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THE GROWTH SUPPRESSING ACTIVITY OF SPATHODEA CAMPANULATA BARK ON C6 & U87MG INVOLVE INDUCTION OF APOPTOSIS AND CELL CYCLE ARREST

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ABSTRACT

The anticancer activity of plant extract has already reported. We evaluated the cytotoxic and apoptosis effect of bark extract of *Spathodea campanulata*, on glioma cell lines. It was found that extract significantly decreased viable cell and caused apoptosis, which is further confirmed by morphologically. Gel electrophoresis study shows fragmented DNA in the form of multiple bands and Flow cytometric analysis revealed appreciable number of cells in early & late apoptotic stages. A significant change in the mitochondrial membrane potential was obtained in the BESC treated glioma cells with MMP shift assay. The cells were arrested mostly in G0/G1 phase of cell cycle.

KEYWORDS: Bark**,** *Spathodea campanulata,* Glioma, Apoptosis, Cell Cycle.

INTRODUCTION

Cancer has become the most dominant disease in our society that continues to demand action. The burden of cancer continues to grow globally and become 2nd leading cause of death. It is estimated 9.6 million deaths globally; about 1 in 6 deaths is due to cancer (WHO 2018). There are various types of cancer responsible for global death in which Gliomas are one of them. Gliomas are the most common type of tumors of the central nervous system (CNS) that originate from glial cells. It is classified into four grades (I-IV) according to their molecular

and histological features, with glioblastoma multiforme (GBM, grade IV) being the most aggressive, invasive, and difficult-to-treat.[1] Glioblastomas exhibit a high rate of growth, high vascularization, and are considered to be highly infiltrative with the lowest survival rate.[2] The American Cancer Society reported an estimated 23,770 cases of brain and other nervous system cancers in 2016 (American Cancer Society, 2016). The malignant glioma are often characterized by invasiveness and angiogenesis, within which angiogenesis facilitates the growth of new blood vessels for development glioma.[3, 4] There is no efficient drug or therapy is present for the treatment of GBM. However, a combination of surgery and radiotherapy additionally alkylation chemotherapy with temozolomide (TMZ) has only modestly increased the survival of glioma patients to a median of 14.6 months.[5] In previous researches, it is stated that Doxorubicin has an anti-glioma effect. The blood-brain barrier (BBB) prevents the entry of doxorubicin to overcome this, liposome encapsulation is adapted which brings the same problem many side effects, and reappearance of symptoms after discontinuation.[6, 7, 8] Therefore, major attention is being given to look for better and safer plants derive products which might be a promising therapeutic modality for gliomas.

India has a rich history of traditional systems of medicine which is a strongly based plant and its plant-derived product. Spathodea campanulata is an African plant that is used in tropical and subtropical areas for ornamental purposes as well as for the treatment of various types of diseases.[9] Different parts of S. campanulata have been used for the treatment of various types of cancer. In this study, we investigated the effects of bark extract of *Spathodea campanulata* (BESC) in glioma cells because of its high fatality rate for brain tumors. Therefore, we evaluated the anti-glioma activity on C6 & U87MG cell lines.

MATERIAL 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-diphenil tetrazolium bromide], 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

Glioma cell lines C6 & U87MG were 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 glioma 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. 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

C6 & U87MG 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 IC_{50} values were determined for the all the carcinoma cells.

Morphological Studies for Detection of Apoptosis

Fluorescence Microscopy

C6 & U87MG cells ($1x10^6$) 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

C6 & U87MG cells ($1x10^6$) 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 glioma 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

C6 & U87MG cells were treated with IC_{50} dose of BESC for 14 hrs then cells were resuspended in 500µl of lysis buffer (50 mMTris- 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.[11]

Mitochondrial membrane potential (Δψm) assay

C6 & U87MG ($1x10^6$) 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 C6 & U87MG cells ($1x\ 10^6$) 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 Annexin- FITC binding buffer provided in apoptosis kit (Sigma). Again after centrifuging at 2000 rpm at 4°C, the cell pellets were dissolved in Annexin- FITC binding buffer containing Annexin- 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.[12]

Cell Cycle Arrest Study

To assay the stage of cell cycle arrest in a flow cytometry, C6 & U87MG 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).[12]

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

MTT assay of BESC showed persuading apoptotic effect on all the glioma cell lines C6 and U87MG. The OD value is continuously diminished with time and the concentration dependent manner of BESC (25, 50, 100, and 200 µg). The IC50 estimation of BESC treatment for 24hrs were determined to be **20.73 µg/mL** for C6, and **60.75 µg/mL** for U87MG cells respectively.

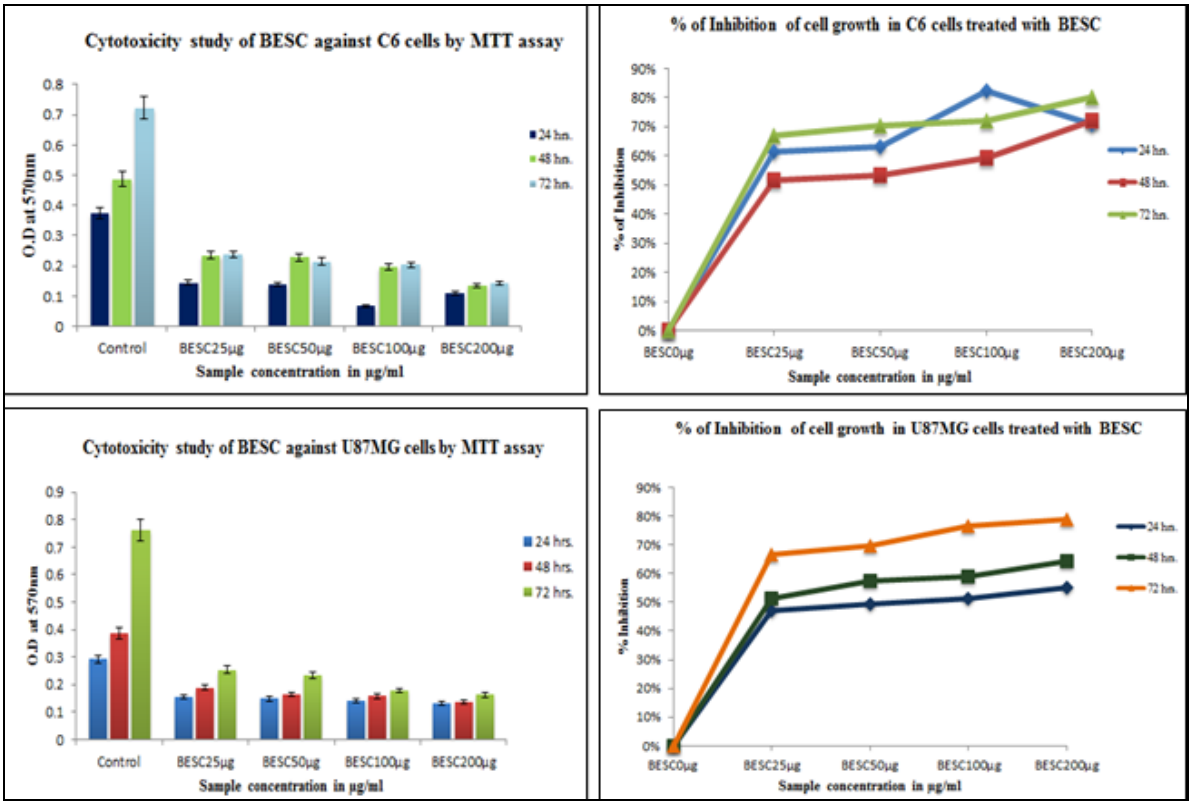

Fig.1.0 Histogram showing the significant decrease in OD value of C6 & U87MG cells after treating with BESC. Treated cells showed dynamically increment in % of inhibition with respect to time and concentration. The IC_{50} value of treated cells for 24hrs was calculated to be 20.73µg/mL for C6 and 60.75 µg/mL for U87MG cells respectively.

Morphological study by Fluorescence Microscope

The IC_{50} value of BESC treated with C6 & U87MG cell line which was stained with red and orange dye i.e. Ethidium bromide and acridine orange respectively. In this study BESC treated cell shows nuclear cleavage were seen and condensed chromatin which indicate beginning of apoptotic processes whereas control showed normal morphology.

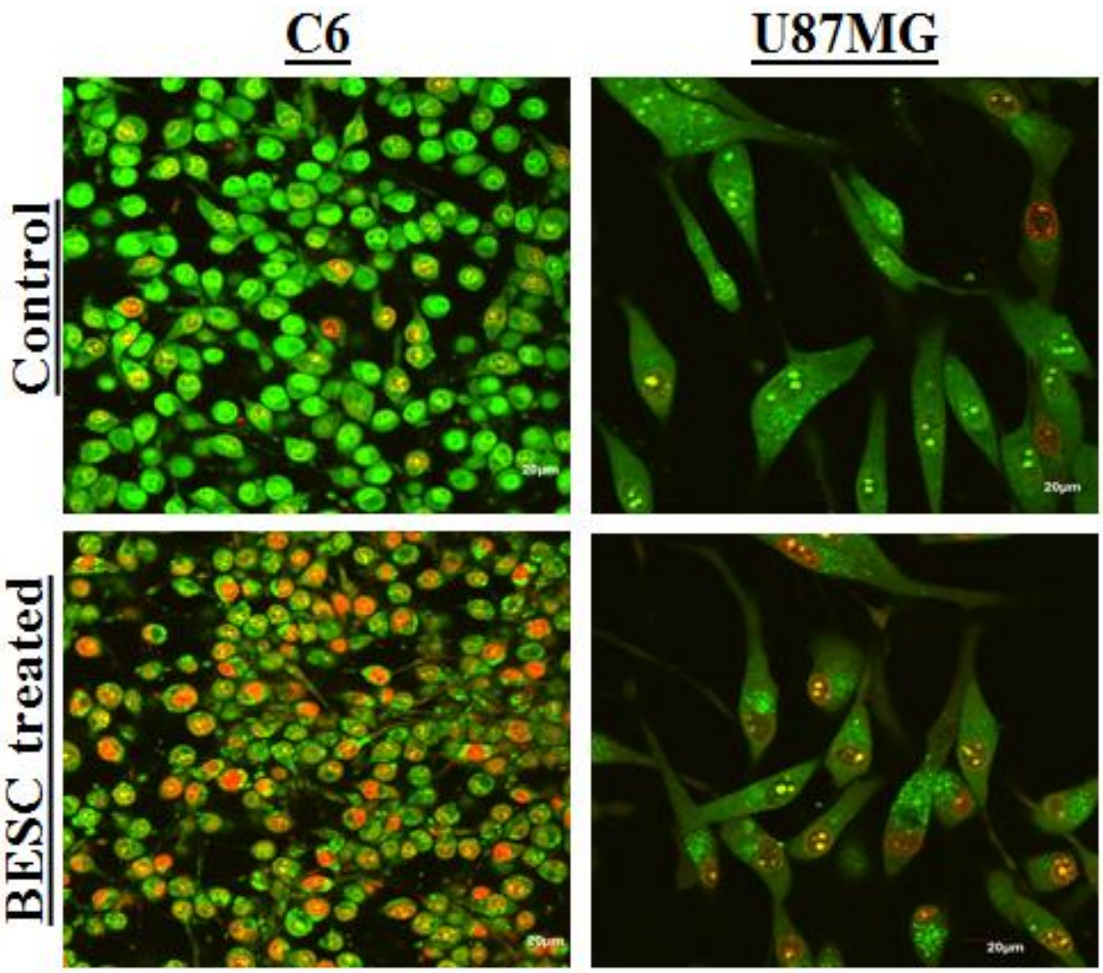

Fig.2.0 showing fluorescence microscopic image of C6 and U87MG cell line treated with IC_{50} dose of BESC. The untreated cell showed unblemished cellular morphology and bright green fluorescence whereas the treated cells show an orange-red colour, suggesting the event of Apoptosis.

Morphological study by confocal Microscope

BESC shows a significant effect on C6 and U87MG cell line after 24 hrs. of treatment with IC_{50} value. The control cells were intact morphology whereas treated cells has deteriorated of nucleus and formation of apoptotic body. The dye PI binds to double stranded DNA, but is excluded from cells with intact plasma membranes. On account of unblemished morphology

PI unfit to get enter in the cell though in apoptotic cell are incapable, the PI get effectively move inside the cell.

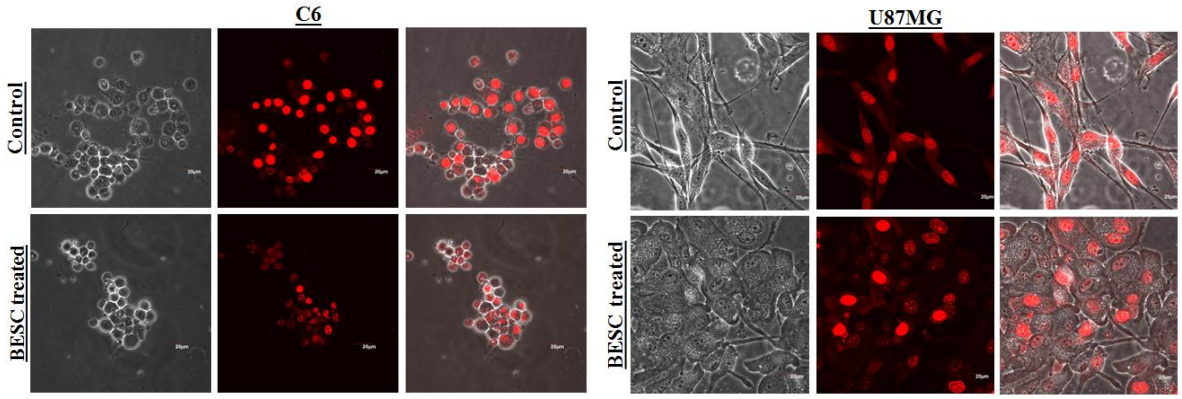

Fig.3.0 Showing Confocal microscopic images of control vs treated cell line of c6 and U87MG with IC_{50} dosage. The Untreated (control) cell shows intact plasma membrane whereas the treated cells show mangle cell morphology due to cleaved nuclei which mark the apoptosis process.

Agarose gel electrophoresis study

The DNA tests were confined from both control and untreated cells. The controls cells showed complete DNA without break (C6 and U87MG), whereas in treated cells showed multiple band in single lane that simply suggest DNA is fragmented. Therefore, it demonstrates BESC induce apoptotic action in C6 and U87MG cell line.

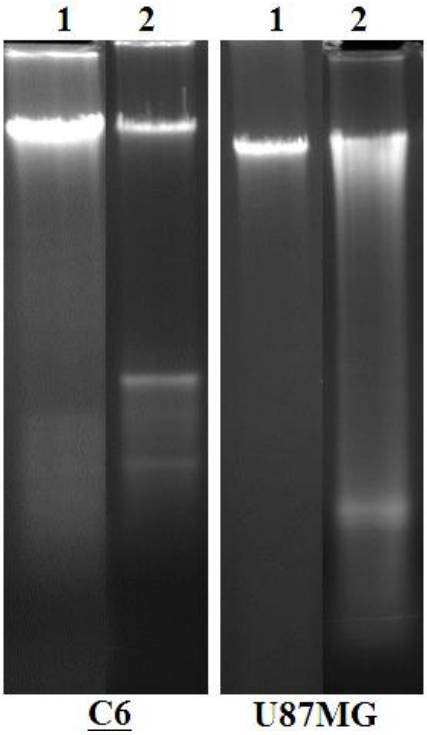

Fig.4.0: showing agarose gel images of control vs treated cell line. Lane 1 represents control of C6 and U87MG respectively. Lane 2 represents BESC treated with IC_{50} dosage on C6 and U87MG cell line respectively.

Detection of mitochondrial membrane potential (Δψm) assay

Mitochondrial membrane potential is play a very important role in synthesis of ATP in the cell. Interruption of mitochondrial film potential is a reliable measure for the studying of the apoptosis. Both cell C6 and U87MG cells were treated with IC_{50} dose of BESC which demonstrated lost Mitochondrial Membrane Potential (Δψm). The JC-1 dye is a lipophilic, cationic dye which can enter in the live cell and form JC stock that give red fluorescence (P3). In contrast, apoptotic cell JC-1 stain can't endure in the mitochondria because of dysfunction of mitochondrial membrane potential; consequently, demonstrating green fluorescence (P4) signifies apoptotic cells. An effective transmembrane move of 5.1% to 86.3% C6 cells were treated with the IC_{50} dose of BESC for 24 hours.

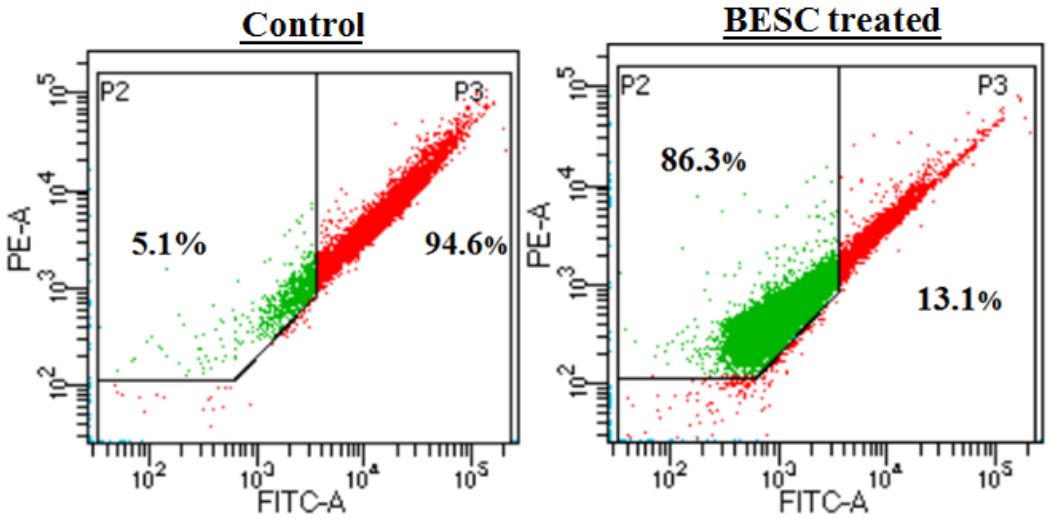

Fig.5.0 showing flow cytometric analysis of mitochondrial membrane potential (Δψm) on C6 cell line in both control and BESC treated cells respectively after 24 hrs. of treatment. A significant decrease in red fluorescence was observed in C6 cell line.

Detection of Apoptosis by Flow Cytometry

In the flow cytometric analysis, dual labelling procedure, in which annexin V FITC and propidium iodide, was used. Lower left (LL) quadrant (annexin V-/PI-) is viewed as the number of live cells, lower right quadrant (LR) (annexin V+/PI-) is considered as the cell populace at an early apoptotic stage, upper right (UR) quadrant (annexin V+/PI+) speaks to the cell populace at late apoptotic stage an extraordinary upper right (UR) and upper left (UL) quadrant (annexin V-/PI+) is considered as necrotic cell populace. Flow cytometric data analysis affirms that treatment with BESC with IC_{50} dose for 24 hrs. Demonstrate apoptotic inducing property from 4.3% to 12.6% in C6 cell line.

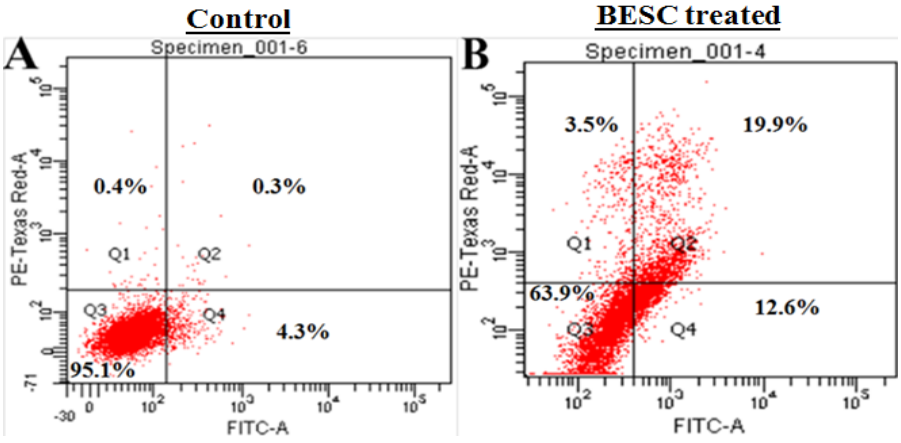

Fig.6.0 showing dual parameter of flow cytometric analysis of control and BESC treated of C6 cells stained with Annexin V FITC and propidium iodide, which signify the presence of early apoptotic cell in left quadrant.

Study of Cell Cycle Arrest by Flow Cytometry

DNA content is the most oftentimes estimated element of the cell. Estimation of DNA content uncovers cell, gives data on cell position in the cell cycle and furthermore permits one to appraise recurrence of apoptotic cells that are portrayed by fragmentary DNA content. Flow cytometric evaluation showed significant expands in DNA content in G1 phase of the cell cycle from 58.9% to 81.4% in C6, whereas in U87MG DNA expand from 23.8% to 52.8%. This observation clearly signifies the growth of C6 and U87MG cell line is inhibited by arresting the cell populations in the sub-G0/G1 as well as G2/M phase of the cell cycle.

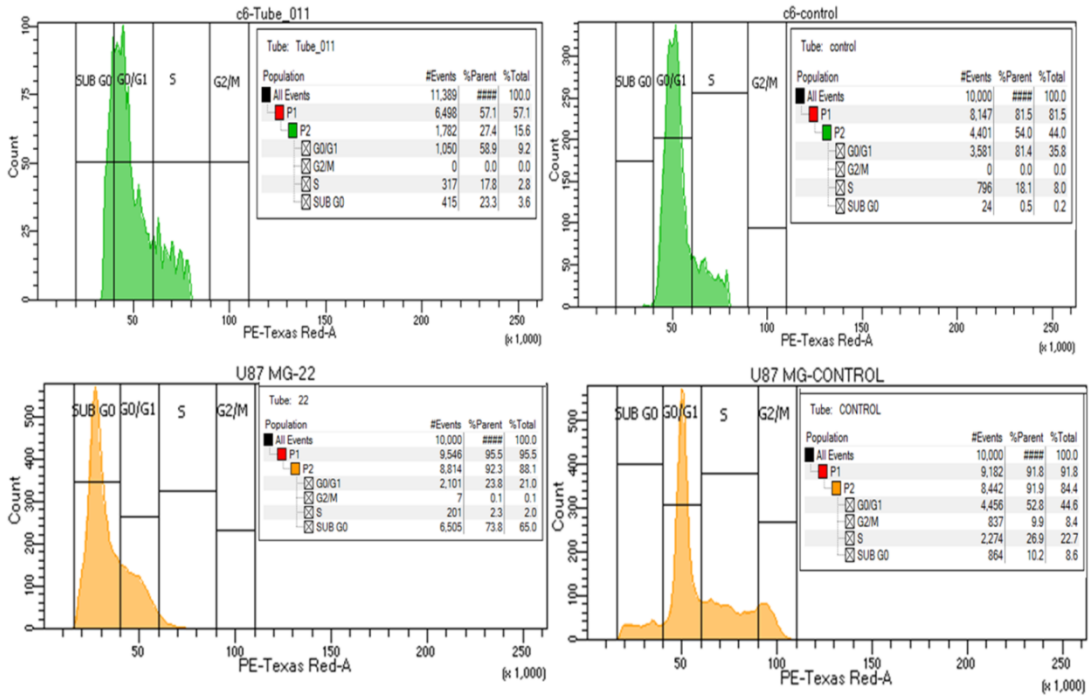

Fig.7.0 showing flow cytometric evaluation of cell cycle phase, controls vs treated BESC at IC_{50} dosage on C6 and U87MG cell line after 24 hrs of treatment. Peaks represent the increase in DNA content of the cell in G1 phase that demonstrate BESC significantly inhibit the growth of C6 and U87MG cell line.

DISCUSSION

Cancer has become a significant problem worldwide, which attracts the attention of the researcher for the discovery of the potent drug. The production of new drug is still remaining challenging because of its side effect and drug resistance. The selection of natural method helps us to overcome from such problem. Indeed, natural products provide a helpful resource for efficient drug development. Our plant of interest is *Spathodea campanulata* which has been used in the treatment of various ailments. We investigated the anti-glioma effect of bark

extract of *Spathodea campanulata* in C6 and U87MG cell line. The anti-glioma effect BESC is determined by the help of MTT assay which showed the cytotoxicity to the glioma cell line (fig.1.0). Further the apoptotic activity is revealed by the fluorescence and confocal microscopic images. The fluorescence microscopic images clearly showed green fluorescence in untreated cell with normal physiology whereas in treated cell a red fluorescence was appeared, it's due to incorporation of dye into the cell. It simply represents the BESC induce apoptosis in glioma cell (C6 and U87MG) line (fig.2.0). In addition, the similar result is obtained from confocal microscopy BESC treated cells C6 & U87MG cell showed disrupted cell morphology and several signs of apoptosis whereas the untreated cells were with complete morphology (fig.3.0). The investigation is further carried out by agarose gel electrophoresis. BESC treated cells showed cleaved DNA that contain multiple bands in single lane that simply represent the digestion of DNA, whereas the untreated control cells showed single and thick DNA bands in a UV transilluminator (fig.4.0). It is typical indication of apoptosis in which BESC are able successfully degrade the genome of the glioma cell. Another study is based on mitochondria membrane potential that is used for the study of apoptosis. Due to loss of mitochondria membrane potential, the cationic dye JC cannot retain in the mitochondria.[13] JC-1 staining of C6 cells treated with BESC showed a promising effect (fig.5.0). There is a shift in transmembrane potential from red to green fluorescence. Therefore, it represents a change in the transmembrane potential which remarks the activation of apoptosis. PS externalization is another approach to study apoptosis by the use of double labelled staining technique with Annexin-V and PI. The translocation of phosphatidylserine molecules to outer surface of the cell allows binding the Annexin-V which is labelled with FITC.[14] Dual staining technique 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 BESC supporting the fact that apoptosis was triggered by the treatment with BESC in C6 cells (fig.6.0). The checkpoint of cell cycle plays vital role in cell regulation. It guides the abnormality in the cell and ensures the proper passage of cells through the different phases of cell cycle. It was known that loss of regulation of these checkpoints resulted into transformation and progression of cancer cells.[17] So, Cell cycle analysis revealed that BESC treated cell showed cell cycle arrest in G0/G1 phase as well as in G2/M phase of cell cycle (fig.7.0) that might trigger apoptosis.

CONCLUSION

The results of the present investigation revealed the cytotoxic effect of bark extract of Spathodea campanulata (BESC) on glioma cell line C6 & U87MG. BESC induced cytotoxicity involved DNA fragmentation, transmembrane shift, phosphatidylserine externalization, and cell cycle arrest. These all are the hallmark of apoptosis which induced cell death pathways and significant promise for future cancer therapeutics. Exploring the medicinal value of such indigenous plants to combat the chronic diseases will be beneficial for the human society. However, furthermore study should be done and identify the active compound which are responsible for anti-glioma activity on cancer cells. This result possibility and hope of developing natural and novel anti-cancer drug.

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Conflict of interest

The authors declare that they have no conflict of interests.

REFERENCES

1. Louis DN, Perry A, Reifenberger G, Von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P, Ellison DW., The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathological*, 2016; 131(6): 803-20.
2. Giakoumettis D, Kritis A, Foroglou N., C6 cell line: the gold standard in glioma research. *Hippokratia*, 2018; 22(3): 105.
3. Takano S, Kamiyama H, Tsuboi K, Matsumura A., Angiogenesis and antiangiogenic therapy for malignant gliomas. *Brain Tumor Pathology*, 2004; 21(2): 69-73.
4. Kamiyama H, Takano S, Ishikawa E, Tsuboi K, Matsumura A., Anti-angiogenic and immunomodulatory effect of the herbal medicine "Juzen-taiho-to" on malignant glioma. *Biological and Pharmaceutical Bulletin*, 2005; 28(11): 2111-6.
5. Rai R, Banerjee M, Wong DH, Mc Cullagh E, Gupta A, Tripathi S, Riquelme E, Jangir R, Yadav S, Raja M, Melkani P., Temozolomide analogs with improved brain/plasma

ratios–Exploring the possibility of enhancing the therapeutic index of temozolomide. *Bioorganic & Medicinal Chemistry Letters*, 2016; 26(20): 5103-9.

6. Von Hoff DD, Layard MW, Basa P, DAVIS Jr HL, Von Hoff AL, Rozencweig M, Muggia FM. (Risk factors for doxorubicin-lnduced congestive heart failure). *Annals of Internal Medicine*, 1979; 91(5): 710-7.
7. Treat LH, Mc Dannold N, Zhang Y, V ykhodtseva N, Hynynen K., Improved anti-tumor effect of liposomal doxorubicin after targeted blood-brain barrier disruption by MRI-guided focused ultrasound in rat glioma. *Ultrasound in Medicine & Biology*, 2012; 38(10): 1716-25.
8. Roy S, Deb N, Basu S, Besra SE., Apoptotic activity of ethanolic extract of *Moringa oleifera* root bark on human myeloid leukemia cells via activation of caspase cascade. *World J Pharm Sci*, 2014; 3(10): 1138-56.
9. Queiroz AC, Contrera FA, Venturieri GC. (The effect of toxic nectar and pollen from Spathodea campanulata on the worker survival of Melipona fasciculata Smith and Melipona seminigra Friese, two Amazonian stingless bees *Hymenoptera: Apidae: Meliponini*). *Socio-biology*, 2014; 61(4): 536-40.
10. Herrmann M, Lorenz HM, Voll RE, Grünke M, Woith W, Kalden JR. (A rapid and simple method for the isolation of apoptotic DNA fragments). *Nucleic Acids Research*, 1994; 22(24): 5506.
11. Roya S, Besra SE, De T, Banerjee B, Mukherjee J, Vedasiromoni JR. (Induction of apoptosis in human leukemic cell lines U937, K562 and HL-60 by *Litchi chinensis* leaf extract via activation of mitochondria mediated caspase cascades). *The Open Leukemia Journal*, 2008; 1(1).
12. Jacobson, M D. (Programmed cell death in animal development). *The Cell*, 1997; 88: 347-354.
13. Sivandzade F, Bhalerao A, Cucullo L. (Analysis of the mitochondrial membrane potential using the cationic JC-1 dye as a sensitive fluorescent probe). *Bio-protocol*, 2019; 9(1).
14. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C. (A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V). *Journal of Immunological Methods*, 1995; 184(1): 39-51.
15. Wising C, Azem J, Zetterberg M, Svensson LA, Ahlman K, Lagergård T. (Induction of apoptosis/necrosis in various human cell lineages by Haemophilus ducreyi cytolethal distending toxin). *Toxicon*, 2005; 45(6): 767-76.

16. Shi Y. (A structural view of mitochondria-mediated apoptosis). *Nature structural biology*, 2001; 8(5): 394-401.
17. Deb N, Hansda A, Dutta S, Pattanaik A, Besra S.E., *Lawsonia Alba* Leaves Induce Apoptosis and Cell Cycle Arrest in B16F10 Melanoma Cells. *IJPPS*, 2018; 10(5): 96-101.