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ANTI TUMOR ACTIVITIES OF SECRETION EXTRACT OF *BELLAMYA BENGALENSIS* IN HUMAN HEPATOCELLULAR CARCINOMA CELL LINES IS MEDIATED BY CASPASE-DEPENDENT APOPTOSIS AND CELL CYCLE ARREST

Abhishek Ranjan Besra[2], Krishnendu Pal[1], Santanu Basu[3], Shila Elizabeth Besra[1*]

[1]Central Instrumentation Division, CSIR, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Kolkata-700032, West Bengal, India.

[2]Indian Institute of Management Lucknow (IIM L), Prabandh Nagar, IIM Road, Lucknow-226013.

[3]Employee State Insurance Hospital Sealdah, 301/3, A.P.C. Road, Kolkata –700009, West Bengal, India.

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***Correspondence for Author**
Dr. Shila Elizabeth Besra
Central Instrumentation Division, CSIR, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Kolkata-700032, West Bengal, India.

ABSTRACT

Anti leukemic activity of secretion extract of *Bellamya bengalensis* f. Annandalei is already reported. We evaluated the cytotoxic and apoptosis inducing effect of secretion extract of *Bellamya bengalensis* (SEBB) on hepatocellular carcinoma cell lines and NO assay on mouse murine macrophage cell line. It was found that SEBB significantly decreased the metabolically viable cells and that SEBB caused apoptosis, as confirmed by morphologically and by Flowcytometric analysis shows appreciable number of cells in early & late apoptotic stages and cells are arrested in the sub-G1 & G1 phases of cell cycle. The SEBB induces nitric oxide in RAW264.7 cells but when used in combination with recombinant interferon-γ (rIFN-γ), it shows a marked enhancement in NO production in murine macrophages RAW264.7 cells. Elevated NO production was significantly inhibited by pre-treatment with PDTC and N^GMMA, thus SEBB indicates a role of immunomodulation.MTT assay in RAW264.7 cells revealed that insignificant reduction and apoptosis is mediated through activation of caspase 3.

KEYWORDS: Hepatocellular Carcinoma, *Bellamya bengalensis*, Secretion, Apoptosis, Nitric Oxide.

1. INTRODUCTION

Drug discovery against cancer is ventured throughout the world especially from the natural products.[1] 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 northern Bihar, the flesh of *Bellamya bengalensis* is used as a traditional medicine against arthritis.[2] Hepatocellular carcinoma (HCC) is one of the most common malignant tumours worldwide. Secretion extract of *Bellamya bengalensis* f. Annandalei have been traditionally used for many ailments. Liver is a versatile organ of the body that regulates internal chemical environment.[3] Hepatocellular carcinoma (HCC) is one of the most common malignant tumours worldwide. HCCs are phenotypically (morphology and microscopy) and genetically heterogeneous tumours, possibly reflecting the heterogeneity of etiological factors implicated in HCC development, the complexity of hepatocytes functions and the late stage at which HCCs usually become clinically symptomatic and detectable.[4, 5] Hepatocarcinogenesis is a multi-factor, multi-step and complex process.[6] Malignant transformation of hepatocytes may occur, regardless of the etiological agent, through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation, immune response, and oxidative DNA damage.[7-8] Nitric oxide (NO), a short-lived gaseous radical, is a potent multifunctional reactive metabolite that is a major effector molecule of immune cells against tumor cells and pathogens[9] Secretion extract of *Bellamya bengalensis* f. annandalei (SEBB) has been investigated against human hepatocellular carcinoma HepG2 and Huh-7 cell line. The NO assay was studied on RAW264.7 cell line activated with murine rIFN-γ and SEBB induced effects is also examined.

2. MATERIALS AND METHODS

2.1 Chemicals

DMEM and RPMI 1640 medium with L-glutamine, Fetal calf serum (FCS), Trypsin (Gibbco, USA), HEPES, Annexin-V FITC apoptosis detection kit, Ethidium bromide, Propidium iodide, Acridine orange, PDTC (Pyrrolidine dithiocarbamate), N^G-MMA (N^G-monomethyl-$_L$-arginine), murine rIFN-γ are purchased from Sigma (St. Louis, MO, USA), Sorafenib tosylate (Brand name: Nexavar, Bayer & Onyx Pharmaceuticals), Penicillin- Streptomycin (Biowest,

Germany), Gentamycin (Nicholas, India), MTT [3-(4,5-dimethylthiozol-2-il)-2, 5-2, 5-dipheniltetrazoliumbromide], RNase (SRL), Greiss reagent is purchased from MP Biomedicals. All the other chemicals and reagents are of analytical grade and purchased locally.

2.2 Cell Culture

Hepatocellular carcinoma cell line HepG2, Huh-7 and murine macrophage cell line RAW264.7 cells are obtained from National Facility for Animal Tissue and Cell Culture, Pune, India. The cells are cultured and routinely maintained in DMEM and RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum.[10] 1% penicillin - streptomycin and were incubated at 37 °C in a humidified atmosphere containing 5% CO2 inside a CO2 incubator. During the sub culturing of cells the adherent property can be diminished by adding 1x Trypsin solution in the cell.

2.3 Extraction and Preparation of Test Sample

Live adult *Bellamya bengalensis* f. annandalei, family Viviparidae is collected commercially from the Howrah 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 and centrifuged for 10 min (4°C) at 1600 g to precipitate the residual debris. It is partially purified by Amicon ultra filtration unit. After filtration the sample was lyophilized to its powder form. 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.

2.4 Cytotoxicity Study by MTT assay

HepG2 and Huh-7 cells ($1x10^5$) were seeded in 96-well plates and incubated inside a CO_2 incubator for 24 h before treatment. The cells were treated with different concentrations (5, 10, 25, 50, 100µg/ml) of SEBB for 24, 48 and 72 hours and graphs are plotted against control cells & Sorafenib Tosylate (Std.) treated cells. The cell growth inhibition studies were performed by MTT assay. For MTT assay the absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually at 595 nm) by microplate manager (Reader type: Model 680 XR Bio-Rad laboratories lnc.).[11] IC50 values for both the cell lines were determined for 24hrs.

2.5 Morphological Studies for Detection of Apoptosis

HepG2 and Huh-7 cells ($1x10^6$) were seeded in sterile cover slip for adherence and incubated for 24hrs. After adherence fresh media was added than cells were treated with IC_{50} dose of SEBB for 24 hrs was observed using the light and fluorescence microscope for morphological changes. The untreated control cells and SEBB treated cells were washed twice with PBS and then stained with acridine orange and ethidium bromide in 1:1 ratio. The cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.[12] Photographs were taken in 45X magnification in Leica Fluorescence microscope.

2.6 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.[13-14] The HepG2 and Huh-7 cells ($1x10^6$) were treated with 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 CaCl2 2H2O; 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.[12]

2.7 Study of Cell Cycle Arrest by Flow Cytometric Analysis

To assay the stage of cell cycle arrest in a flow cytometer.[15] $1x10^6$ cells were treated with SEBB (IC_{50} dose) for 18 hrs. Cells were washed with PBS, fixed with methanol and kept at -20 °C for 5 min. The cells 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) (12)

2.8 Caspase-3 Assay

The assay was performed using a Caspase-3 Assay kit, Colorimetric (Sigma) according to the manufacturer's protocol.[13] HepG2 and Huh-7 cells ($1x10^{7-8}$) 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 x g for 5 min at 4 °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 µl 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 20,000x g for 15 min at 4 °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 °C. The absorbance was read at 405 nm and the results were calculated using a pnitroaniline calibration curve.[12]

2.9 Toxicity study on RAW264.7 cells by MTT assay

RAW264.7 cells ($1x10^5$) were seeded in 96-well plates and incubated inside a CO_2 incubator for 24 h before treatment. The cells were treated with three different concentrations of each of SEBB, i.e., 25, 50 and 100µg/ml for 24 and 48 hours. The cytotoxicity study was performed by MTT assay with slight modifications.[12] 20 µl of MTT (5 mg/ml) was added to each well and then the plate was allowed to incubate for 3 h at 37 °C in 5% CO_2 incubator. 100 µl of DMSO was added to each well to dissolve the formazan crystal formed and the O.D values were recorded at 492 nm.

2.10 Nitric oxide (NO) assay in non- primed and rIFN-γ primed RAW264.7 cells

RAW264.7 cells ($1x10^6$) were seeded in 96-well plates and incubated for 24 h before stimulation and treatment. The cells were stimulated for 6 h in the presence of rIFN-γ (10 U/ml). rIFN-γ-stimulated cells were treated separately with SEBB with 25, 50 and 100 µg/ml) for 18 h. In addition, to study the effect of the extract on non-primed RAW264.7 cells, the cells ($1x10^6$) were treated with different concentrations of SEBB separately for 24 h without rIFN-γ-stimulation and incubated at 37 ˚C in an atmosphere of 5% CO_2. NO synthesis was measured by a microplate assay plate.[16] 100 µl of each culture supernatant was allowed to react with 100 µl of Griess reagent (1% sulfanilamide / 0.1% *N*-(1-naphthyl)-ethylenediamine

dihydrochloride / 2.5% H_3PO_4) at room temperature for 10 min. The absorbance values were recorded at 540 nm.

2.11 NO assay with PDTC in rIFN-γ stimulated RAW264.7 cells

It is well known that PDTC, an anti-oxidant compound, inhibits activation of NF-κB.[17] As an approach to determine the signaling mechanism of the extract on NO production, the influence of PDTC, NF-κB inhibitor, on SEBB treated rIFN-γ primed RAW264.7 cells was examined. RAW264.7 cells ($1x10^6$) were seeded in 96-well plates for 24 h before stimulation. The cells were activated for 6 h in the presence of rIFN-γ (10 U/ml) with or without PDTC (100 μM). The cells were then treated with SEBB 25, 50 and 100μg/ml for 18 hrs. The NO assay was performed using Griess reagent as described previously. The O.D values were read at 540 nm.

2.12 NO assay with N^GMMA in rIFN- γ stimulated RAW264.7 cells

N^GMMA is the specific inhibitor of NO production in the $_L$-arginine-dependent pathway.[18] To define if the signaling mechanism in SEBB-induced NO production participates in the $_L$-arginine-dependent pathway in RAW264.7 cells**.** Before stimulation with rIFN-γ (10 U/ml), RAW264.7 cells ($1x10^6$) were seeded in 96-well plates and incubated inside a CO_2 incubator for 24 h. The cells were activated in the presence of rIFN-γ with or without N^GMMA (10 mM) for 6 h. The cells were then treated separately with SEBB with 25, 50 and 100 μg/ml, for 18 h. Synthesis of NO was determined by performing the NO assay with the culture supernatants. The O.D values were recorded at 540 nm with microplate reader and comparison was done.

2.13. Statistical Analysis

Statistical analysis was done by Student's *t*-test. $P < 0.05$ was considered as significant.

The percentage cell inhibition was calculated by the following formula: - %Cell inhibition= 100 × (O.D of control- O.D of treated)/O.D of control O.D= Optical Density.

The percentage cell viability was calculated by the formula: - Viable cells (%) = (Total number of viable cells per ml/Total number of cells per ml) × 100.

3. RESULTS

3.1 Cytotoxicity study by MTT assay

In the viability study by MTT assay, there was a significant reduction in a time and concentration dependent manner in the O.D. values compared to that of the untreated control cells after treating the cells with SEBB at the concentration of 5µg, 10µg, 25µg, 50µg and 100 µg for 24, 48, 72 hrs in HepG2 and Huh-7 hepatoma cell lines for 24, 48, 72 hrs. Sorafenib Tosylate (2.09µg/ml) is used as standard for HepG2 and Huh-7. Both the studies showed an inhibition in cell growth in a concentration- dependent manner. The IC $_{50}$ value of SEBB was determined for the HepG2 and Huh-7 cell lines. The IC_{50} value of HepG2 cell line was 12.88µg/ml and Huh-7 cell line was 22.988µg/ml and graph was plotted accordingly.

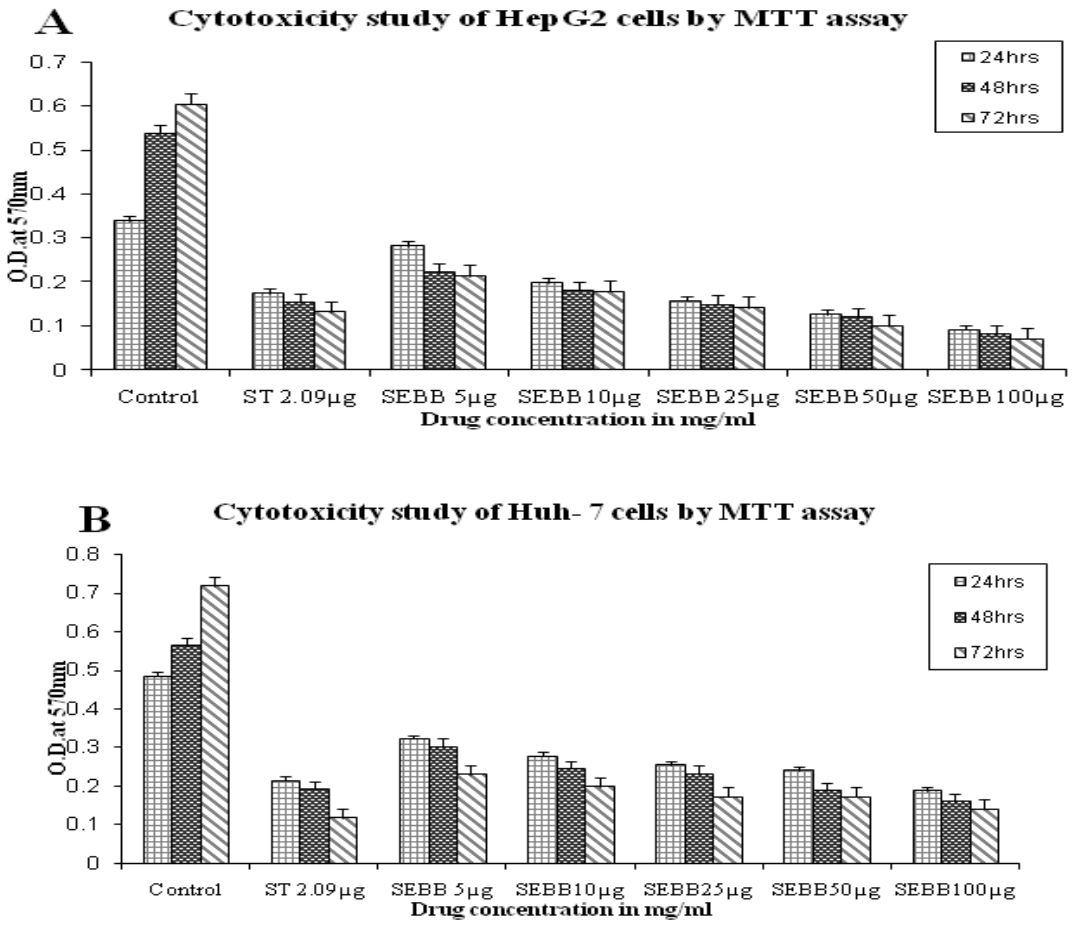

Fig1: Histogram A and B shows the effect of SEBB on cell cytotoxicity by MTT assay on HepG2 and Huh-7 cell lines after 24, 48, & 72 hrs. The O.D.at 570 nm is compared to the untreated cells and Sorafenib Tosylate treated cells. Reduction in the O.D. at 570 nm was observed in a time and concentration dependent manner. Data are mean ± S.E.M. denotes significant decrease in O.D. at 570 nm from control values p<0.05.

3.2 Morphological Studies for Detection of Apoptosis

The effect of SEBB to produce cell death in the HepG-2 and Huh-7 cell lines is observed by inducing apoptosis. After the fluorescence microscopic study, the fluorescent image showed that untreated control cells, having intact nuclei being stained with only acridine orange gives green fluorescence. But treated hepatoma cells with condensed chromatin, fragmented nuclei were stained with both acridine orange and ethidium bromide. Morphologically this indicates that the treatment with SEBB causes apoptotic change in the hepatoma cells compared to the untreated control.

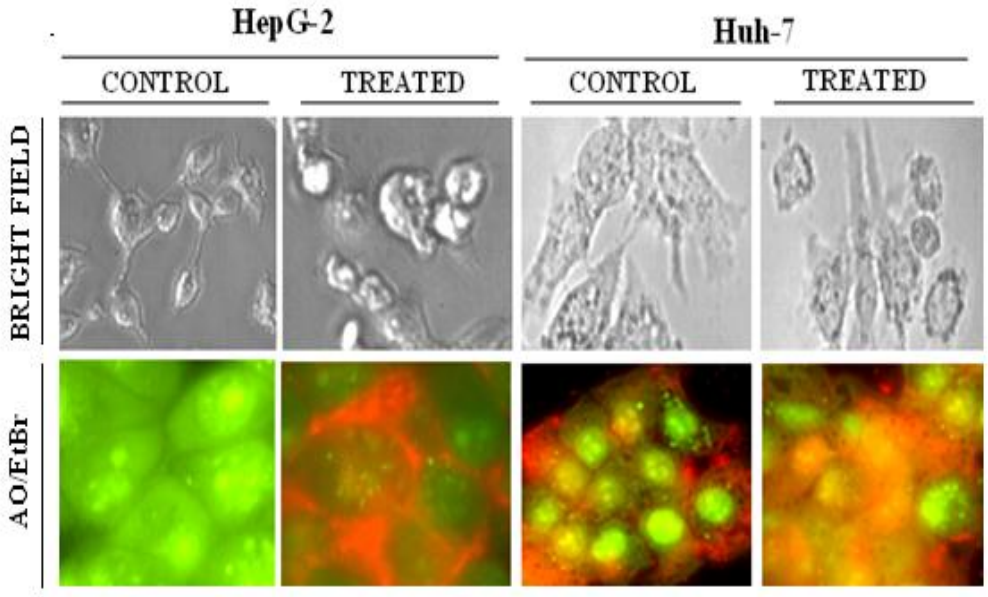

Fig.:2 Light and Fluorescence microscopic images of SEBB treated HepG-2 and Huh-7 cells. The control cells were with intact nuclei and gave bright green fluorescence whereas treated cells showed intense orange-red fluorescence showing signs of apoptosis.

3.3 Detection of Apoptosis by Flow Cytometric Analysis

In the flow cytometric analysis, double labeling 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 extreme upper right (UR) & upper left (UL) quadrant (Annexin V-/PI+) is considered as necrotic cell population. Flow cytometric data analysis revealed that after 18 h in control 9% and 9.8% where as treatment with, IC_{50} dose of

SEBB, 27.2% and 33.2% of HepG2 and Huh-7 cells were in LR quadrant (early apoptotic stage) (Fig. 3).

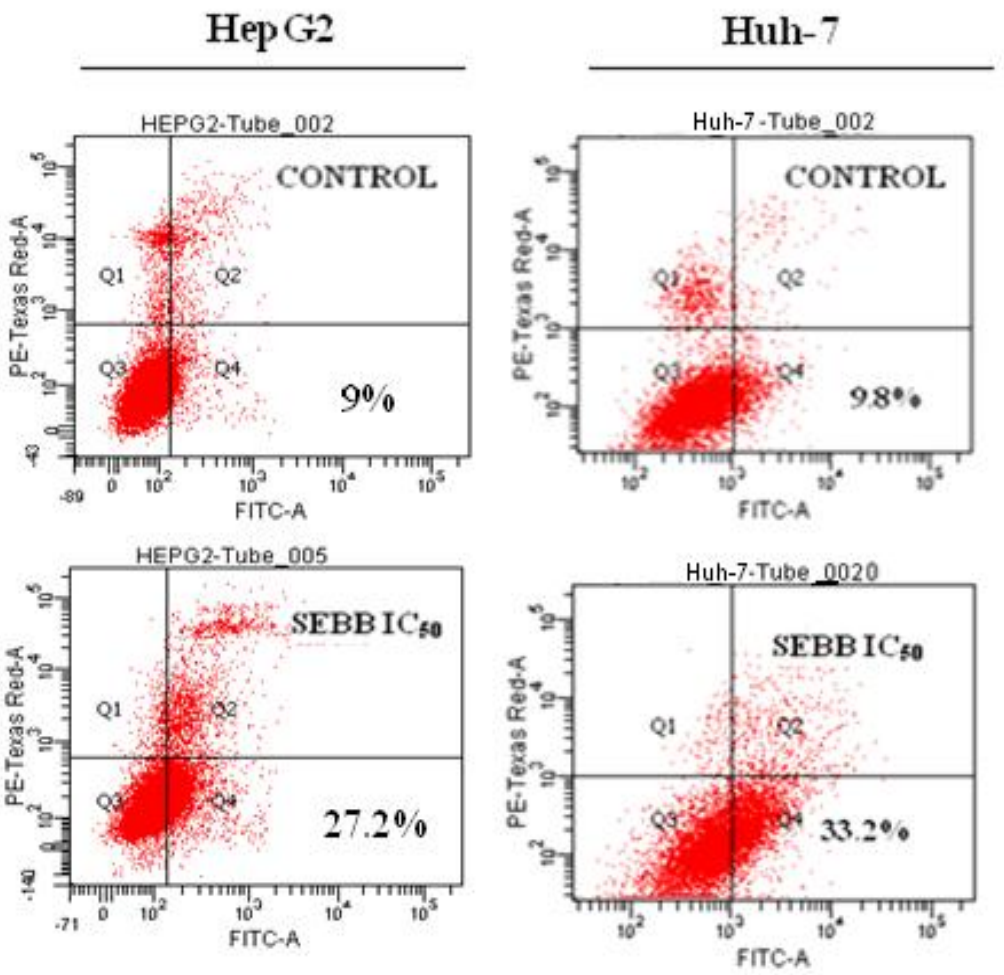

Fig. 3: Detection of apoptosis by Flow cytometric analysis in control and SEBB treated of HepG2 and Huh-7 cells respectively after 18 hrs treatment at IC_{50} doses with SEBB. Staining was done with Annexin V FITC and Propidium iodide. Dual parameter dot plot of FITC-fluorescence (*x*-axis) vs. PI-fluorescence (*y*-axis) shows logarithmic intensity.

3.4 Study of Cell Cycle Arrest by Flow Cytometric Analysis

Flow cytometric analysis showed that after 18 hrs treatment of Hep-G2 and Huh-7 with SEBB at IC_{50} dose, G0/G1 peak was changed. DNA content increased in treated cells than that of control (For HepG2 cells 68.6% against 57%and for Huh-7 cells 63.3% against 52.6% 63.3%) both in G0/G1 phase, after SEBB treatment. These result indicated that drug treatment arrested the cell cycle of the cells mostly at G0/G1 phase (Fig.4).

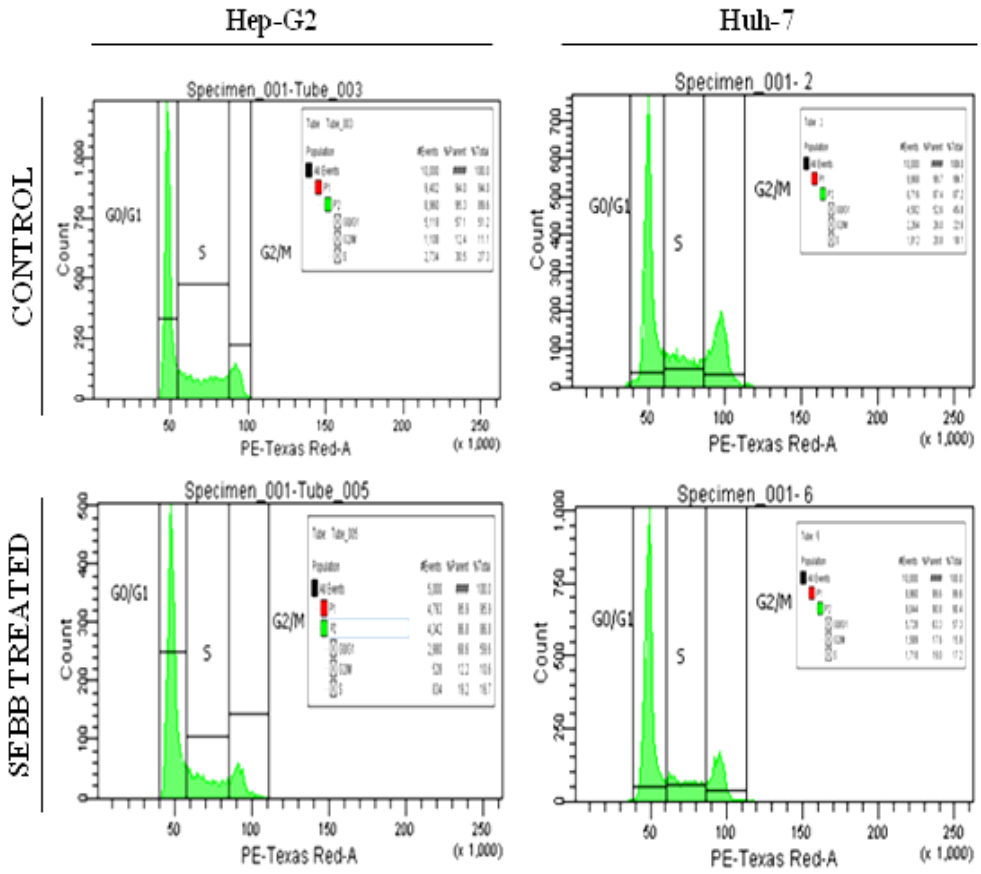

Fig. 4: Flow cytometric analysis of cell cycle phase distribution in controls and treated SEBB at IC50 dose on HepG2 and Huh-7 cells after 24 hrs treatment. Histograms represent various contents of DNA with actual number of cells (x-axis denotes fluorescence intensity of PE- Texas red and y-axis denotes count.

Caspase 3 Assay

Cell death can be two types: apoptosis and necrosis. Caspases plays an important role in apoptotic pathway causing cell shrinkage and nuclear condensation (Nancy and Lazebnik 1998). Caspase-3 production increased in HepG-2 1.38 fold after 24hrs treatment by SEBB at IC_{50} dose. In case of Huh-7 cell line the increases was 1.43 fold, both the hepatocellular carcinoma cells when compared with that of the untreated control cells (Fig. 5).

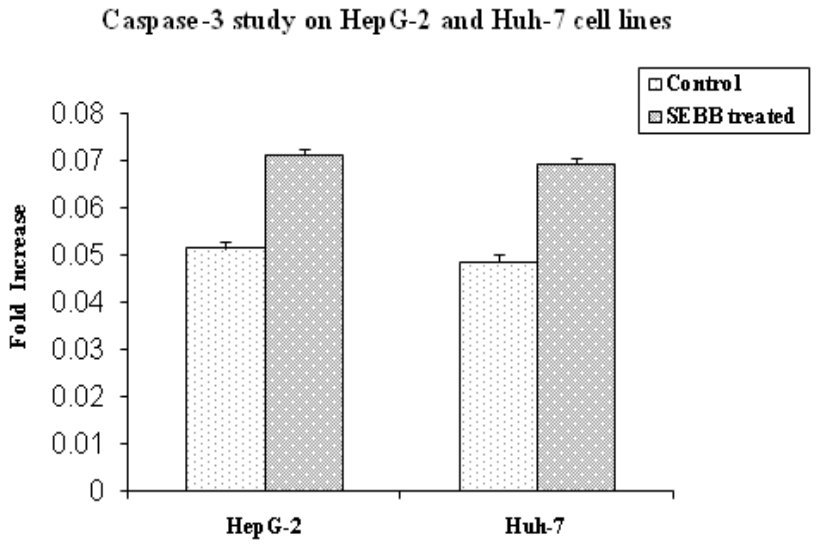

Fig.5 Fold increase in caspase 3 production in HepG-2 and Huh-7 cell lines after SEBB treatment for 24 hrs at IC_{50} dose with respect to control.

3.5 Toxicity study in RAW264.7 cells by MTT assay

Treatment with SEBB (Fig.6) for 24h did not show any cytotoxic effect on the RAW264.7 cells. However, when treated for 48 hrs it significantly enhances the cell growth than control cells as observed by the MTT assay.

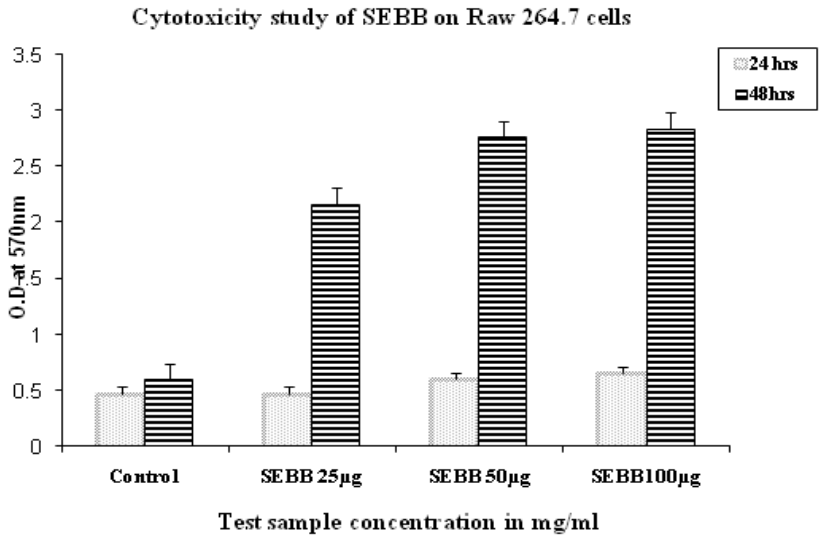

Fig.6: Histogram shows the effect of SEBB on cell cytotoxicity by MTT assay on Raw264.7 cell line after 24 and 48 hrs. The O.D.at 570 nm is compared to the untreated control cells. Data are mean ± S.E.M.* denotes significant increase in O.D. at 570 nm from control values $p<0.05$.

3.6 Nitric oxide (NO) assay in resting and rIFN-γ primed RAW264.7 cells

NO production was slightly elevated in resting RAW264.7 cells treated alone with SEBB. However, when the cells were activated with rIFN-γ for 6 h and then treated separately with SEBB (Fig.7), NO production was markedly enhanced compared with that of non-primed conditions.

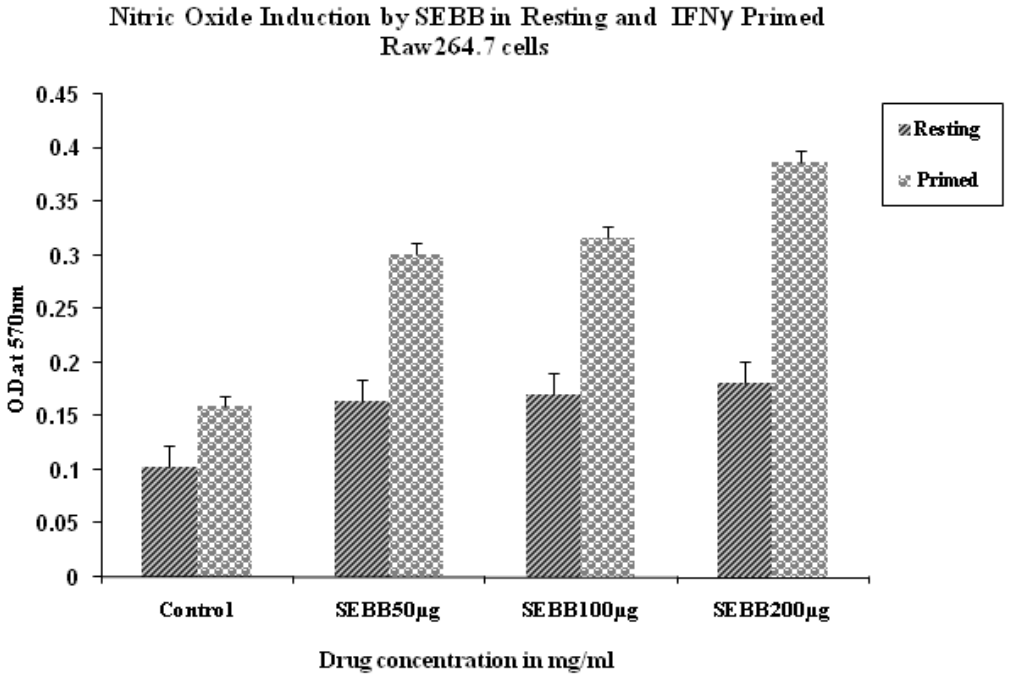

Fig.7: Experiments showed that SEBB itself was able to induce NO production in resting RAW264.7 cell line. But when SEBB was used in combination with recombinant interferon-γ (rIFN-γ), there was a marked cooperative induction of NO production in murine macrophage RAW264.7 cells.

3.7 Effect of PDTC in SEBB & rIFN-γ stimulated RAW264.7 cells

Pre-treatment with PDTC to the rIFN-γ plus SEBB stimulated (Fig.8) RAW cells caused significant block in the production of Nitric Oxide.

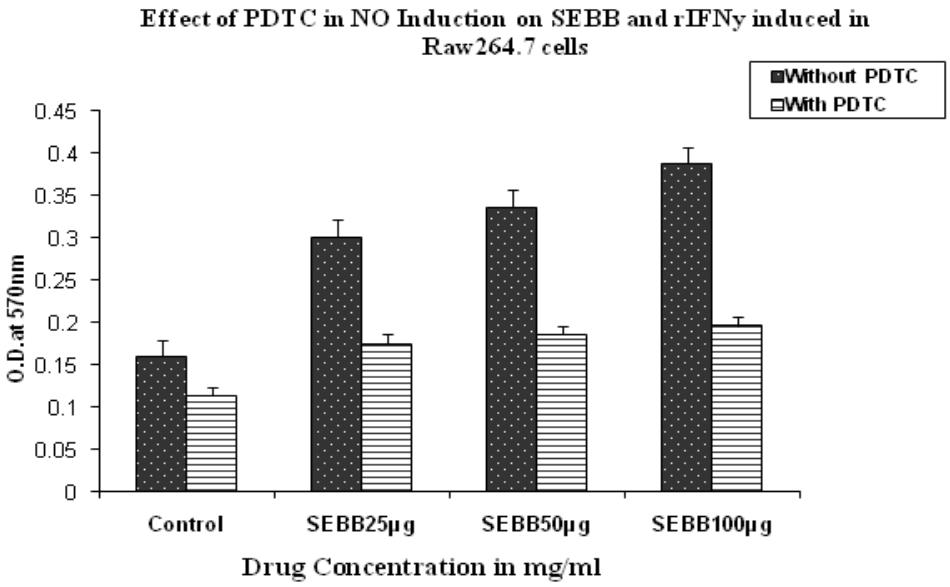

Fig. 8: Histogram represents the effect of PDTC on SEBB induced NO production in rIFN-γ activated murine macrophage RAW264.7 cell line.

3.8 NO assay with *NGMMA* in rIFN-γ stimulated RAW264.7 cells

The production of NO by rIFN-γ plus SEBB (Fig.9) in RAW264.7 cells was significantly decreased due to the pre-treatment of N^{G}MMA.

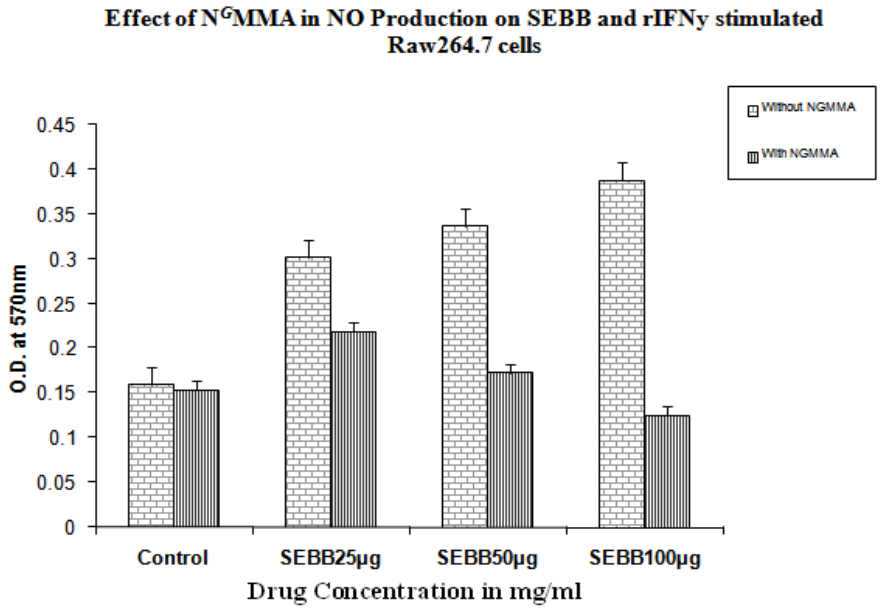

Fig. 9: Histogram represents effect of N^{G}MMA on SEBB induced NO production in rIFN-γ stimulated macrophages RAW264.7 cells.

1. DISCUSSION

Chemoprevention ,which refers to the use of non-toxic chemical substances to inhibit, delay and/ or reverse cellular events associated with carcinogenesis, is regarded as a promising alternative strategy to therapy for the management of cancer.[19] In our previous study SEBB showed a significant antiproliferative, cytotoxic and apoptogenic activity against human myeloid leukemic cell lines.[20] The present study reveals that the secretion extract of the *Bellamya Bengalensis* f. annandalei (Kobelt, 1908) possesses highly significant anti-hepatocellular carcinoma activity. The anti- proliferative and the cytotoxic activities of SEBB have been supported by the observations in MTT assays. SEBB inhibit the growth and metabolic activities of Hep G2 and Huh-7 cell line in a concentration & time dependent manner and IC_{50} dose of both the cell lines were determined. However, treatment with SEBB on RAW264.7 cells for 24 and 48 h did not show any cytotoxic effect but it significantly enhances the cell growth than control cells as observed by the MTT assay.These finding reveals that the SEBB preferentially acts on Hep G2 and Huh-7 human liver carcinoma cells.

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. Light and Fluorescence microscopic images clearly showed nuclear disintegration of SEBB treated both the Hep-G2 and Huh-7 cells compared with that of the untreated control cells when stained with acridine orange and ethidium bromide. In the first stage of apoptosis the changes in the membrane composition leads to extracellular exposure of PS residues which bind Annexine-V, a natural ligand, in a calcium dependent manner.[21, 22] Membrane changes leading to PS exposure occur readily in apoptotic cells, while the cell lose membrane integrity later in the apoptotic process and expose DNA. So using a DNA binding dye PI in tandem with flurochrome conjugated Annexin-V, apoptotic cells and necrotic cells can be discriminated by Flowcytometric.[21] So, the flowcytometer by this double straining provides clear detection of three populations of cells (viable, apoptotic & necrotic). Thus, the early apoptotic cells bind only Annexin-VFITC and late apoptotic cells with both Annexin –V FITC and PI & viable cells do not take any of the dye and so FSC and SSC are absolutely different for all cell types.[23] For further evidence in support of the apoptogenic activity of SEBB was obtained from the flow cytometric data analysis. 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.[24-26] 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 HepG2 and Huh-7 cells.

Flowcytometric determination of cell cycle phase distribution has further confirmed the SEBB causes cell cycle deregulation in both the cell lines. Cell cycle analysis revealed that treatment with SEBB arrested both the HepG2 and Huh-7 cell populations in the G0/G1 phase of cell cycle. Caspases, a family of cysteine proteases have emerged as key enzymes in the regulation of apoptotic pathway. Activation of Caspase leads to the classical form of apoptotic pathway.[27] Caspase-3 assays showed increase in the activities of caspase-3 after SEBB treatment in HepG2 and Huh-7 cells. From the above performed experiments it can be confirmed that SEBB possesses anti- Hepatocellular carcinoma activity in both the HepG2 and Huh-7 human liver carcinoma cell lines.

Treatment with SEBB on RAW264.7 cells for 24 and 48 h did not show any cytotoxic effect but it significantly enhances the cell growth so, we studied the NO assay because induction of NO production and gene expression by activated macrophages can lead to cytostatic and cytotoxic effects on malignant cells.[28-30] The pivotal role of NO in the tumoricidal activity of macrophages has now been well established.[31] In this study, it was demonstrated that SEBB themselves able to induce NO production in RAW264.7 cell line. But when SEBB was used in combination with recombinant interferon-γ (rIFN-γ), there was a marked cooperative induction of NO production in murine macrophages RAW264.7 cells. The elevated production of NO by rIFN-γ plus SEBB treated RAW264.7 cells were markedly inhibited by pre-treatment with PDTC, an inhibitor of NF-κB. Nuclear factor kappaB (NF-kappaB), a transcription factor, plays an important role in the regulation of immune and inflammatory responses. NF-κB proteins are involved in the activation of an exceptionally large number of genes in response to infection, inflammation and other stressful situations. The inducible form of nitric oxide synthase (iNOS) is also under the control of NF-κB.[32-35] Signal transduction pathway of NO production has been previously reported as LPS stimulation of rIFN-γ-primed macrophages induces NF-κB activation.[36] Since observations showed that pre-treatment with PDTC blocked NO production by resting and rIFN-γ-stimulated, SEBB treated RAW264.7 cells, these findings might explain that SEBB influence NO production via the NF-κB signaling pathway. The results of this study suggest that SEBB may provide a second signal for induction of NO production in RAW264.7 cells. N^GMMA, an analog of $_L$-arginine, inhibited SEBB induced NO production by resting as well as rIFN-γ-activated RAW264.7 cells. The strong inhibition of NO production by N^GMMA indicates that the signaling mechanism in SEBB induced NO production participates in the $_L$-arginine-dependent pathway in RAW264.7 cells.

CONCLUSION

Our results demonstrated that the SEBB acted as an accelerator of activation of RAW264.7 cells by rIFN-γ via a process involving $_{L}$-arginine-dependent NO production and that SEBB elevated NO production via activation of NF-κB signaling pathway. SEBB was found to have significant anti-cancer activity against hepatocellular carcinoma cell lines- HepG2 & Huh-7 and significant immunomodulatory activity of SEBB was observed in cooperation with rIFN-γ in RAW264.7 cell line. These findings point to the possibility and hope of developing novel and potential anti-cancer agents derived from SEBB with minimum or less side effects.

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