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IN-VITRO ANTI-CANCER EFFICIENCY OF SILIBININ AGAINST U7MG GLIOBLASTOMA CELLS

Arpana Sharma*
Jai Prakash Mishra**
Gautam Singh Dhaked****
Rakesh Jhanwar^
Neelu Jain^^

ABSTRACT

Glioblastoma is grade IV tumor with poor prognosis due to its high proliferative and migration behavior. Glioblastoma attack the glial cells, which are nurse cells that provide nourishment to the other cells in the brain. Silibinin is a bioactive flavonoid present in fruits and vegetables reported to suppress migration in numerous cancer cells. In the present study, the effects of Silibinin were investigated in glioma cells namely, U87MG. The results showed that Silibinin significantly inhibited the cell viability. Further, the anti-cancer potential was shown by decrease in growth and proliferation at low micromolar concentrations. Also, there was considerable increase in the percent dead cells. A distinct modification in the cell morphology was observed in U87MG cells treated with Silibinin. These findings suggested that Silibinin has anti-cancer role leading to suppression of growth of glioma cells.

Keywords: MTT, proliferation, Silibinin, Trypan blue, U87MG.

INTRODUCTION

Glioblastoma multiforme (GBM) or gliomas is a grade IV highly malignant and fast growing tumor attacking the glial cells **[1]**. Glioblastoma is a rare but fatal disease whose risk of diagnosis increases with age and accounts for nearly one-half (49%) of all malignant tumors in all ages combined, and non-malignant meningioma, which accounts for more than one-half

***Arpana Sharma** is Assistant Professor at the Department of Biotechnology (Life Sciences), Mewar University, Gangrar, Rajasthan-312901. She holds PhD in LifeSciences (2019) on the topic, "To Study the Role and Mechanisms of Glutamate Receptor in Anti-Cancer Effects of Phytochemicals on Glioblastoma" from Central University of Gujarat. Corresponding author e-mail: arpanacug@gmail.com.

****Jai Prakash Mishra** is Professor at the Central University of Gujarat, Gandhinagar, Gujarat, 382030, India.

*****Gautam Singh Dhaked, ^Rakesh Jhanwar & ^^Neelu Jain** are Assistant Professors at Mewar University, Gangrar, Chittorgarh, Rajasthan- 312901, India

(54%) of non-malignant tumors **[2]**. GBM is aggressive tumor as it has ability to migrate and invade into other parts of the brain **[3]**.

Various growth factors act as ligand leading to activation of the complex network of the signalling pathways which translates the signals into Ras-Raf-MAPK-ERK activation resulting in cell viability and growth **[4]**.Hence targeting these signalling pathways is a key therapeutics. Many inhibitors and blocking agents against ERK have been used with the conventional treatment strategy including chemotherapy against glioblastoma **[5]**. So far, targeted therapy has not accomplished satisfactory results in glioblastoma.

Many dietary components present in foods and beverages we consume have contributed to control nearly 30% of cancer burden of developed industrialized countries **[6]**. The phytochemical components which are found in these beverages act as the next defensive approach which illustrates most potent molecules acting as the chemo-preventive agents. These can be used as therapeutics which can selectively kill the cancer cells, particularly brain tumor **[7]**. Cancer chemotherapy is related with toxic side effects, high cost, has led to limited utility of those compounds. In comparison, natural compounds isolated from the dietary sources, having no side effects and very cost effective, have become more appealing than the synthetic molecules in the treatment strategy **[8]**.

Phytochemicals are the secondary metabolites of plant origin which have been grouped into many classes like triterpenoids, flavonoids, catechols, sulphated carbohydrates and tannins **[9]**. Flavonoids are one type of phytochemicals which modulate cancer properties and have been observed in inhibition of cancer **[10].**

Silibinin (**Figure 1**), which is the active component of silymarin complex also called as milk thistle, is extracted from the *Silybum marianum* comprises of flavonoids **[11]**. The plants are native to the Mediterranean regions of Europe, North Africa, and the Middle East. Its usage has not been reported in the traditional ayurvedic texts. It has many additional health benefits as clinical studies have shown that it can act as a chemopreventive agent on many cancer types, can target cyclin dependent pathways and cause cell cycle arrest **[12]**.

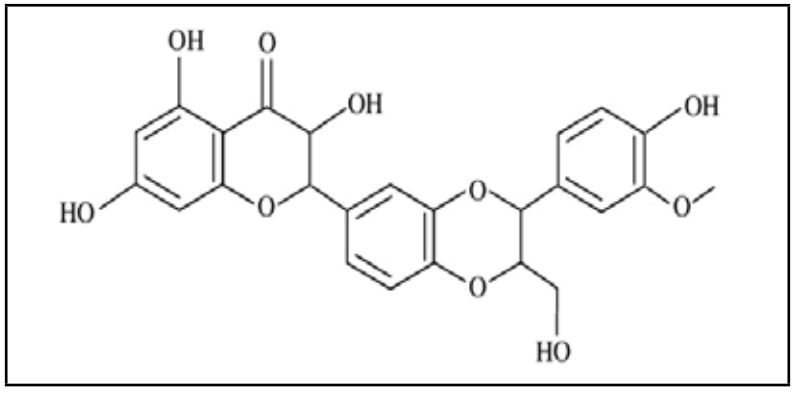

Figure 1: Chemical structure of Silibinin

Hence, in the present study we have taken U87MG a glioblastoma grade IV tumor as the cancer model and used Silibinin to target hallmarks of cancer properties like cell growth,

proliferation, and migration. The findings of present study may pave new roads in understanding use of Sb phytochemicals in the treatment of glioma.

MATERIALS AND METHODS

Materials and Reagents

Silibinin, dimethyl sulfoxide (DMSO), trypan blue reagent (0.4%) were obtained from St. Louis, MO, USA. MTT 3-(4, 5-dimethythiazol2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were from Invitrogen (Carlsbad, CA, USA).

Cell culture and culture conditions

The human glioblastoma cell line U87MG was purchased from the NCCS, Pune. It was routinely maintained in modified eagle's medium (MEM) with glutamine supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution. All the drug stocks were prepared in DMSO. All the drug treatments were performed without glutamine conditions.

MTT assay for cell viability

Approximately 8000 cells/well of U87MG were seeded in 96 well plates and treated with different concentration of phytochemicals from 2.5 to 100 µM and DMSO in control in without glutamine media for 48 hours. Absorbance was measured at 570 nm in microplate reader as described earlier (Synergy H1 Hybrid Reader, BioTek, USA) **[10].**

Trypan blue dye exclusion assay

Approximately 1×10^5 per 60 mm cells were seeded, treated with either DMSO alone or desired concentrations of Silibinin for 48 hours. After trypsinization, cells mixed 10 µL (0.4%) trypan blue. Live and dead cells were counted using a phase contrast microscope (Zeiss, Germany) as described earlier **[13]**.

Study of Morphological Alterations

After 48 hours of incubation of U87MG cells with treatment of all the drugs, cells were viewed using an inverted microscope (Zeiss, Germany) under 100 X objectives. The images were then compared to assess the effect of various treatments on the morphology of cells.

Statistical analysis

Statistical analyses were undertaken using GraphPad Prism version 6.0. Experiments were repeated two to three times. Student t-test was done to indicate the statistical analysis. The differences were considered significant when $p < 0.05$ and indicated in the figures. For densitometry analysis, ImageJ software was used.

RESULTS AND DISCUSSION

Silibinin inhibits cell viability of U87MG glioma cells

U87MG cells were treated with different concentrations ranging from 2.5 to 100 µM of Sb **(Figure 2)** for 48 hours and checked for cell viability in glutamine conditions. For assessing cell viability, MTT assay was performed. The respiring and viable cells have mitochondrial dehydrogenase enzyme which reduces the yellow color to purple color formazon crystals.

The intensity of the color correlates with proper functioning of the mitochondria suggesting the presence of viable cells and hence cytotoxicity of the phytochemical on the cells can be measured **[13]**. Sb significantly inhibited the viability of U87MG cells in a dose dependent manner at 48 hours **(Figure 2)**. Based on the screening studies, the effective doses for Sb (25, 50 and 100 μM) were taken for the further experiments.

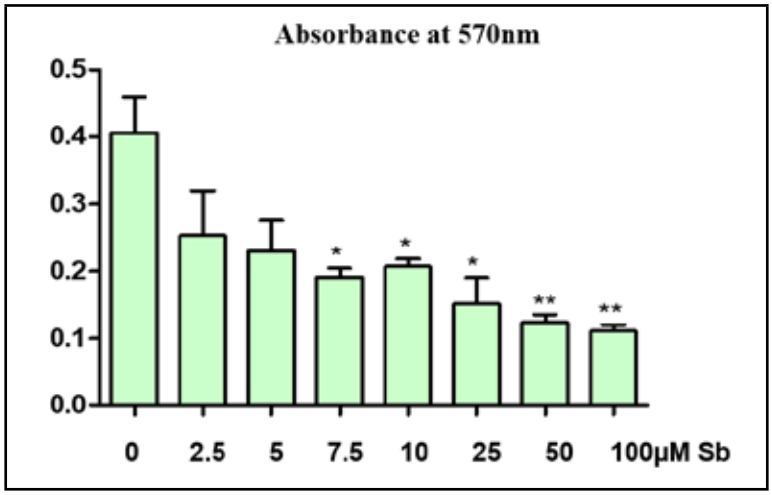

Figure 2: Effect of Silibinin (Sb) on cell viability on U87MG cells. Cells were treated with the indicated concentration of 0-100μM of Sb for 48 hours. The data are presented as means for triplicate samples for each treatment. Student's t-test was done. Bars;SE;*P<0.05;P<0.01 vs. the control.

Since ages, Silibinin has been used as hepatoprotective agents an effect which has been attributed to antioxidant properties **[14]**. It has many additional health benefits as clinical studies have shown that it can act as a chemopreventive agent on many cancer types, can target cyclin dependent pathways and cause cell cycle arrest **[15]**, and promote tumor apoptosis. There was no report demonstrating the effect of Silibinin on U87MG glioma growth and proliferation. So in the present study we investigated the effects of Silibinin on U87MG.

Silibinin inhibits cell growth and increases cell death in U87MG cells

The anti-cancer efficiency of any compound can be assessed by checking its effect on cell death and proliferation. To evaluate the effects of Sb on the growth of human cancer cells, the growth inhibitory potential of these compounds was determined in human glioblastoma U87MG cells. After the screening studies and deciding the doses and time-point, we next performed the trypan blue assay to check the effect of Sb on cell growth and death. Dye exclusion methods are routinely used to measure cell viability, with trypan blue exclusion test being one of the most common procedures. Trypan blue is a vital stain. The test is based on the principle that live cells have integral undamaged functional membrane and hence do not take the dye, whereas dead cells have broken damaged cell membrane so take the dye from the surrounding medium. Non-viable cells have distinct blue colour when observed under microscope, whereas viable cells are visible as unstained. This results in the ability to

distinguish between viable and non-viable cells **[16]**. The cells were treated with 25, 50 and 100 µM of Sb for 48 hours in minimum essential media.

For Sb, a significant dose dependent decrease was found in live cell number control to 66.7 % for 50 µM (<0.05) and 40.3% at 100 µM (<0.001)**(Figure 3B)**. The total cell number also decreases considerably to 66.7% and 45.1% (<0.001)**(Figure 3A)**. There was increase in percent dead cells from control 0.5% to 4% (< 0.001) **(Figure 3C)**.

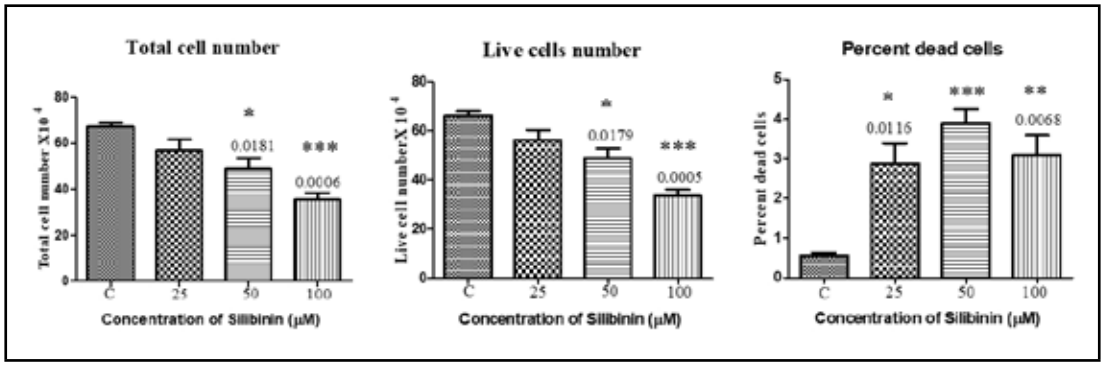

Figure 3 : Desease in Cell Number

Morphological analysis of Silibininon U87MG cells

In U87MG cell line, control cells treated with DMSO alone showed a branchy, elongated, slender and clustered polygonal shape which are seen as aggregated cells, and considered as the normal cell growth effect as observed under the light microscope. The most visible changes in cell morphology were observed after treatment with Sb (50 µM) **(Figure 4A)** and Sb (100µM) **(Figure 4B)** after 48 hour. After Sb treatment the cell morphology changed markedly to round, shrunken, branching between cells disappeared and showed a decrease in their number in proliferation **(Figure 4C)** and increase in dead cells as compared to control **(Figure 4D)**.

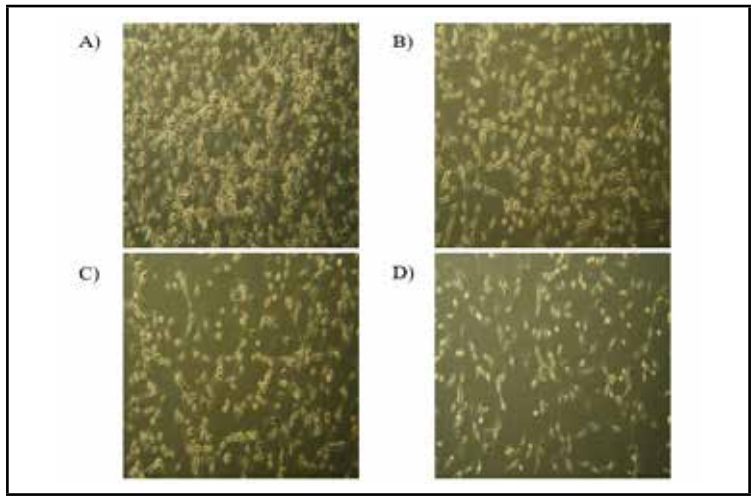

Figure 4 : Change in Cell Morphology

CONCLUSION

The current study focuses on the role of Silibinin on growth in the glioblastoma (GBMs) cells. We checked the effect of Silibinin on U87MG cells, a grade IV glioblastoma cell line. There was a dose-dependent decrease in the live cell number and increase in the percent dead cells as shown by trypan blue dye exclusion assay. When seen in microscope, we could observe that there were morphological changes in the cells which are the indicators of cells dying in the presence of Sb. Further studies are needed to check the cause of decrease in the cell proliferation. Overall, this is a preliminary study of Silibinin on U87MG cells showing it as a potential candidate for anti-cancer therapy.

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