine, it has been mentioned that snail flesh is used as medicines for the cure of a number of ailments such as conjunctivitis and gastro intestinal disorders. In the northern Bihar, the flesh of *Bellamya bengalensis* is used as a traditional medicine against arthritis.³ But no work has been done so far in the field of cancer with this fresh water snail. The present communication is an approach to study the anti-proliferative, cytotoxic and apoptotic activity of secretion extract of *Bellamya bengalensis* f. *annandalei* (SEBB) family Viviparidae has been investigated against three myeloid cell lines.

MATERIALS AND METHODS

Chemicals

The following chemicals were used: RPMI 1640 medium (Gibco, USA), Fetal bovine serum (FBS), HEPES, L-glutamine, Penicillin- Streptomycin (Bio-west, Germany), Gentamycin (Nicholas, India), Ara-C (Arabinofuranosyl Cytidine), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], Ethidium

bromide and Acridine orange, Annexin V- FITC apoptosis detection kit, RNase, Propidium iodide, Caspase-3 were purchased from Sigma (St. Louis, MO, USA), Caspase- 9 assay kit (U.S. Biological, USA), Trypan blue, Proteinase -K, Agarose medium EEO(SRL, India) and other chemicals and reagents were of analytical grade and purchased from local firms.

Cell cultures

U937 (human leukemic monocyte lymphoma cell line), K562 (human myelogenous leukemia cell line) and HL-60 (human promyelocytic leukaemia cell line) obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. The cells were cultured and routinely maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100µg/ml), Gentamycin (100µg/ml) and were incubated at 37°C in a humidified atmosphere containing 5% CO₂ inside a CO₂incubator.

Extraction and Preparation of Test Sample

Live adult *Bellamya bengalensis* f. *annandalei*, family Viviparidae was collected commercially from the Kolkata market and authenticated by Dr. R. Venkitesan Scientist-'C' O/C Mollusca Section, Zoological Survey of India-Kolkata 53. Authentication No: **F.No.229-10/98-Mal (1)**. Secretion was collected from the snail in a cold room, and then it was centrifuged for 10 min (4°C) at 1600 g to precipitate the residual debris, then it was partially purified by Amicon ultra filtration unit. After filtration the sample was lyophilised to powder form. It was kept in a glass container, sealed with parafilm and stored at -20°C. Stock solution (1 mg/ml) of secretion extract of *Bellamya bengalensis* f. *annandalei* has been designated as SEBB



and dissolved in PBS from where desired concentrations were prepared for the experiment.

Cell Growth Inhibition Study and Cytotoxicity Study

U937, K562. HL-60 cells (1×10^5) were seeded in 96- well sterile plates each and were treated with different concentrations (10, 25, 50, 100 μ g/ml) of SEBB for 24, 48 and 72 h and graphs are plotted against control cells & Ara-C (Std) treated cells. The cell growth inhibition studies were done by Trypan blue exclusion method⁴ and the cytotoxicity studies were performed by MTT assay. For cell growth inhibition study compound microscope is used. For MTT assay the absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually at 492 nm) by microplate manager (Reader type: Model 680 XR Bio-Rad laboratories Inc)⁵. IC_{50} Values for all the 3 cell lines are determined for 24 hrs.

Morphological Studies for Detection of Apoptosis

Fluorescence Microscopic Studies

U937, K562 & HL-60 cells (1×10^6) treated with 3 different IC_{50} doses of SEBB (corresponding to each cell line) for 24 hrs were observed using a fluorescence microscope for morphological changes. The untreated control cells and SEBB treated cells are harvested separately (centrifuged at 1000 rpm for 5 min) pellet washed twice with PBS and then stained with acridine orange (100 μ g/ml) and ethidium bromide (100 μ g/ml). The cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.⁶

Study of Phosphatidylserine (PS) Externalization

PS externalisation was examined after treating the cells (1×10^6) with IC_{50} doses of SEBB for 24 h under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystems, Heidelberg, Germany). The untreated and the SEBB treated cells were harvested separately, washed with ice cold PBS then Annexin V FITC binding buffer (10mM HEPES, 140mM NaCl and 2.5mM $CaCl_2 \cdot 2H_2O$; pH 7.4) respectively and they were then stained with 5 μ l of Annexin V FITC for 10 min at room temperature. The cells were mounted on slides and the images were captured to observe the cells undergoing early apoptosis.⁶

Detection of apoptosis by DNA fragmentation and agarose gel electrophoresis

U937, K562 & HL-60 cells (1×10^6) were treated with IC_{50} (24hrs) doses of each cell line of SEBB and with standard anti-cancer drug AraC (100 μ g/ml) for 24 hrs. The cells were harvested and washed twice with PBS. The cells were resuspended in 500 μ l of lysis buffer (50mM Tris-HCl, pH -8.0, 10mM EDTA, 0.5% SDS), 10 μ g of Proteinase- K was added and lysis was done by incubation at 50 °C for 1h and 37°C overnight respectively. DNA extraction was done by following the general phenol-chloroform extraction procedure.⁷ To detect the DNA fragments, the isolated DNA samples were mixed with loading dye and

were subjected to 1% agarose gel electrophoresis for overnight at 20 Volt using ethidiumbromide. DNA fragmentation was observed in UV transilluminator.

Detection of Apoptosis by Flow Cytometric Analysis

In order to investigate the type of cell death induced by SEBB, flow cytometric analysis was done by performing dot plot assay.⁸⁻⁹ The leukemic cells (1×10^6) were treated with individual IC_{50} dose of SEBB for 18 hrs. The cells were pelleted down, centrifuged at 2000 rpm for 8 min at 4 °C and washed with Annexin -V- FITC binding buffer (10mM HEPES, 140 Mm NaCl and 2.5mM $CaCl_2 \cdot 2H_2O$; pH 7.4). The cell pellets were dissolved in Annexin V FITC binding buffer containing Annexin-V- FITC and Propidium iodide. After 15 min incubation in dark at room temperature flow cytometric analysis was done. All data were acquired with a Becton-Dickinson FACS Caliber single laser cytometer. Flow-cytometric reading was taken using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). Live statistics were used to align the X and Y mean values of the Annexin-V FITC or PI stained quadrant populations by compensation. Data analysis was performed with Cell Quest (Macintosh platform) program.⁶

Study of Cell Cycle Arrest by Flow Cytometric Analysis

To assay the stage of cell cycle arrest in a flow cytometer¹⁰, 1×10^6 cells were treated with SEBB (individual IC_{50} dose) for 18 hrs. Cells were washed with PBS, fixed with methanol and kept at -20 °C for 3 min. They were then resuspended in cold PBS and kept at 4 °C for 90 min. Cells were pelleted down, dissolved in PBS, treated with RNase for 30 min at 37 °C and stained with Propidium iodide and kept in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined on FACS (Becton Dickinson FACS Fortessa multi laser cytometer), fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson).⁶

Caspase-9 Assay

The assay was performed using a Caspase-9, Apoptosis Detection, Colorimetric BioAssay Kit, (US Biological) according to the manufacturer's protocol. U937, K562 and HL-60 cells (1×10^7 -⁸) were treated with individual IC_{50} doses of SEBB for 24 h. The cells were pelleted down and resuspended in 50 μ l of cell lysis buffer (supplied with the kit) and incubated on ice for 10 min. After centrifuging at 10,000x g for one min, the supernatants (cytosolic extract) were transferred to fresh tubes and kept on ice and the caspase-9 assay was performed according to the supplied kit protocol. 50 μ l of 2X reaction buffer (containing 10mM DTT) was added to each sample. 5 μ l of LEHD-pNA substrate (4mM) (200 μ M final concentration) was added and incubation was done at 37 °C for 1-2 h. Absorbance was read at 405 nm and calculations were thereby done.⁶

Caspase-3 Assay

The assay was performed using a Caspase-3 Assay kit, Colorimetric (Sigma) according to the manufacturer's protocol. U937, K562 and HL-60 cells (1×10^7) were treated with individual IC_{50} doses of SEBB for 24 h. The untreated control and the treated cells were pelleted down by centrifugation at $600 \times g$ for 5 min at $4^\circ C$. Supernatants were removed and the cell pellets were washed with 1 ml of PBS. The cells were again centrifuged and the supernatants were removed completely. The cell pellets were suspended in 100 μ l of 1X lysis buffer (50mM HEPES, pH 7.4, 5mM CHAPS, 5mM DTT) and incubated on ice for 20 min. The lysed cells were centrifuged at $20,000 \times g$ for 15 min at $4^\circ C$ and the supernatants (cell lysates) were analysed for the caspases-3 activity according to the manufacturer's protocol. Cell lysates were incubated with 2mM Caspase-3 substrate (Ac-DEVDpNA) in 1X assay buffer (20mM HEPES, pH 7.4, 2mM EDTA, 0.1% CHAPS, 5mM DTT) for 90 min at $37^\circ C$. The absorbance was read at 405 nm and the results were calculated using a p-nitroaniline calibration curve.⁶

Statistical Analysis

All the data are expressed in terms of percentage decrease from the control values. Statistical analysis was done by Student's *t*-test. $P < 0.05$ was considered as significant.

RESULTS

Cell Growth Inhibition Study and Cytotoxicity Study

SEBB significantly inhibited the growth of U937, K562 and HL-60 cells compared with that of the control cells after 24, 48 and 72 h of treatment in a concentration-dependent manner (Fig. 1). In the MTT assay, there was significant concentration dependent reduction in the O.D values after treating the U937, K562 and HL-60 cells with 10, 25, 50, 100 μ g/ml of SEBB for 24, 48 and 72 h compared to the control cells (Fig. 1). These observations provided proof for cytotoxic nature of SEBB. The IC_{50} calculated after MTT assay is **22.21 μ g/ml (U937), 10.3 μ g/ml (K562), 12.79 μ g/ml (HL-60)**.

Morphological Studies for Detection of Apoptosis

Fluorescence Microscopic Studies

Fluorescence microscopic observations of the SEBB-treated (individual IC_{50} dose/ h) U937, K562 & HL-60 cells stained with ethidium bromide and acridine orange (colour-red or orange), revealed the presence of apoptotic cells (early and late) as compared to the untreated control cells stained with only acridine orange (colour-green) (Fig.2). Arrays of nuclear changes were observed including chromatin condensation and apoptotic body formation that are indicative of an apoptotic process comprising of both early and late apoptotic stages.

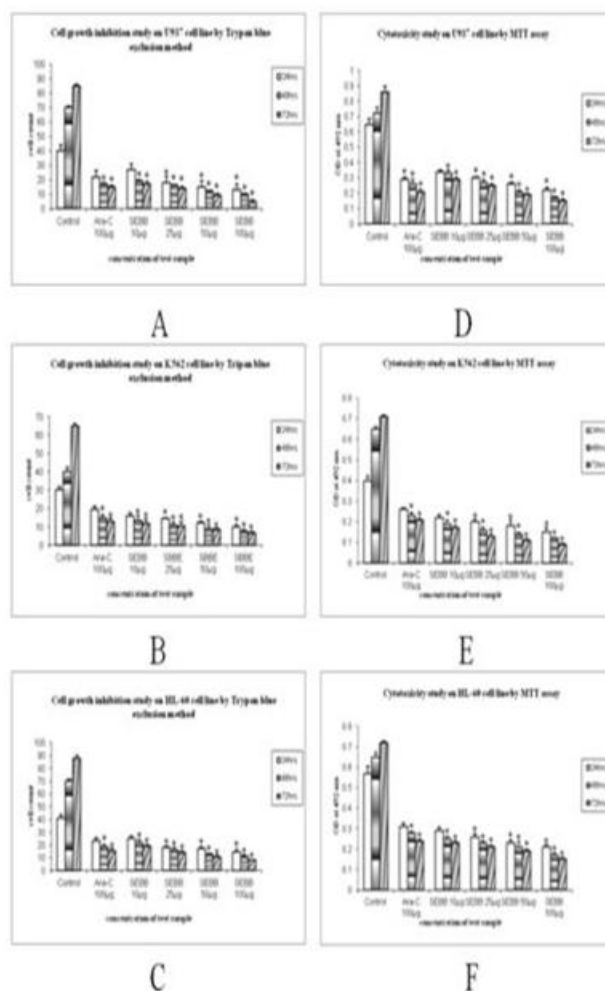


Figure 1: The effect of SEBB (10, 25, 50, 100 μ g/ml) on cell growth inhibition (A-C) and cytotoxicity studies (D-F) in U937, K562 and HL-60 cells. Cell count studies after 24, 48 and 72 h. of SEBB (10, 25, 50, 100 μ g/ml) treatment in U937 cells (A), K562 cells (B) and HL-60 cells (C). Cytotoxicity studies by performing MTT assays after SEBB (25, 50, 100, μ g/ml) treatment for 24, 48 and 72 h. in U937 cells (D), K562 cells (E) and HL-60 cells (F). Ara-C (100 μ g/ml) was used as standard reference anti-cancer drug.

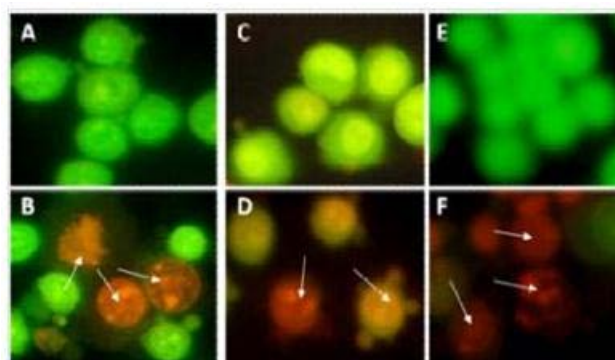


Figure 2: Fluorescence microscopic images of untreated control U937 (A), K562 (C) and HL-60 (E) and SEBB treated U937 (B), K562 (D) and HL-60 (F) cells. The control cells were with intact nuclei and gave bright green fluorescence whereas treated cells showed intense orange- red fluorescence showing signs of apoptosis.

Study of Phosphatidylserine (PS) Externalization

PS is predominantly accumulated in the inner leaflet of plasma membrane of living cells but in the apoptotic cells, Phosphatidylserine is translocated from inner to outer leaflet of plasma membrane. Treatment with SEBB caused externalisation of PS that after Annexin V FITC binding has given green fluorescence (Fig. 3).

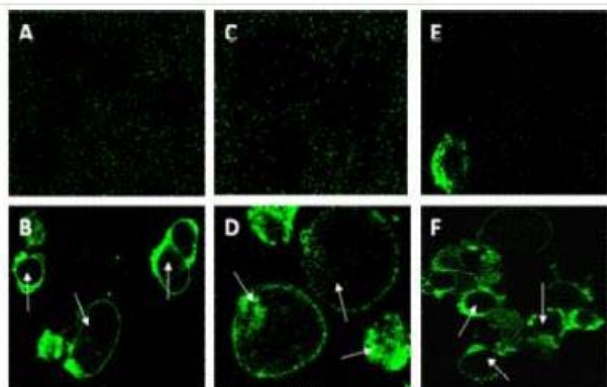


Figure 3: Fluorescence microscopic images of untreated control U937 (A), K562 (C), HL-60 (E) cells and SEBB treated U937 (B), K562 (D) HL-60 (F) cells. The SBBE treated cells showed green fluorescent rings of externalized Phosphatidylserine indicating sign of apoptosis after 24 hrs of treatment.

Detection of apoptosis by DNA fragmentation and agarose gel electrophoresis

The gel pattern of the DNA samples isolated from untreated control U937, K562 and HL-60 cells showed intact DNA bands whereas the gel pattern of the DNA samples isolated from SBBE (IC₅₀ dose) treated U937, K562 and HL-60 cells showed degraded DNA bands in the form of ladders (Fig. 4). So, the observations confirmed that the treatment with SBBE caused apoptosis in all the three human leukemic cells.

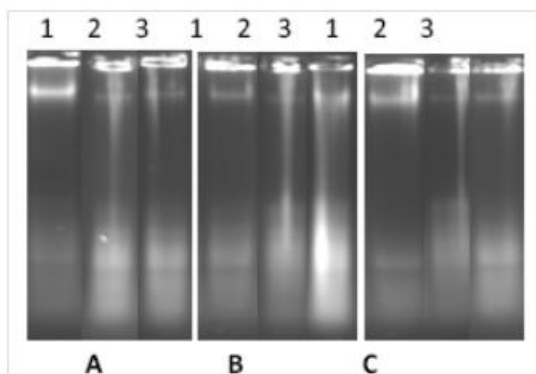


Figure 4: The gel pattern of DNA samples isolated from U937 cells (A) untreated control (lane 1), treated with 100µg/ml of standard anti-cancer drug, Ara-C (lane 2) and cells treated with IC₅₀ dose of SBBE (lane 3), K562 cells (B) untreated control (lane 1), treated with 100µg/ml of standard anti-cancer drug, Ara-C (lane 2), treated with IC₅₀ dose of SBBE (lane 3) and HL-60 cells (C) untreated control (lane 1), treated with 100µg/ml of standard anti-cancer drug, Ara-C (lane 2), cells treated with IC₅₀ dose of SBBE (lane 3). Treatment with both the standard anti-cancer drug, Ara-C and SBBE showed distinct DNA ladder

formation indicating the process of apoptosis in all the three human leukemic cell lines.

Detection of Apoptosis by Flow Cytometric Analysis

In the flow cytometric analysis, double labelling technique, using annexin V FITC and propidium iodide, was utilized. Lower left (LL) quadrant (annexin V-/PI-) is regarded as the population of live cells, lower right quadrant (LR) (annexin V+/PI-) is considered as the cell population at early apoptotic stage, upper right (UR) quadrant (annexin V+/PI+) represents the cell population at late apoptotic stage and upper left (UL) quadrant (annexin V-/PI+) is considered as necrotic cell population. Flow cytometric data analysis revealed that after 18 h of treatment with IC₅₀ dose of SBBE, 41.76% of U937, 81.23% of K562 and 75.12% of HL-60 cells were in LR quadrant (early apoptotic stage) and 8.99% of U937 and 14.59% of K562 and 23.81% HL-60 cells were in UR quadrant (late apoptotic stage) (Fig.5).

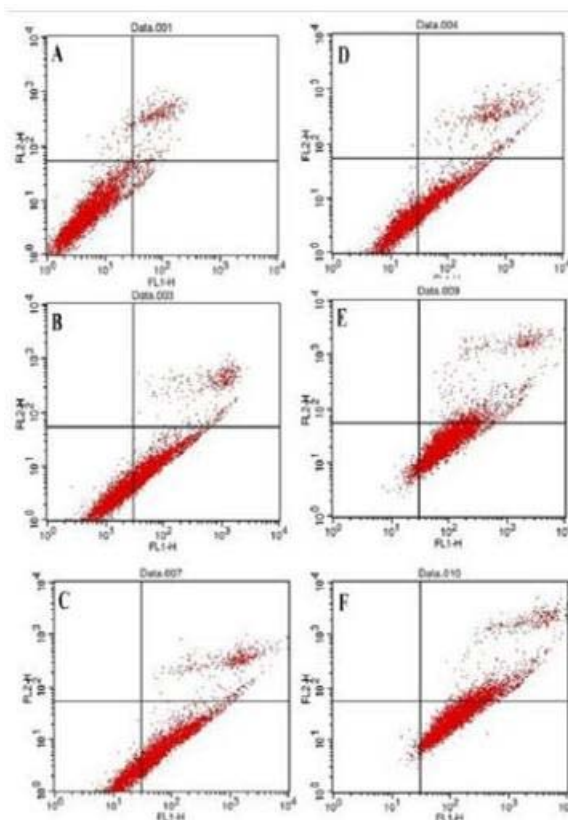


Figure 5: Detection of apoptosis by Flow cytometric analysis control (A, B & C) and treated (D, E & F) U937, K562 & HL-60 cells respectively after 18hrs treatment at IC₅₀ dose with SEBB. Staining was done with Annexin V FITC and Propidium iodide. Dual parameter dot plot of FITC-fluorescence (x-axis) vs. PI-fluorescence (y-axis) shows logarithmic intensity.

Study of Cell Cycle Arrest by Flow Cytometric Analysis

Flow cytometric analysis showed that after 24 h treatment of U937 with SEBB at IC₅₀ dose, sub-G1 peak was changed. DNA content increased greater than two-fold in treated cells (19.79 against 8.88%), but decreased in G1 phase (35.76% against 58.29%). In case of K562 cell line, DNA content increased in sub-G1 phase (24.61

against 18.41%) as well as in G₁ phase (37.32% against 34.01%) after SEBB treatment. In case of HL-60 cell line, DNA content increased greater than three-fold in sub-G₁ phase (21.32% against 6.40%) but decreased in G₁ phase (37.64% against 63.1%). These results indicated that drug treatment arrested the cell cycle of both the cell lines at sub-G₁ phase (Fig 6).

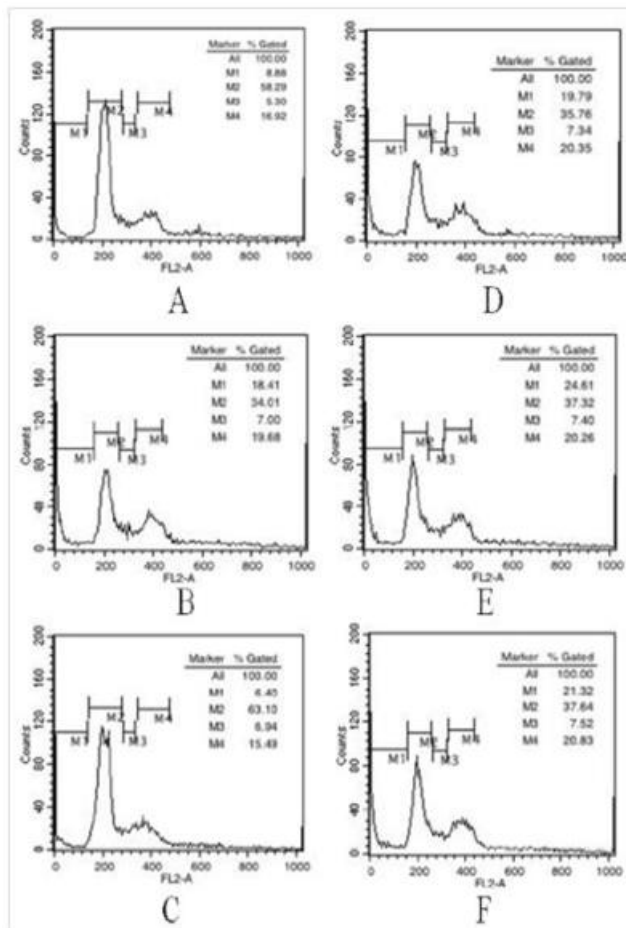


Figure 6: Flow cytometric analysis of cell cycle phase distribution in control (A) and treated (D) U937, in control (B) and treated (E) K562 & in control (C) and treated (F) HL-60 cells after 18 h treatment at IC₅₀ dose of SEBB. Histograms represent various contents of DNA with actual number of cells (x-axis denotes fluorescence intensity of Propidium iodide and y-axis denotes count).

Caspase 9 Assay

Caspase-9 is one of the main initiator caspases and has been linked to the mitochondrial death pathway. To investigate whether the treatment with SEBB induced apoptosis *via* intrinsic pathway, caspase-9 assay was performed in U937, K562 and HL-60 cells. The experiments revealed increase in the caspase-9 activity in the SEBB (IC₅₀ dose) treated U937 (1.83 fold increase), K562 (2.2 fold increase) and HL-60 cells (2.28 fold increase) compared with that of the untreated control U937, K562 and HL-60 cells respectively (Fig. 7), supporting the fact that apoptosis induced by the SEBB treatment might be mediated through the intrinsic pathway.

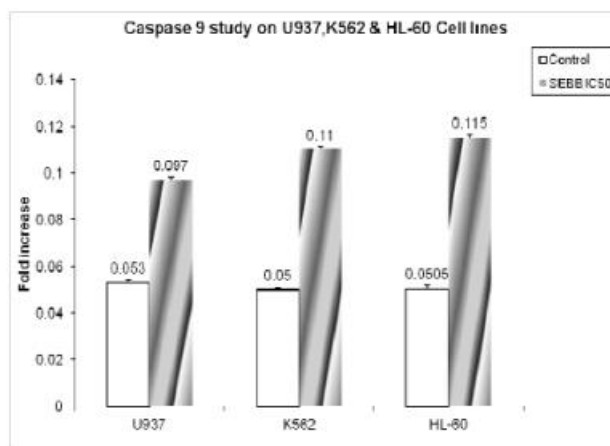


Figure 7: Fold increase in Caspase 9 productions in U937, K562 & HL-60 cell lines after SEBB treatment for 24 h at IC₅₀ dose with respect to control.

Caspase 3 Assay

Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. The primary target of the caspase-9 is procaspase-3, one of the most deleterious effector caspases. To observe whether caspase-9 activated caspase-3 after SEBB (IC₅₀ dose) treatment, caspase-3 assay was performed in U937, K562 and HL-60 cells. Caspase-3 activation was clearly observed in SEBB treated U937 (1.59 fold increase), K562 (2.14 fold increase) and HL-60 (2.05 fold increase) cell when compared with that of the untreated control cells (Fig. 8).

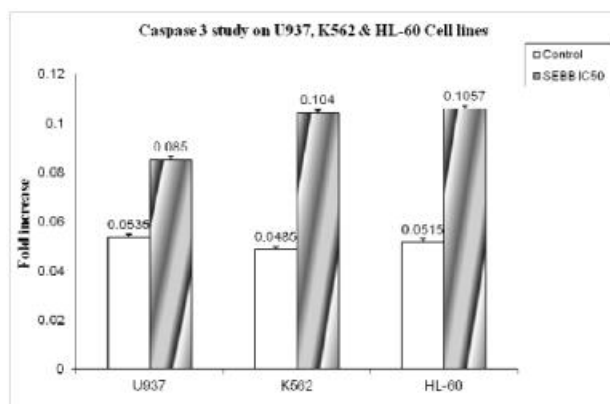


Figure 8: Fold increase in Caspase 3 productions in U937, K562 & HL-60 cell lines after SEBB treatment for 24 h at IC₅₀ dose with respect to control.

DISCUSSION

The search for improved cytotoxic agents continues to be an important line in the discovery of modern anticancer drugs.¹¹ This is not surprising because, in most developed countries and, to an increasing extent, in developing countries, cancer is amongst the third most common causes of death and morbidity.¹² The fate of cancer cells primarily depends on the proliferation control i.e., DNA replication and apoptosis induction through the coordinated cell cycle regulation.¹³ Apoptogenic activities of SEBB treated cell were supported by the observations in cell growth inhibition studies and in MTT assays respectively. The process of apoptosis is characterized by

several morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. Fluorescence microscopic images clearly showed nuclear disintegration of SEBB treated leukemic cells compared with that of the untreated control cells when stained with acridine orange and ethidium bromide. Externalization of PS from inner leaflet to outer leaflet of the membrane is the hallmark of early phase of apoptosis. Externally translocated PS binds with annexin-V in a concentration dependent manner.¹⁴ Confocal microscopic images of treated U937, K562 and HL-60 cells showed bright green fluorescent rings of externalized PS, supporting the fact that treatment with SEBB induced apoptosis in the leukemic cells. Further evidence in support of the apoptogenic activity of SEBB was obtained from the gel patterns of Agarose gel electrophoresis. SEBB treated cells showed degraded DNA bands in the form of ladders, a typical indication of apoptosis, whereas the untreated control cells showed intact DNA bands when observed in UV transilluminator. To confirm the apoptosis or necrosis using, dual staining with Annexin V FITC and propidium iodide in dot plot assay made it possible to identify live, early apoptotic and late apoptotic cells.^{15, 16} Experiments showed increased number of cells in the early and late apoptotic stage after treatment with SEBB implying the fact that apoptosis was triggered by the treatment with SEBB in U937, K562 and HL-60 cells. So, the observations indicated that the treatment with SEBB was inducing apoptosis in all the leukemic cells. Cell cycle analysis revealed that treatment with SEBB arrested the U937 and HL-60 cell populations in the sub-G₁ phase and K562 cell population in both the sub-G₁ & G₁ phase of cell cycle. There are two major apoptotic pathways known to date, initiated by either the mitochondria (the 'intrinsic' pathway) or the cell surface receptors (the 'extrinsic' pathway). Mitochondria mediated apoptosis occurs in response to a wide range of death stimuli, including activation of tumor suppressor proteins (such as p³⁸ and p⁵³) and oncogenes (such as c- Myc), DNA damage, chemotherapeutic agents, serum starvation, and ultraviolet radiation.¹⁷ In the intrinsic pathway, diverse proapoptotic signals converge at the mitochondrial level triggering caspase-9 activation initiating a downstream Caspase cascade through the complex formation with Apaf-1, d ATP, and pro-caspase-9 in the cytosol, which ultimately lead to the activation of the executioner caspase-3 and finally cell death.¹⁸⁻²³ Caspase-9 and caspase-3 assays showed increase in the activation of caspase-9 and caspase-3 respectively after SEBB treatment in U937, K562 and HL-60 cells.

CONCLUSION

It may be concluded that, secretion extract of *Bellamya bengalensis* f. *annandalei* (SEBB) possesses potent antineoplastic agent, which is anti proliferative, cytotoxic and apoptogenic against human myeloid leukemic cells (U937, K562 & HL-60) mitochondria mediated intrinsic

pathway. Further process of isolation & purification of the active anticancer molecule from SEBB are in progress.

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