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THE GROWTH SUPPRESSING EFFECTS OF LEAF EXTRACT OF *BISCHOFIA JAVANICA* BLUME ON GLIOMA CELLS

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ABSTRACT

Natural products have long been known as one of the most excellent sources of lead drug candidate in drug discovery. Nowadays, a large number of Indian medicinal plants have been utilized for the treatment of a variety of diseases. Leaves of *Bischofia javanica* have been traditionally used for many ailments including cancer. Anti-leukemic activity of the leaf extract was already reported on human leukemic cell lines The present study investigated the cell cycle arrest and apoptosis activity of methanolic extract of *Biscofia javanica* Blume on glioma cell lines. The extract significantly inhibited cell viability in a time and concentration dependent manner in both the glioma cells. Morphological study showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies and degraded DNA bands in carcinoma cells than control. Cell cycle phase arrest was observed in the G0/G1 phase and MMP shift assay exhibited significant change in the methanolic extract of *Biscofia javanica* (MEBJ) treated cells. These findings suggest that metabolic activity of both the glioma cells were suppressed by extract and possesses induction of apoptosis and cell cycle arrest.

KEYWORDS: *Bischofia javanica*, Glioma, Cell Cycle, Apoptosis.

INTRODUCTION

The most common malignant tumour of the brain are gliomas, accounting for over 80% of all primary brain malignancies.[1] Plant as enormous resource, an effective strategy for the treatment and prevention serves the mankind. *Bischofia javanica* Blume (Euphorbiaceae), commonly known as Bishop Wood, is widely distributed in India over the Sub-Himalayan region, Orissa, and south-West Coast from Konkan to Nilgiris.[2] Traditionally, the bark is used for the treatment of tuberculosis, stomach ulcer, mouth ulcer, and athlete's foot.[4] Leaves are used in the treatment of stomachache and the leaf juice for cancerous wounds.[5] The major phytoconstituents are tannin, β amyrin, betulinic acid, friedelan-3α-ol, epifriedelinol, friedelin, luteolin and glucoside, quercetin, beta-sitosterol, stigmosterol, and ursolicacid.[6] The ethanolic extract of the leaves has also been shown to possess antimicrobial activity.[7] The bioassay-guided fractionated compounds including betulinic acid and its derivatives, betulonic acid, 3β-O-(Z)-coumaroyl betulinic acid, and 3β-O-(E)-coumaroyl betulinic acid, from the chloroform extract of the bark of BJ have been found to act as catalytic inhibitors of topoisomerase II activity with IC_{50} values ranging from 0.38 to 58 μM.[8] Anti-inflammatory and analgesic potency of BJ were studied by the authors in a previous research work.[9] Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis.[10] The anti-leukemic activity of BJ for cancer on leukemic cell lines U937, K562, and HL60 was reported. Therefore, present study is an approach to explore the cell cycle arrest and apoptosis activity of the methanolic extract of *Bischofia javanica* (blume) leaf which has been investigated against glioma cell lines C6 and U87MG.

MATERIALS AND METHODS

Plant Material

The leaves of *Biscofia javanica* were collected from Melli region of East Sikkim, India, in the month of June 2005. The plant material was identified and authenticated at Botanical Survey of India (BSI), Sikkim branch and the herbaria were preserved in the institutional museum: HPI /LS/ No.12.

Preparation of the Plant Extract and Phytochemical Analysis

The collected leaves were shade dried for 15 days and ground in a laboratory grinder. The powdered leaves were extracted with methanol in soxhlet apparatus at 50-60° C. After

exhaustive extraction, the extract was concentrated by distilling the solvent for further use. The concentrated extract was kept in desiccators. The yield was determined as 5.0% w/w. The methanolic extract of *Biscofia javanica* (MEBJ) was dissolved in phosphate buffer saline and employed for anti-gliomas studies. Phytochemical analysis of the methanol extract was subjected to phytochemical analysis for constituent identification using a standard protocol.[11]

Chemicals and reagents

DMEM medium with L-glutamine, Fetal Calf serum(FCS), Phosphate buffer saline (PBS), Trypsin (Gibco, USA), Penicillin- Streptomycin, Gentamycin, HEPES, Ethidium bromide, Acridine orange, Propidium iodide, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide(MTT), JC-1, Proteinase K, RNase (SRL), Isoamyl alcohol, phenol, chloroform, Dimethyl sulfoxide (DMSO), Sodium hydroxide, Agarose, Sodium bicarbonate, Ethanol, Methanol etc.

Cell Culture

Glioma Cell lines: C6 and U-87 MG were obtained from National Centre for Cell Science, Pune, India. The cells were cultured and routinely maintained in DMEM medium supplemented with 10% heat inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100µg/ml), Gentamycin (100µg/ml) and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 inside a CO_2 incubator. Both C6 and U-87MG cells are adherent in nature. During sub culturing of the cells, the adherent property can be diminished by adding 1x Trypsin solution in the cell.

Cytotoxicity Study

C6 and U87 MG ($1x10^5$) cells (100µl of cell suspension per well) were seeded in 96-well plates and incubated inside a CO_2 incubator for 24 hours before treatment. The cells were treated with MEBJ in doses of 25 µg to 200µg for a period of 24,48 and 72 hours at 37ºC in a humidified atmosphere containing 5% CO_2 in air, the untreated cells served as control. After treatment, 20 µl of MTT (3mg/ml) was added to each well. The plate was then allowed to incubate for 3-4 hours at 37°C in CO2 incubator. The reaction resulted in the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a purple formazan product. The MTT--formazan product was dissolved by added 100µl of DMSO. The O.D values were recorded at 570 nm by microplate manager (Reader type: Model 680 XR Bio-Rad laboratories lnc). The IC_{50} value of MEBJ on both the glioma cells were determined.

Fluorescence microscope Study

C6 and U87 MG glioma cells ($1x10^6$) were treated with different IC_{50} doses of MEBJ for 24 hours and observed using a fluorescence microscope for determining morphological changes. After 24 hours, the untreated control cells and MEBJ treated cells were harvested separately and washed with PBS and then stained with 100µg/ml of Acridine orange and ethidium bromide in a ratio of 1:1. The cells were then immediately mounted on slides and observed under a fluorescence microscope at a magnification of 60X for the morphological determination of the cells undergoing apoptosis.

Confocal microscope Study

C6 and U87 MG glioma cells ($1x10^6$) were treated with different IC_{50} doses of MEBJ for 24 hours and observed using a confocal microscope for determining morphological changes. After 24 hours, the untreated control cells and MEBJ treated cells were harvested separately and washed with PBS and then stained with 10µg/ml of Propidium iodide. The cells were then immediately mounted on slides and observed under a confocal microscope at a magnification of 60X for the morphological determination of the cells undergoing apoptosis.

Agarose Gel electrophoresis study

C6 and U87 MG glioma cells ($1x\ 10^6$) were treated with IC_{50} dose of MEBJ for 24 hrs. After trypsinization cells were harvested and washed twice with PBS. The cells were resuspended in 500µl of lysis buffer [1(M) Tris Hcl, 5(M) NaCl, 20% Triton X], 10µg/ml of Proteinase - K was added and kept for incubation at 55ºC for 30 minutes and at 37°C overnight. After incubation was done by following the general phenol-chloroform extraction procedure and kept at -20°C overnight. After centrifugation, DNA precipitates were washed with 70% ethanol, dried and evaporated at room temperature and suspended in TE buffer at 4ºC overnight. To detect the DNA fragments the isolated DNA samples were electrophoresing overnight at 20V in 1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator and Chemi Doc ^{TM} MP Imaging System, Bio-Rad.

Mitochondrial Membrane Potential (Δψm) Study using JC-1

C6 and U-87 MG cells were treated with MEBJ with IC_{50} dose for 18 h to assay the mitochondrial membrane potential of the cell in a flow cytometry. Cells were washed with PBS, pelleted down and dissolved in prewarmed PBS. 200 µM JC-1 stain was added and the samples were incubated at 37 °C for 15 min. Shift in the mitochondrial membrane potential was determined by 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) with the help of BD FACS Diva software (Becton Dickinson).

Cycle arrest Analysis

C6 and U87 MG glioma cells (1x 10^6) were treated with IC_{50} dose of MEBJ was taken as standard drug for 18 hours. Cells were washed with PBS, fixed with chilled methanol and kept at 4°C for 90 minutes. Cells were pelleted down, dissolved in cold PBS, treated with RNase for 30 min at 37 °C and stained with Propidium iodide and transferred to FACS tube. Cell cycle phase distribution 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).

STATISTICAL ANALYSIS

Student's t test was used for statistical analysis and $p<0.005$ was considered significant.

The percentage of cell growth was calculated by the following formula.

% Cell inhibition= 100x (O.D of control – O.D of the treated)/ O.D of control

Where O.D refers to 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) x100.

RESULTS

Cytotoxicity Study

In cytotoxicity study by MTT assay, MEBJ showed significant reduction in the O.D values of treated C6 and U-87 MG cells after 24,48 &72 hours as compared to the control cells. The observations showed inhibition of cell proliferation in a time and concentration dependent manner. The IC_{50} value of MEBJ was **15 µg** and **12µg** for C6 and U87 MG cells respectively.

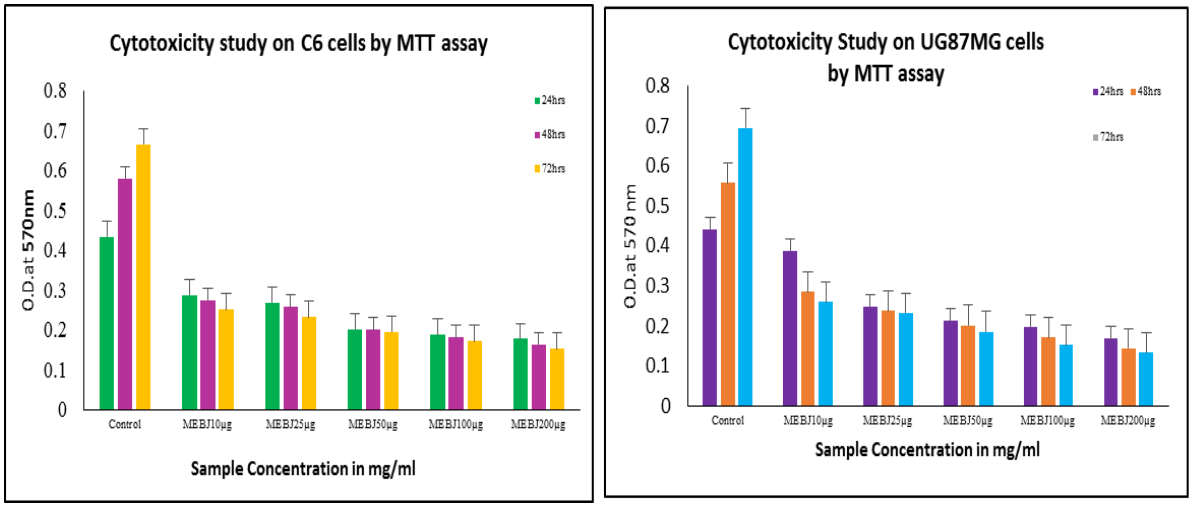

Fig.1: Histogram shows the effect of MEBJ on C6 and U87 MG cell lines after 24,48 and 72hrs. Reduction in the O.D at 570 nm is observed in a time and dose dependent manner. Data are S.E.M ± mean.

Fluorescence Microscope Study

Fluorescence microscopic observations of IC_{50} dose of MEBJ treated C6 and U-87 MG glioma cells stained with ethidium bromide and acridine orange revealed the presence of apoptotic cells (both early and late) as compared to the untreated control cells. Arrays of nuclear changes were observed including chromatin condensation and apoptotic body formation that are the indication of occurrence of apoptosis.

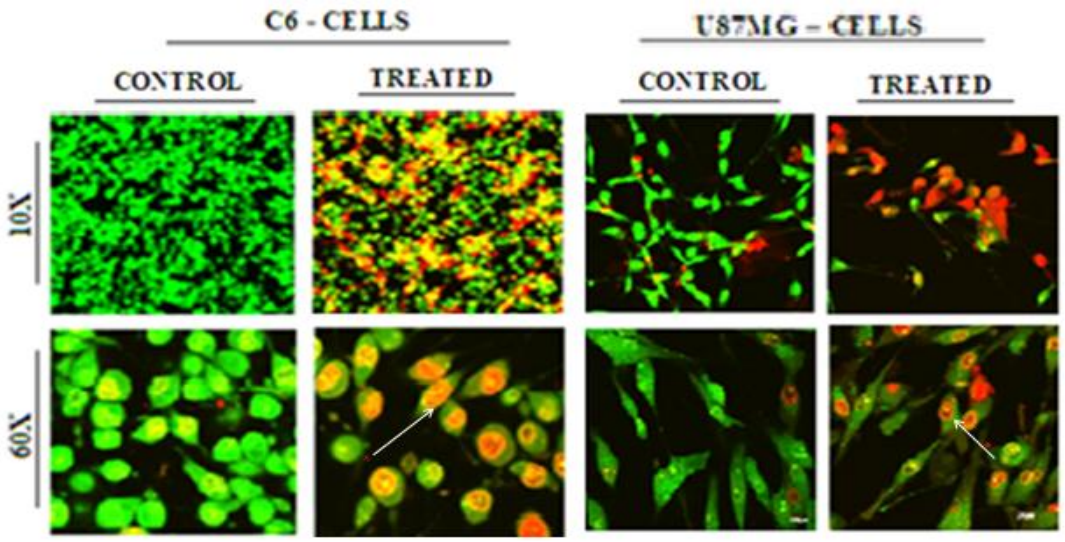

Fig.2: Fluorescence microscopic images C6 and U-87 MG cell lines control & treated with IC_{50} dose of MEBJ. Both the cell lines were stained with Acridine orange &

Ethidium bromide. Both the treated cells showed apoptosis indicated by arrow. The images were captured in 10X & 60X.

Confocal microscope Study

Confocal microscopic observations of IC_{50} dose of MEBJ treated C6 and U-87 MG glioma cells stained with propidium iodide, revealed the presence of apoptotic cells as compared to the untreated control cells. Arrays of nuclear changes were observed including chromatin condensation and apoptotic body formation that are the indication of occurrence of apoptosis.

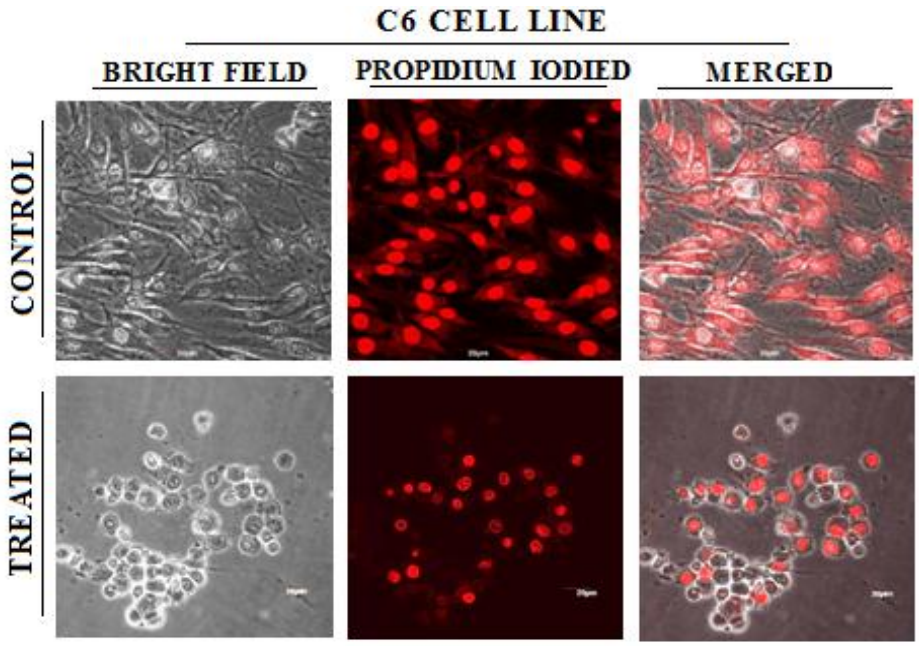

Fig.3: The untreated control C6 cells showed intact nuclei whereas treated with IC_{50} value of MEBJ cells represent the fragmented nuclei and showing the formation of apoptotic bodies after 24 h stained with propidium iodide (PI).

Agarose gel electrophoresis study

After Agarose gel electrophoresis, the DNA samples isolated from the untreated control C6 and U87MG cells showed intact DNA bands whereas the DNA samples from C6 and U87MG glioma cells treated with MEBJ showed fragmented DNA bands in the form of ladders. So, the observations confirmed that the treatment with MEBJ, caused apoptosis in C6.

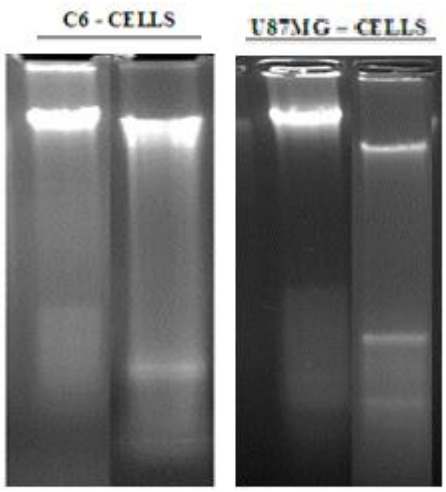

Fig. 4: The gel pattern of DNA samples isolated from untreated control C6 & U87MG glioma cells. C6 cells treated with IC_{50} dose of MEBJ. Treatment with the MEBJ showed distinct DNA ladder formation indicating the process of apoptosis in rat glioma cell line.

Detection of Mitochondrial Membrane Potential (Δψm) using JC-1

Depolarization in mitochondrial membrane potential was observed by staining treated and untreated cells with JC1 dye. The depolarization led to a transmembrane shift from red to green fluorescence leading to the release of Cytochrome C. A significant transmembrane shift of 8.7 to 19.9(for C6 cell) and 9.9 to 16.4(for U-87 MG) in the mitochondrial membrane potential from red to green fluorescence was observed when C6 and U87MG cells were treated with the IC_{50} dose of EFESS for 18 hours respectively.

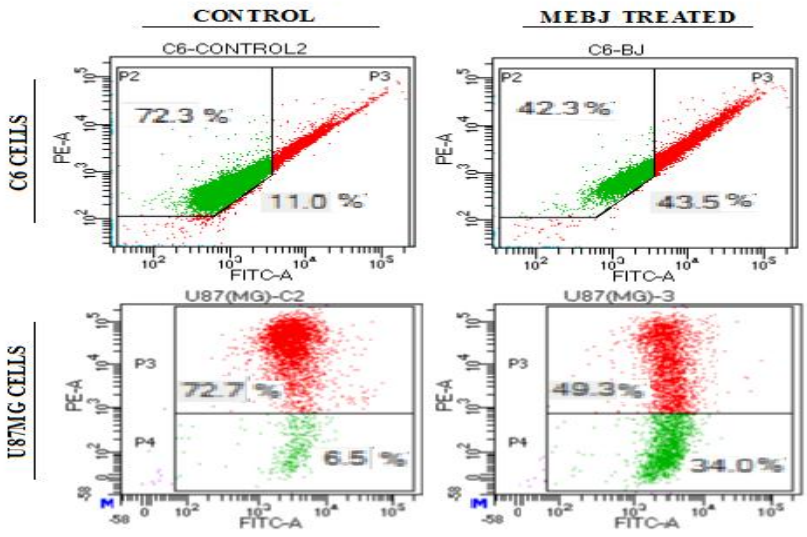

Fig.5. Showing flow cytometric analysis of mitochondrial membrane potential (Δψm) on C6 and U87MG cells, both the control and MEBJ treated cells respectively after 24 hrs.

of treatment. A significant decrease in red fluorescence was observed in both the glioma cell line.

Study of Cell Cycle Arrest by Flow Cytometric Analysis

Flow cytometric analysis showed that after 24-hour treatment of C6 with MEBJ at IC_{50} dose, the DNA content increased in G1 phase (against). In case of U-87 MG cell line the DNA content increased in G1 phase (against) after treatment with MEBJ. The results indicated that drug treatment arrested the cell cycle of both the cell lines at G0/G1 phase.

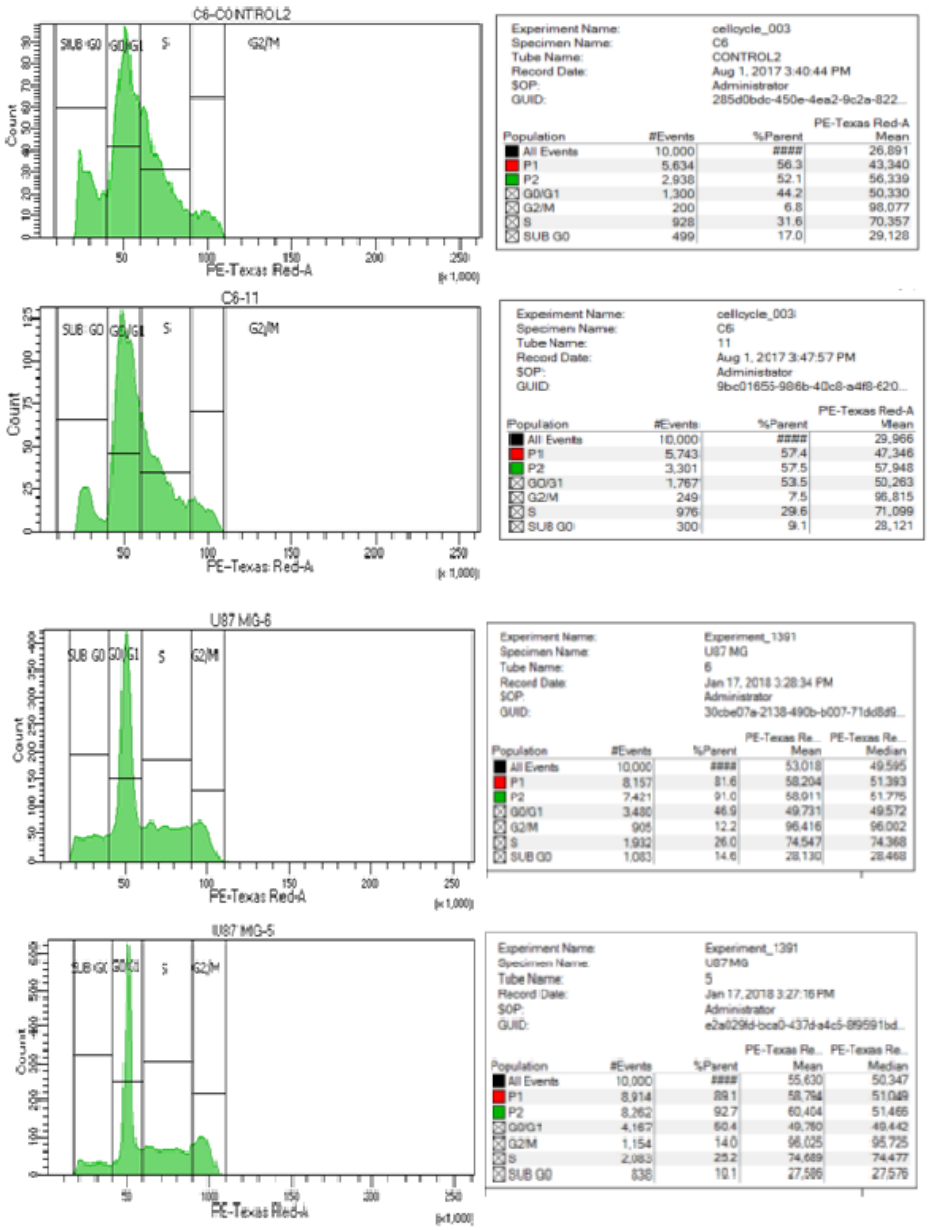

Fig 6: Flow cytometric analysis of cell cycle phase distribution in control (A &C) and treated (B & D) of C6 & U-87 MG cells respectively after 18 hrs treatment at IC_{50} dose

of MEBJ. 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).

DISCUSSION

Cancer is a disease characterized by uncontrolled proliferation of cells. Tumor development is accelerated by disruption of the balance between cell proliferation and cell death, which is maintained through regulation of various signal transduction pathways. 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 for the management of cancer. A vast variety of naturally occurring substances have been shown to protect against experimental carcinogenesis and it is becoming increasingly evident that phytochemicals of different plants possess marked cancer chemo-preventive properties.[12] Numerous experimental, epidemiological, and clinical studies have revealed that NSAIDs are having promising anticancer activities.[13] The mechanism responsible for the anti-tumor activity of NSAIDs is still unknown. But the antineoplastic effects of NSAIDs may also include activation of apoptosis, inhibition of angiogenesis, or direct inhibition of cancer cell growth by blocking signal transduction pathways responsible for cell proliferation.[14]

Despite of remarkable progress in the discovery and development of novel cancer therapeutics, cancer remains the second leading cause of death in the world. Research indicates that most cancers are caused by a dysfunction of many genes coding for proteins such as growth factors, growth factor receptors, anti-apoptotic proteins, transcription factors, and tumor suppressors, all of which constitute a target for cancer treatment.[15] Gliomas account for the vast majority of malignant adult brain tumors. Even though tremendous effort has been made to optimize treatment of patients with high-grade glioma, the prognosis remains poor, especially for patients with glioblastoma.[16] The most standard care for GBM is surgical resection followed by radiotherapy and Temozolomide (TMZ) based chemotherapy. The drugs used in current chemotherapy of brain cancer like TMZ have certain side effects such as myelosuppression and cerebral edema. Apoptosis is characterized by metabolic and morphological features that distinguish the process from necrosis. These features include chromatin condensation and DNA cleavage by endogenous endonuclease and membrane phospholipid changes followed by cell segmentation into apoptotic bodies.

During the process of apoptosis, plasma membrane integrity is maintained and secretion of lysosomal enzymes remains low, thus limiting traumatic damage to surrounding tissue.[17]

The present work reveals that the leaf extract of *Biscofia javanica* possess potent anti-proliferative activity against glioma cell lines C6 and U-87 MG. The cytotoxicity study was performed in C6 and U87MG cells and it was found that MEBJ showed significant decrease in O.D value of treated cells in a concentration dependent manner which indicates anti-proliferative activity. Further the morphological study of C6 and U87MG cell treated with MEBJ was also conducted by fluorescence microscopy and confocal microscopy. The fluorescence microscopic images by Acridine orange and ethidium bromide denoted that the two glioma cells treated with MEBJ showed cell shrinkage, membrane blabbing, nuclear fragmentation and condensation of chromatin which are the hallmark of apoptosis. Apoptogenic activity of MEBJ was further evidenced from the confocal microscopic images of the treated glioma cell lines C6 when compared with that of the untreated control cells. After MEBJ treatment, the cells showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei. Further in support of the apoptotic activity, agarose gel electrophoresis was done and the observations showed that MEBJ showed ladder like degraded DNA band in the gel pattern whereas the untreated control cells showed intact DNA band when observed in U.V trans illuminator and Chemi Do^{cTM} Imaging System, Bio-Rad. Cell cycle analysis revealed that treatment with MEBJ arrested in C6 and U87MG cells, cell populations in the G0/G1 phase of cell cycle. Mitochondrial membrane potential was measured using JC-1, a cell-permeable, cationic carbocyanine dye that exists in a monomeric form that on entering the cytoplasm emits a green fluorescence. Subsequently, on entering the mitochondria it forms J aggregates and emits a red fluorescence. The ratio of red/green fluorescence represents loss of mitochondrial potential. JC-1 staining of C6 and U87MG cells treated with MEBJ shows a significant shift in the transmembrane potential from red to green fluorescence. The observation suggests a change in the transmembrane potential, thereby reflects the occurrence of apoptosis. From all the above performed experiment it can be confirmed that MEBJ possesses anti-cancer potential on C6 and U87MG, glioma cell lines. Hence, this research work has explored the potent anticancer activity of leaves of MEBJ on mouse glioma as well as on human glioblastoma cell lines and rationalized its ethno-medicinal use for cancer.

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CONFLICT OF INTEREST

The authors proclaim that they have no conflict of interest.

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