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APOPTOSIS INDUCING POTENTIALTY OF SPATHODEA CAMPANULATA BARK AGAINST HEPATOCELLULAR CARCINOMA: HEPG2 CELLS

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ABSTRACT

Bark extract of *Spathodea campanulata* (BESC) showed potential activity on glioma cells in our previous study but in its activity on hepatocarcinogenesis of hepatoma cells have not been reported so far. Therefore, in this present study we investigated the apoptosis activity of bark extract of *Spathodea campanulata* (BESC) on hepato-carcinoma cells. BESC significantly inhibited cell viability in a time and concentration dependent manner in the HepG2 cells. Morphological study showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies in the BESC treated cells. Agarose gel electrophoresis and detection of apoptosis by flow cytometric analysis had been also performed to ensure the appreciable number of cells in early & late apoptotic stages of HepG2 cells. Cell cycle phase arrest was observed in the G0/G1 phase and MMP shift assay exhibited significant change in the BESC treated cells. The good performance of *S. campanulata* towards hepatocellular carcinoma (HCC) cell line confirms it could be used as a natural source of anti-tumor agents with minimum side effects.

KEYWORDS: Hepatocellular carcinoma, *Spathodea campanulata*, Bark, Apoptosis.

INTRODUCTION

The liver is the vital organ of the body which performs an immeasurable number of physiological functions. For the proper function of the body, the liver must have to keep healthy. It has a great role in circulatory, digestive, endocrine, immune system, etc. Any abnormality leads to disrupt all the related function resultant an immunocompromised circumstance that makes our body 'infection house'. Among them, liver cancer is one of the main reasons. More than 800,000 individuals are diagnosed to have this disease every year all through the world. Liver cancer is also a leading cause of cancer deaths worldwide, accounting for more than 700,000 deaths each year (ACS, 2019). There are 782,000 death occur in the year of 2018 due to liver cancer (WHO, 2018). Hepatocellular carcinoma (HCC) is one of the most common, aggressive, and highly resistant forms of tumors in the world.[1,2]

The exact reasons for hepatocarcinogenesis are unknown. Previous studies showed that the HCC is a multifactor and multistep process which has several risk factors, which include liver cirrhosis, hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, alcohol addiction, fatty liver, etc. and also environmental factor including toxins (Aflatoxin).[3,4,5,6] Influence to these factors the p53 gene gets mutated resulting in prevention in the tumor suppression mechanism which initiates the hepatocarcinogenesis.[7] Till now there is no conventional treatment of HCC so there is an urgent need for efficient therapeutics.

Many researchers have found that there are natural antitumor compounds that induce apoptosis with minimum toxicity and side effect. In India plant is the crown of the Indian traditional system that solves the mystery of many diseases, the use of the plant for the treatment of various types of disease is an ancient practice. Bark extract of Spathodea campanulata (BESC) has been investigated against human hepatocellular carcinoma HepG2 cell line. However different parts of Spathodea campanulata have been used for several ailments including anti-inflammatory, analgesic,[8] diabetes,[9] antioxidant, antimicrobial,[10] with cardio[11] and nephroprotective[12] effects. Be that as it may, no work on hepatocellular carcinoma has been accounted for up until this point. The present investigation is an approach to study the apoptosis-inducing activity of Bark extract of *Spathodea campanulata* (BESC) against Hepatocellular carcinoma (Hep-G2).

MATERIALS AND METHODS

Chemicals

The following chemicals were used: DMEM medium, Foetal bovine serum (FBS), trypsin (Gibco, USA), HEPES, L- glutamine, Penicillin- Streptomycin (Bio-west, Germany), Gentamycin (Nicholas, India), MTT [3-(4,5-dimethylthiozol-2-il)-2,5-2,5-dipheniltetrazoliumbromide], Ethidium bromide and Acridine orange, Annexin V- FITC apoptosis detection kit, RNase, Propidium iodide, were purchased from Sigma (St. Louis, MO, USA), Proteinase k (SRL), DMSO (dimethylsulphoxide),Chloroform, isoamyl alcohol, Methanol (Merk), and all other chemicals and reagents were of analytical grade and procured locally.

Cell culture

HCC cell lines HepG2 was purchased from the National Facility for Animal Tissue and Cell Culture, Pune, India. The cells were maintained in DMEM medium (Gibco, USA), supplemented with 10% heat inactivated FCS, 100U/ml Penicillin (Biowest, Germany), and 100mg/ml Streptomycin (Biowest, Germany). Cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO2 in air. In all the experiments untreated HCC cells were termed as control group.

Plant Material

The bark of *Spathodea campanulata* (family: Bignoniaceae) was collected from the park of Kasba, Gariahat, Kolkata, India in the month of May, 2015. The bark of this plant was authenticated by Dr.V. P. Prasad, Scientist - D, Central National Herbarium, Botanical Survey of India, Ministry of Environment & Forests, and Government of India at Howrah, West Bengal. A voucher specimen No is CNH/Tech. II/2015/30/309 was deposited in the Cancer Biology & Inflammatory Disorder Division, CSIR, Indian Institute of Chemical Biology, Kolkata.

Preparation of extracts

The barks of *Spathodea campanulata* were harvested during dry season and air dried, then grinded into powder (200gm) and soaked in about 350 ml petroleum ether (Merck) in room temperature and was repeated 2 times. Petroleum ether extract was obtained. Then, it was dissolved in 500 ml of methanol (Merck) in room temperature for 7 days with occasional shaking. The extract was concentrated in a Buchi rotary evaporator at 40°C and stored at room temperature for further in-vitro study. The methanol free extract of *Spathodea*

campanulata bark was yield 19.4 g. Stalk solution was prepared as 1mg/ml in PBS from here desired concentrations (25,50, 100,200µg/ml) was used for *in-vitro* experiments.

Detection of Cytotoxicity by MTT assay

HepG2 cells ($1x10^5$) were separately seeded in 96-well sterile plates for 24, 48 and 72 hrs. All the treated cells were grown in humidified atmosphere containing 5% CO2 in an incubator at 37℃ and the untreated cells were considered as control. After desired incubation 20µl of MTT (4-5mg/ml in PBS as a stock solution) was added to each well and incubated again for 3 to 4hrs at 37℃. The MTT assay is a colorimetric assay for assessing the metabolic activity of the cells or cell viability of NADPH dependent cellular oxidoreductase enzymes, and represents number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT, which is yellow in colour, to insoluble purple coloured formazan. The intensity of the colour was measured at 570nm by micro-plate manager (Reader type: Model 680XR Bio-Rad Laboratories Inc.). The IC50 values were determined for the all the carcinoma cells.

Morphological studies for detection of apoptosis

Light Microscopy Study

To investigate the effect of BESC on cell morphology 1×10^6 cells (HepG2) were seeded in DMEM media supplemented with 10% FBS. After 24 hrs, the cells were treated with IC_{50} dose of BESC and then the cells were then observed under a light microscope (Leica DMI 4000 B) at a magnification of 40X.

Fluorescence microscopy study

HepG2cells ($1x10^6$) were treated with different IC50 doses of BESC (corresponding to each cell line) for 24 hrs and observed using a fluorescence microscope for determining morphological changes. The untreated control cells and BESC treated cells were harvested separately (centrifuged at 1000 rpm for 5 min), the pellets were washed twice with PBS and then stained with 100µg/ml of acridine orange (Sigma, USA) and 100µg/ml of ethidium bromide (Sigma, USA) in a ratio of 1:1. The cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.

Confocal microscopy study

HepG2cells ($1x10^6$) were treated with different IC_{50} doses of BESC for 24 hrs. After 24hrs the untreated control cells and BESC treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10µg/ml of Propidium iodide (Sigma) for 5 min. After mounting on slides the cells were observed to determine the differences in nuclear morphology between the untreated and BESC treated HCC cells under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystem, Heidelberg, Germany) installed with an inverted microscope (LeicaDM-7RB).[10] Images for Propidium iodide was acquired from UV laser line using 450 nm band-pass filters for UV for images.

Agarose gel electrophoresis study

HepG2 cells were treated with IC_{50} dose of BESC for 14 hrs then cells were resuspended in 500µl of lysis buffer (50 mM Tris-HCl, pH -8.0, 10 mM EDTA, 0.5% SDS), 100µg/ml of proteinase K was added and incubation was done at 50ºC for 1 h and 37ºC overnight respectively. DNA extraction was done by following the general phenol-chloroform extraction procedure and kept at -20ºC overnight. After centrifugation, DNA precipitates were washed with 70% ethanol, dried and evaporated at room temperature and dissolved in TE buffer (pH 8.0) at 4ºC overnight. To detect the DNA fragments, the isolated DNA samples were electrophoresed overnight at 20 V in 1% Agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator.[13]

Mitochondrial membrane potential (Δψm) assay

HepG2 ($1x10^6$) cells were treated with BESC with desired dose and untreated as control for 24 hours to assay the mitochondrial membrane potential activity of cell in a flow cytometer. Cell were washed with PBS, pelleted down and eventually stained with JC-1 stain. The sample were incubated at 37ºC for 15 min. Change in the mitochondrial membrane potential was determined by FACS (Becton Dickinson FACS Fortessa 4 leaser cytometer), Fluorescence detector equipped with 520 nm argon laser light source and 623 nm band pass filter (liner scale) with the help of BD FACS Diva software (Becton Dickinson).

Apoptosis assay

In order to investigate the type of cell death induced by BESC, flow Cytometric analysis was done by performing dot plot assay. The HepG2cells ($1x\ 10^6$) were treated with individual IC_{50} dose (18 hrs) of BESC for 18 hrs. The cells were centrifuged at 2000 rpm for 8 min at 4°C and pelleted down. Then washed with AnnexinV- FITC binding buffer provided in

apoptosis kit (Sigma). Again after centrifuging at 2000 rpm at 4°C, the cell pellets were dissolved in AnnexinV- FITC binding buffer containing AnnexinV- 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 LSR Fortessa 4 laser Cytometry. Flow-Cytometry 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 BD FACS Diva software program.[13]

Cell cycle arrest study

To assay the stage of cell cycle arrest in a flow cytometry, HepG2 cells ($1x10^6$) cells were treated with BESC (IC_{50} dose) for 18 hrs. Cells were washed with PBS, fixed with cold methanol. They were then resuspended in cold PBS and kept at 4 °C for 90 min. Cells were pelleted down, dissolved in cold 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 4 laser cytometer), fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using BD FACS Diva software (Becton Dickinson).[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.

RESULTS

Detection of Cytotoxicity by MTT assay

The methanolic extract of BESC showed a significant effect on liver cell line HepG2. The apoptotic effect was obtained in a concentration (25, 50, 100, and 200 µg) and time (24, 48 & 72hrs) dependent manner. The IC50 estimation of BESC treatment for 24hrs was determined to be 151 µg/mL for HepG2.

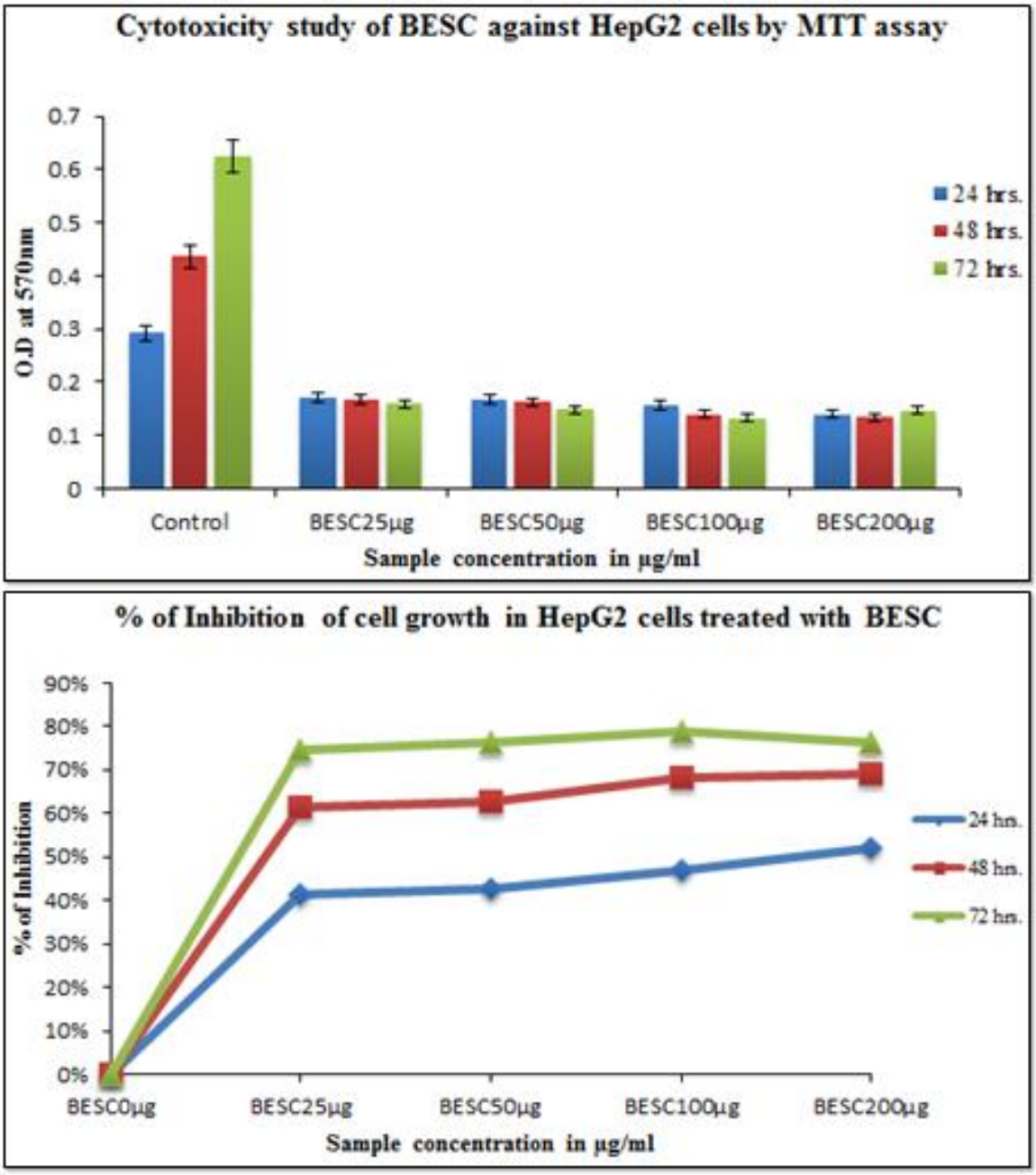

Fig. 1.0: Histogram showing the significant decrease in OD value of HepG2 cells after treating with BESC than control. The treated cells showed progressively increase in % of inhibition with time and concentration dependent manner. The IC_{50} value of treated cells for 24hrs was calculated to be 151µg/mL for HepG2 cells.

Morphological study by light microscope

Light microscopic images clearly show membrane disintegration in HepG2 cells treated with IC_{50} dose of BESC compared whereas untreated cells showed healthy morphology.

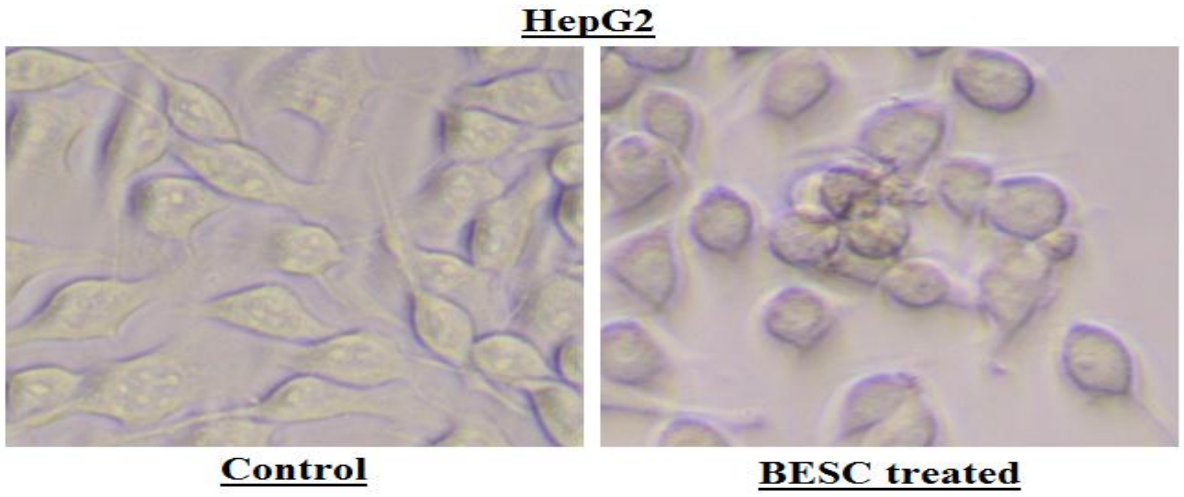

Fig. 2.0: Showing the light microscopic images of control vs treated cell line of hepg2 with ic_{50} dosage. The untreated cells showed intact morphology whereas the treated cells show distorted morphology.

Morphological study by fluorescence microscope

The morphological changes were observed in HepG2 cells treated with BESC with IC_{50} dose and stained with both acridine orange and ethidium bromide. As shown in Figure 2.0 normal live cells were bright green in colour whereas BESC treated HepG2 cells were bright orange in colour with condensed nuclei. In addition, normal cells showed organized structure, while apoptotic cells showed highly condensed chromatin in HepG2 cells treated with BESC. The formation of apoptotic body and chromatin condensation indicates the induction of apoptosis.

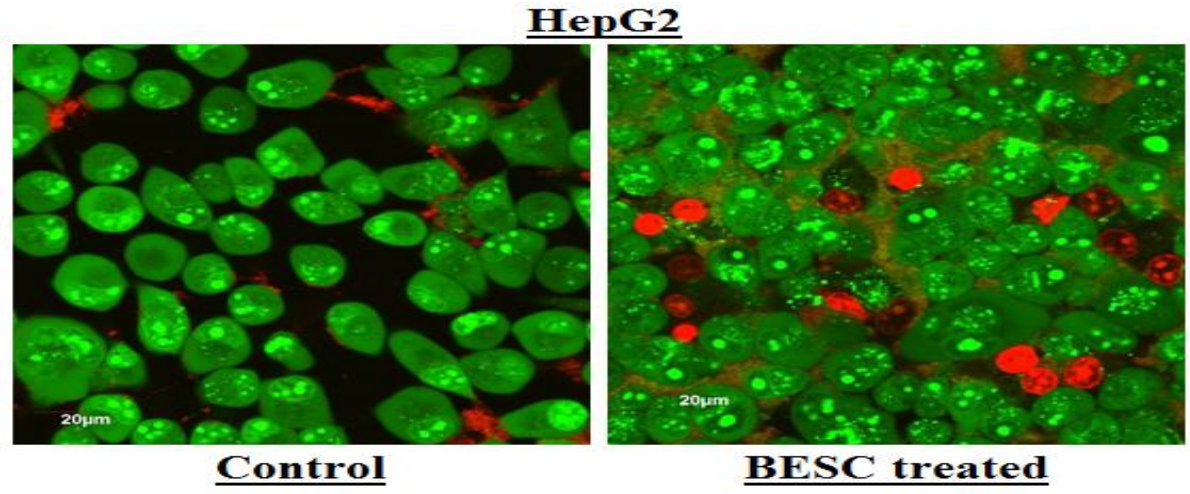

Fig. 3.0: Showing Fluorescence microscopic images of control vs treated cell line of HepG2 with IC_{50} dose of BESC. The untreated cells give a bright green fluorescence whereas the treated cells show an orange-red colour, suggesting the occurrence of Apoptosis.

Morphological study by confocal microscope

BESC showed an apoptotic effect on HepG2 cell line after 24 hrs. of treatment with IC_{50} Value. The cell was stained with PI which binds with ds DNA of distorted cell whereas normal cell prevent the entry of PI. It shows the induction of apoptotic body in HepG2 cell line (fig.4.0).

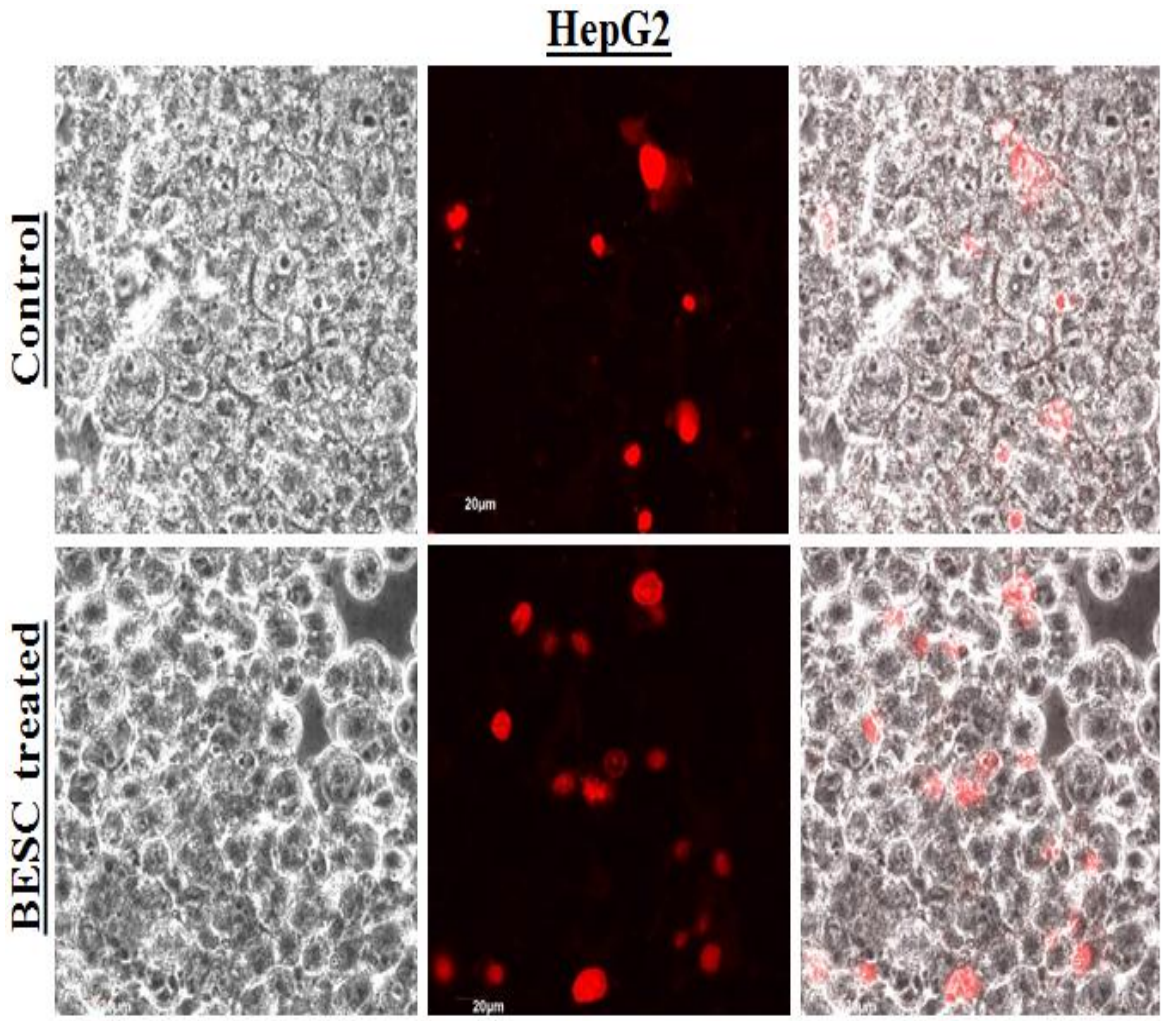

Fig. 4.0: Showing Confocal microscopic images of control vs treated cell line of HepG2 with IC_{50} dosage. The Untreated cell showed complete morphology whereas the treated cells show disrupted morphology due to fragmented nuclei and apoptotic body formation take place.

Agarose gel electrophoresis study

Agarose gel electrophoresis of the DNA samples isolated from both control and treated cell. The untreated HepG2 cells showed intact DNA bands, whereas BESC treated HepG2 cells showed fragmented laddering pattern of DNA. So, the observations demonstrate that the treatment with BESC induce apoptosis (fig.5.0).

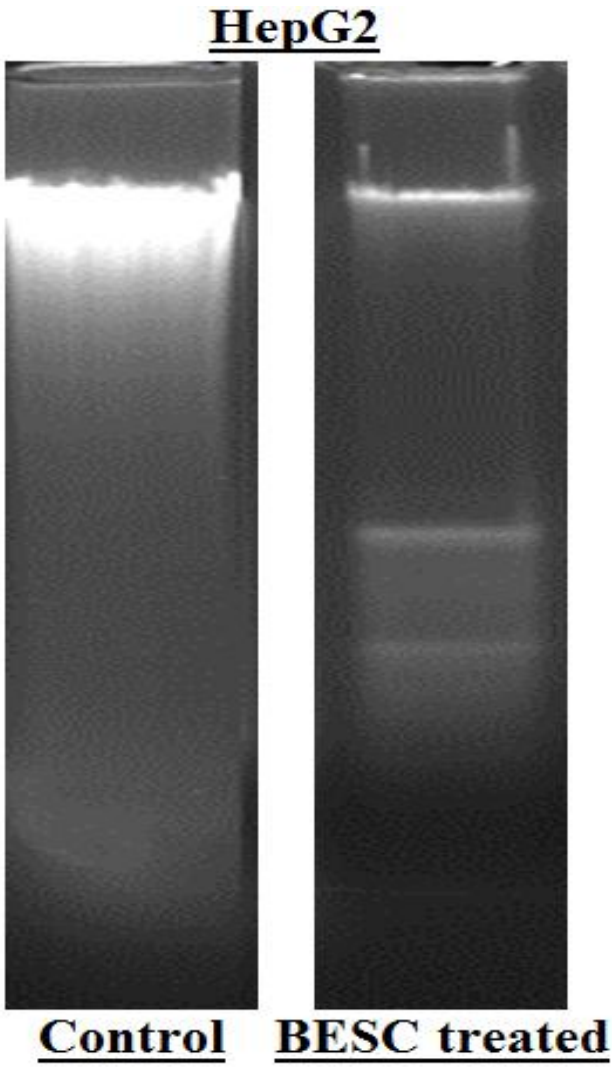

Fig. 5.0: Showing agarose gel study of control and BESC treated of HepG2 cell with IC_{50} dose. The control of HepG2 cell showed thick band of DNA whereas BESC treated cell showed laddering pattern in DNA.

Detection of mitochondrial membrane potential (Δψm) assay

Disruption of mitochondrial membrane potential is another method for studying of the apoptosis. HepG2 cells were treated with desired dose of BESC, showed a loss of Mitochondrial Membrane Potential (Δψm). Due to lipophilic nature of JC-1 dye it cannot endure in the mitochondria of the apoptotic cell because of dysfunction of mitochondrial membrane potential. In addition live cell flourish red pigment (P3) and apoptotic cell showed green fluorescence (P4). An efficient transmembrane shift was obtained from 18.7% to 69.0% was obtained when HepG2 cells were treated with the IC_{50} dose of BESC for 24 hours.

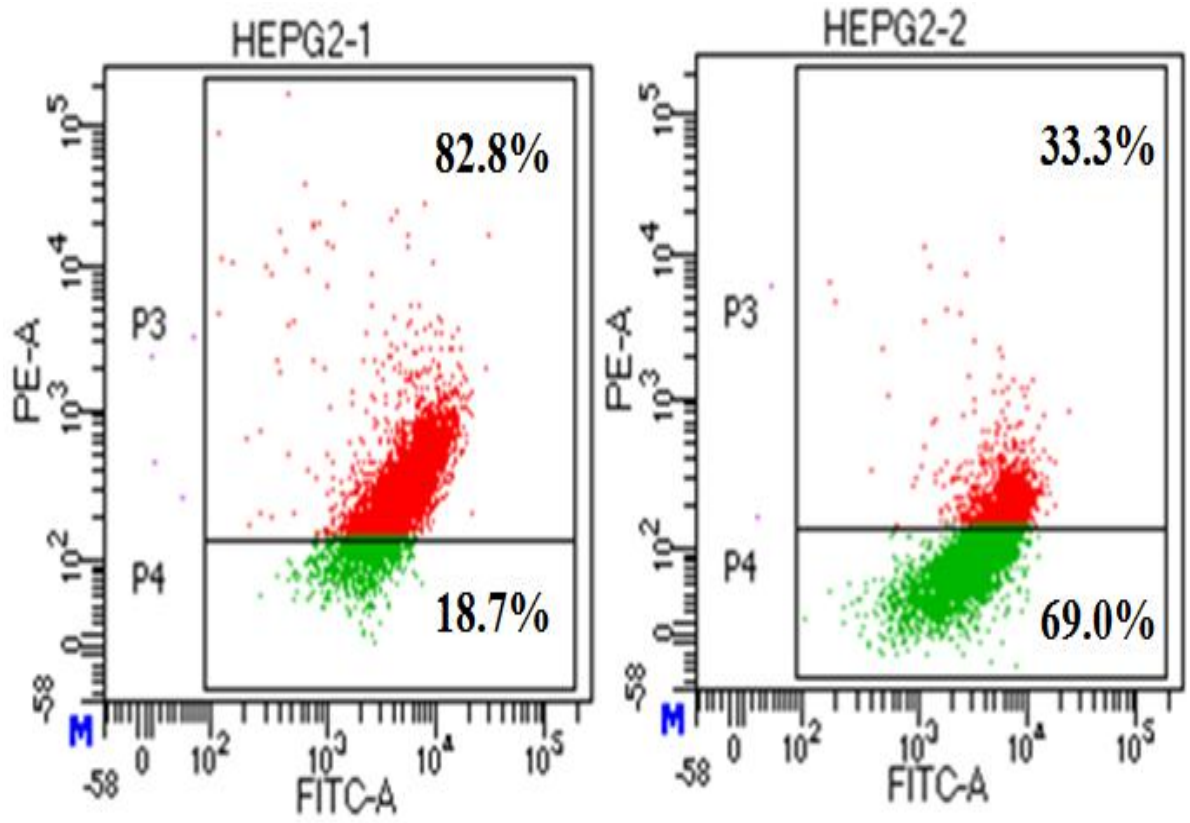

Fig. 6.0: Showing Flow cytometric analysis of mitochondrial membrane potential (Δψm) on HepG2 cell line in both untreated and BESC treated cells after 24 hrs. A significant shift red to green fluorescence was obtained in HepG2 cells.

Detection of Apoptosis by Flow Cytometry

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 extreme upper right (UR) & upper left (UL) quadrant (annexin V-/PI+) is considered as necrotic cell population. Flow cytometric data analysis revealed that after 24 h of treatment with desired dose of BESC for quantification of apoptosis was observed for HepG2 3.4% against 7.5% were in lower right quadrant. Which signify the BESC have apoptotic inducing property on HepG2 cell line (fig.7.0).

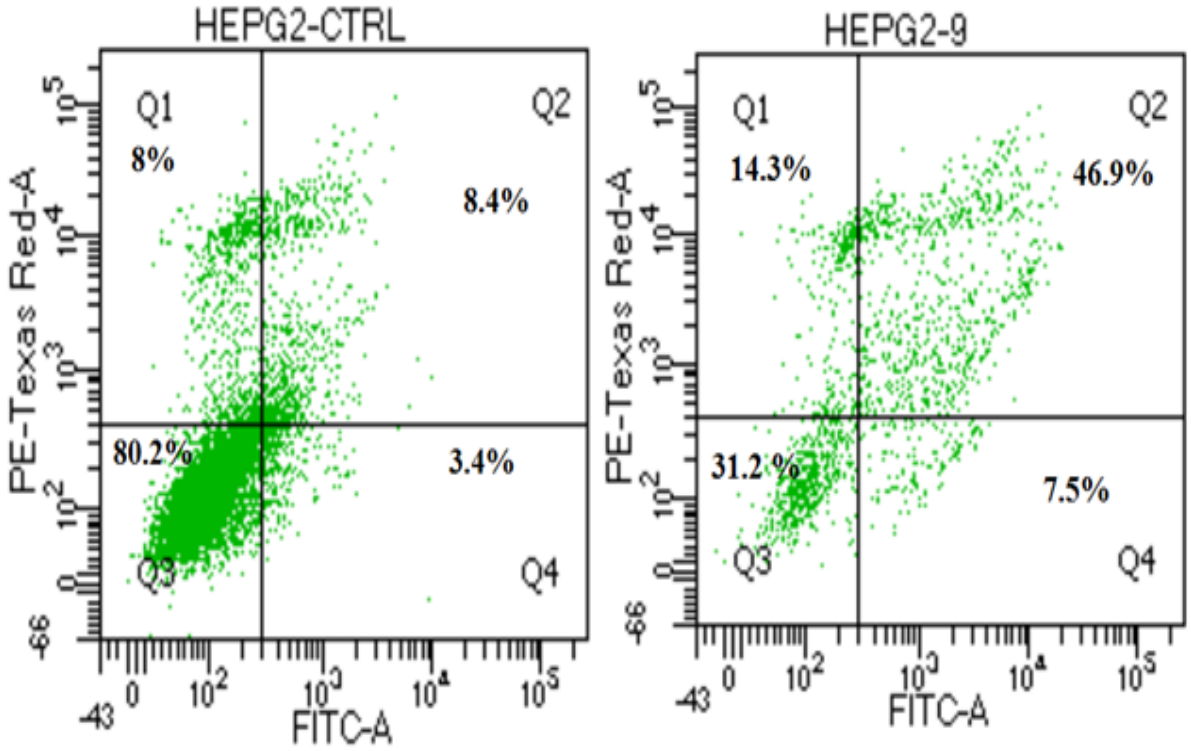

Fig. 7.0: Showing dual parameter of flow cytometric analysis of control and BESC treated of HepG2 cells stained with Annexin V FITC and propidium iodide, Dual parameter dot plot of FITC fluorescence (*x*-axis) *vs* PI-fluorescence (*y*-axis) shows logarithmic intensity.

Study of cell cycle arrest by flow cytometry

The flow cytometry additionally empowers the distinguishing proof during the various phases of the cell cycle. In this manner it likewise assists with deciding the relative cell DNA substance and gives information on cell position in the cell cycle. Flow cytometric evaluation showed significant expands in DNA content in G1 phase of the cell cycle from 45.5% to 69.1% in HepG2. This perception clearly showed the growth of HepG2 cell line is repressed by arresting the cell populations in the sub-G0/G1 as well as G2/M phase of the cell cycle (fig.8.0).

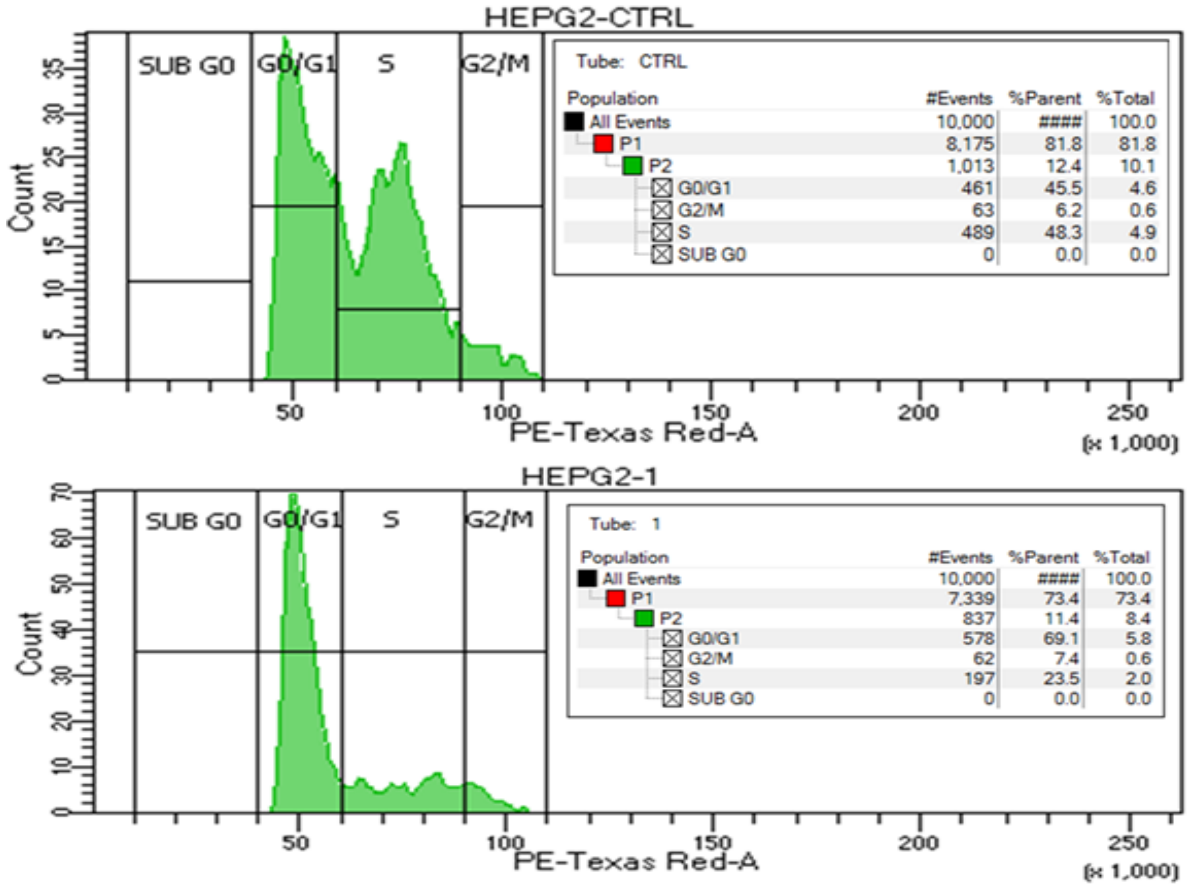

Fig. 8.0: Showing flow cytometric analysis of cell cycle phase distribution in controls and BESC treated with IC_{50} dosage on HepG2 cell after 24hrs of treatment. Histograms represent increase in DNA content with actual number of cells which demonstrating the apoptosis inducing property of BESC on HepG2 cell line.

DISCUSSION

The search for potent anticancer agents is still a dream. Natural product is a significant source of anti-cancer drug. Therefore most of the researchers shifted to the natural sources. Nowadays, active constituents have been isolated and used to treat human tumors. The ethnopharmacological understanding is helpful to forward the search for plants with potent cytotoxic activity.[15] The hepatocellular carcinoma (HCC) is viewed as one of the most common malignant tumors and life-threatening. The present study demonstrates the anti-tumor effect of bark extract of Spathodea campanulata (BESC), methanolic, extracts on the HepG2 cell line. It showed growth suppressing capability against a hepatocellular carcinoma (HCC). BESC significantly showing the cytotoxic effect on the HepG2 cell line. It is due to the induction of apoptosis resulting in DNA fragments into the cell cytoplasm. Cell cycle analysis revealed that treatment with BESC arrested the HepG2. Due to the induction of

apoptosis in a natural way without any Side-effect become an approaching target in both therapeutic and preventive approaches in cancer.

Our findings are further supported by the observations in MTT assays (fig.1.0). BESC significantly inhibited the growth of all the HepG2 cells in a concentration and time-dependent manner. Light microscopic images also revealed membrane disintegration in HepG2 cells which was treated with BESC whereas untreated cells showed intact membrane (fig.2.0). Fluorescence microscopic images clearly showed nuclear disintegration of BESC treated HepG2 cells whereas the untreated cells showed normal morphology. It was stained with acridine orange and ethidium bromide. The untreated cells showed bright green fluorescence with an intact membrane which excluded the entrance of ethidium bromide (fig.3.0). On the other hand, BESC treated cells showed a combination of orange-red fluorescence because apoptotic and necrotic cells could not exclude the dyes and gave a combination of orange-red. So, the observations indicated that the treatment with BESC was inducing apoptosis in the HCC cells. To check the chromosomal morphology, cells were stained with PI and observed under the confocal microscopy. BESC treated cells showed distorted morphology and several signs of apoptosis-like chromatin condensation, nuclear fragmentation and, formation of apoptotic bodies whereas the untreated control cells were with intact nuclei (fig.4.0). Further findings are supported by agarose gel electrophoresis. BESC treated cells showed the fragmented DNA in single-lane whereas untreated cells showed one thick band of DNA (fig.5.0). This simply suggests the BESC is able to successfully degrade the genome of the HepG2 cell line. The loss of mitochondrial membrane potential is a hallmark of apoptosis. In normal cells, the JC-1 dye able to enter into the mitochondria and flourish red pigment. In apoptotic cells, the mitochondrial membrane potential disrupts, and the JC-1 cannot persist within the mitochondria. This depolarization led to a transmembrane shift from red to green fluorescence leading to the release of cytochrome C. Apoptotic cells showing principally green fluorescence are easily differentiated from healthy cells which show red fluorescence. JC-1 staining of HepG2 cells treated with BESC shows a significant shift in the transmembrane potential from red to green fluorescence. Hence, our finding suggests a change in the transmembrane potential, by that triggering apoptosis. Another study is based on the quantitative estimation of cells undergoing early vs late apoptosis. It was further supported by dot plot assay by performing by dual staining with annexin V FITC and propidium (fig.7.0). Phosphatidylserine (PS) externalization is a hallmark of apoptosis and several markers have been discovered for the

study. The translocation of PS molecules to the external surface of the cell permits the binding of Annexin-V which is labelled with FITC.[16] Dual staining technique with annexin V FITC and propidium iodide in dot plot assay made it conceivable to distinguish live, early apoptotic and late apoptotic cells.[17,18] The study showed the increase in the number of early and late apoptosis by the treatment with BESC with IC50 dose in HepG2 cells. The deregulation of checkpoints is one of the main causes of the progression of cancer. It expels the irregularity in the cell and ensures the proper sequence of events of cells through the different phases of the cell cycle. Cell cycle analysis revealed that treatment with BESC arrested the cell populations in the G0/G1 phase of the cell cycle by inducing apoptosis (fig.8.0).

CONCLUSION

The present investigation revealed the methanolic extract BESC, isolated from *Spathodea campanulata* bark inhibited cell growth and induced cell apoptosis in hepatocellular carcinoma (HCC). The further microscopic images of BESC treated cell showed cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies in BESC treated cell which remark the occurrence of apoptosis. DNA fragmentation in agarose gel electrophoresis likewise indicates the cytotoxicity of the BESC. We found that increase in cell populations in the G0/G1 phase of the cell cycle through the cell number early and late apoptotic cell is also increased. The results provide a rationale that BESC can be used to develop as a potential anticancer agent against human hepatocellular carcinoma. Further, it required more in vitro studies on different cell lines to find the active compound that is responsible for anti-cancer activity. It will help us to make it a promising drug for liver cancer patients.

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Conflict of interest

The authors proclaim that they have no conflict of interest.

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