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Effect of Flavonoids on Cell Viability and Glutamate-Induced Migration of U87MG Glioma Cells

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Abstract

Aim: To evaluate anticancer effect of the flavonoids (fisetin, acacetin and silibinin) on glioma cells. **Methods:** U87MG cells, glioblastoma grade IV tumor, were used as the invitro model to assess the anticancer effects of phytochemicals fisetin, acacetin and silibinin. Cell viability assay was performed by MTT. Cell growth and death assay was checked by trypan blue dye assay. Wound healing assay was used to check the migration of the cells. RT-PCR, western and gelatin zymography were performed for MMPs expression. **Results:** We found that number of live cells and cell proliferation were decreased by all three flavonoids and fisetin showed better effects as compared to acacetin and silibinin as performed by MTT and trypan blue assays. Fisetin significantly decreased migration more as compared to acacetin and silibinin as observed in the wound healing assay. Furthermore, we found glutamate promoted cell migration which was inhibited by fisetin in U87MG cells. We found that MMP-2 and MMP-9 expression levels decreased in the presence of fisetin as performed by zymography, RT-PCR and immunoblotting. **Conclusion:** Fisetin significantly decreases cell growth, proliferation and migration and the effects were relatively better as compared to acacetin and silibinin. Fisetin inhibits PCNA, MMP-2 and MMP-9 expression. All these results suggested that fisetin had anticancer capability through influencing the cell viability and migration of U87MG cells.

Keywords
Fisetin, Glioblastoma, Glutamate, Migration, MMPs

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INTRODUCTION:

Glioblastoma multiforme (GBM) or gliomas is a grade IV highly malignant and fast-growing tumor attacking the glial cells or supportive tissues, which provide nourishment and assist in signal transmission. Gliomas generally affect people in the age group of 40-65. It covers 30%-40% of all intracranial tumors [1, 2]. The reason which makes the glioblastoma a grade IV and a very aggressive tumor is its ability to migrate and invade to the other parts of the brain by destroying the components of ECM by proteolytic enzymes which are secreted by the tumor cells like matrix metalloproteinase (MMPs), by killing the neuronal cells and overcoming the physical constraints of space [3].

Fascinatingly, unlike other cancers brain tumors are also challenged by the space constraints due to the cavity of the skull. Brain tumors smartly surmount this problem by killing the healthy brain cells located in the vicinity of the tumors and creating space for

their growth. This work is accomplished by the elevated release of the glutamate which kills the neurons called as excitotoxicity [4-6]. This active destruction is done by glutamate which kills the neuronal cells and creates space for tumor expansion [5]. Also, glutamate plays a crucial role in glioma invasion and migration [6].

Cancer chemotherapy has been modest treatment modality used with both natural and synthetic compounds for the last 30-40 years but many shortcomings are associated with the chemical and synthetic compounds [7]. They have been related with the toxic side effects, high cost and they have to pass through rigorous regulatory process before they come to the public use. This 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 [7]. Many phytochemicals have anti-cancer, anti-bacterial and anti-fungal activities. They inhibit cancer growth, proliferation, cancer metastasis and invasion process of cancer cells and signaling pathways which need for cancer cell survival. Flavonoids are one type of phytochemicals and natural plant products which have many diverse biological activities [8].

Acacetin (5,7-dihydroxy-4-methoxyflavone) is extracted from *Clerodendrum inerme*, a widely available local herb is a flavonoid which has been suggested in many neuropsychiatric disorders as therapeutic application [9]. Acacetin is very widely known for its neuroprotective effects, anti-inflammatory, antioxidant and anticarcinogenic properties [10]. But, the biological targets of acacetin in the brain tumor are yet not clear unknown so it becomes a potent molecule to be used in the brain tumors [11]. Fisetin [2-(3, 4-dihydroxyphenyl)-3, 7-dihydroxychromen-4- one] is present in many vegetables and fruits like grapes, strawberry, cucumber, and persimmon. It has many important properties such as antioxidant, anti-inflammatory and anticancer behavior [12]. Various reports have shown the anticancer activity of fisetin against prostrate, pancreatic and lung cancer. Fisetin is used singly and also in conjunctions with other anticancer agents have shown to enhance the cytotoxic effects against cancer cells [13]. Silibinin which is the active component of silymarin complex also called as milk thistle, is extracted from the silybum marianum, has been used as hepatoprotective agents an effect which has been attributed to antioxidant properties [14, 15]. 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 many pathways and cause cancer death [16].

In the present study we have taken U87MG a glioblastoma grade IV tumor as the cancer model and used different phytochemicals acacetin (Ac), fisetin (Fs), and silibinin (Sb) which are known for their Neuroprotective effects. Also, glutamate plays a key role in glioma invasion and migration. We observed that fisetin (Fs) is the effective phytochemical as compared to acacetin and silibinin. Fisetin was used to target hallmarks of cancer properties like cell growth, proliferation and migration. In this study we also explored the role of fisetin on glutamate mediated involvement in glioma migration. The findings of present study may pave new roads in understanding the role of glutamate and fisetin in the treatment of gliomas.

MATERIALS AND METHODS:

Chemicals and Reagents

Fisetin, acacetin, silibinin, dimethyl sulfoxide (DMSO), trypan blue reagent (0.4%) and mitomycin were from Sigma Aldrich (St. Louis, MO, USA). MTT 3-(4, 5-dimethythiazol2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Invitrogen (Carlsbad, CA, USA). Protease and phosphatase inhibitors cocktails were bought from Roche Molecular Biochemicals (Indianapolis, IN, USA). PVDF membrane was purchased from GE life sciences and ECL was purchased from Merck Millipore. Ethanol was supplied by Merck Biosciences, India. RT PCR kit was from Thermo fisher. Reverse transcriptase, dNTPs and taq polymerase reagents including associated buffers were purchased from Bangalore Genie (India).

Cell culture and stock preparations

U87MG, grade IV human glioblastoma cell line was procured from NCCS, Pune, India. It was routinely maintained in modified eagle's medium (MEM) containing glutamine (2mM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution. All the drug stocks were prepared in DMSO and treatments were performed in without glutamine conditions.

Morphological examination under bright microscopy

For the morphological investigation, U87MG cells were seeded in 60-mm dishes at 1× 10^5cells/ml. The cells were treated with acacetin (10, 20 and 40 µM), fisetin (10, 25 and 50 µM) and silibinin (25, 50 and 100 µM) for 48 hours. Morphological variations were observed based on the visual observations and photographs were taken under a light microscope (Carl Zeiss, Germany).

MTT assay

Approximately 8000 cells/well of U87MG were seeded in 96-well plates and treated with various concentrations of phytochemicals from 2.5 to 100 µM and DMSO in control in without glutamine media for 48 hours. Absorbance taken at 570 nm in microplate reader as described earlier (Synergy H1 Hybrid Reader, BioTek, USA) [17].

Trypan blue dye exclusion assay

Approximately 1×10^5cells per 60-mm were seeded, treated with desired doses of fisetin, acacetin and silibinin for 48 hours. After trypsinization, total cells were harvested and counted based on the visual color variations with trypan blue and phase contrast microscope (Zeiss, Germany) as described earlier [18].

Wound healing migration assay

Cells were seeded in 6-well plates till 70% confluent. Subsequently, cell surface were scratched with a 200 µL plastic pipette tip to generate an identical, cell-free wound area as described previously [19]. The media were replaced with fresh MEM with different concentrations of fisetin (10, 25 and 50 µM) or acacetin (40 µM) and silibinin (100 µM) as per experiment designed.

Gelatin zymography

The gelatin zymography is done to study the activities of MMP-2 and MMP-9. After treatment time was over, conditioned-medium was collected and normalized with the total protein, and samples were run on 10% SDS-PAGE infused with 0.1 mg/mL gelatin and the bands were observed after staining with Coomassie brilliant blue-R250 as described previously [20].

Reverse transcription-PCR

U87MG cells were seeded and treated with desired doses of fisetin. The total RNAs from the cells were removed from cells using Trizol reagent according to the instructions of the manufacturer. The procedure follows the conversion of mRNA into cDNA and amplification. For detection MMP-2, MMP-9 and actin reverse transcription kit was used (Thermofisher) as per the manufacturer's guidelines. The following pairs of primers were used: MMP-2 5'GCGGATGATGCCTTTGCT3' (fwd primer), 5'GCGCGACAGAAGCCGTACTTGCCA3' (rev primer); MMP-9 5'CGGGACAAGCTCTTCGGCTT3' (fwd primer), 5'GAGGTGCCGGATGCCATTCA3' (rev primer) and β-actin 5'AGCCATGTACGTAGCCATCC3' (fwd primer), 5'TCTCAGCTGTGGTGGTGAAG3' (rev primer). The PCR amplification was carried out and melting temperature (Tm) was maintained ±5°C based on the primers' melting temperature provided.

Western blot assay

Fisetin treated (10, 25, 50 µM) cells were collected after 48 hours of incubation by trypsinisation and whole cell lysate was prepared. SDS-PAGE and Western blotting was done as described [21]. Membranes were probed with primary antibodies PCNA, MMP-2 and MMP-9 followed by corresponding HRP-linked secondary antibody IgG mouse/rabbit subsequently detected by ECL

Statistical analysis

Statistical analyses were undertaken using GraphPad Prism version 6.0. Experiments were repeated two to three times. Student's t-test was done to indicate the statistical significance of differences between two groups which was considered significant when $p<0.05$. For densitometry analysis, ImageJ software was used.

RESULTS:

Anticancer activity screening of flavonoids (acacetin, fisetin, silibinin) to evaluate their effect on cell viability of U87MG cells

For the first time, the flavonoids fisetin (Fs), acacetin (Ac) and silibinin (Sb) **(Fig. 1A)** were studied on U87MG cells with concentrations ranging from 2.5 to 100 µM for 48 hours. All the three flavonoids inhibited the viability of U87MG cells in a dose-dependent manner in both presence **(Fig. 1B)** and absence of glutamine **(Fig. 1C)**. We observed that these flavonoids show better effect in without glutamine conditions as compared to the presence of glutamine. Based on the screening studies the effective dose of Ac taken was (10, 20 and 40 µM), for Fs (10, 25 and 50 µM) and for Sb (25, 50 and 100 µM) were taken for the further experiments. Subsequently, further experiments were performed in without glutamine conditions.

Fisetin inhibits cell growth and increases cell death more effectively than acacetin or silibinin in U87MG cells

Subsequently, effective concentrations of fisetin (10, 25 and 50 µM), acacetin (10, 20 and 40 µM) and silibinin (25, 50 and 100 µM) were used in without glutamine conditions. Trypan blue counting showed dose-dependent reduction **($p<0.01$-0.001)** in the total cell number after 48 hours of treatment **(Fig. 2A-C)**. The highest concentrations of fisetin, acacetin and silibinin showed 30.9% **($p<0.01$) (Fig. 2A)**, 4.3% **($p<0.01$) (Fig. 2B)**, and 6% **($p<0.01$) (Fig. 2C)** dead cells, respectively.

Morphological analysis of U87MG cells exposed to flavonoids

After treatment the control cells of U87MG showed a slender elongated appearance with extensions and polygonal shape, which is a characteristic of the

normal astroglial cell morphology. The most noticeable variations in cell morphology were seen after exposure with fisetin (50 µM) after 48 hours as compared to Ac (40 µM) and Sb (100 µM). Fisetin-treated cells were round, shrunken, branching was lost and showed a reduction in their number in proliferation and increase in dead cells as compared to control **(Fig. 2D)**.

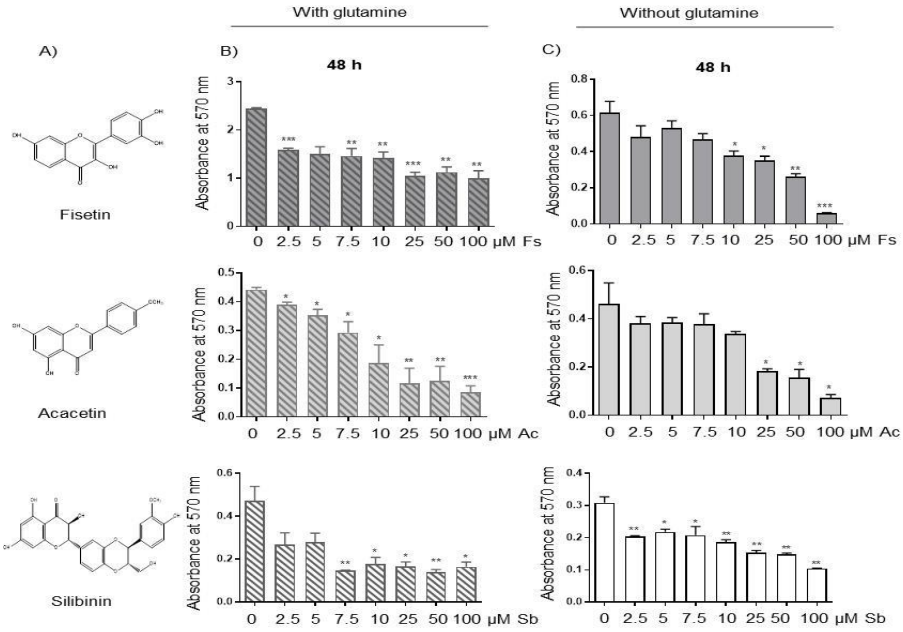

Figure 1: Effect of phytochemicals on cell viability of U87MG cells. For viability, cells were exposed with the indicated concentrations (0-100) µM Fs, Ac and Sb. (A) Chemical structure of Fs, Ac and Sb. Assay was performed in (B) With glutamine (C) Without glutamine, then subjected to MTT assay to check viability. Student's t-test was done. Bars, SE; P *<0.05, **<0.01, *<0.001 as compared to control.**

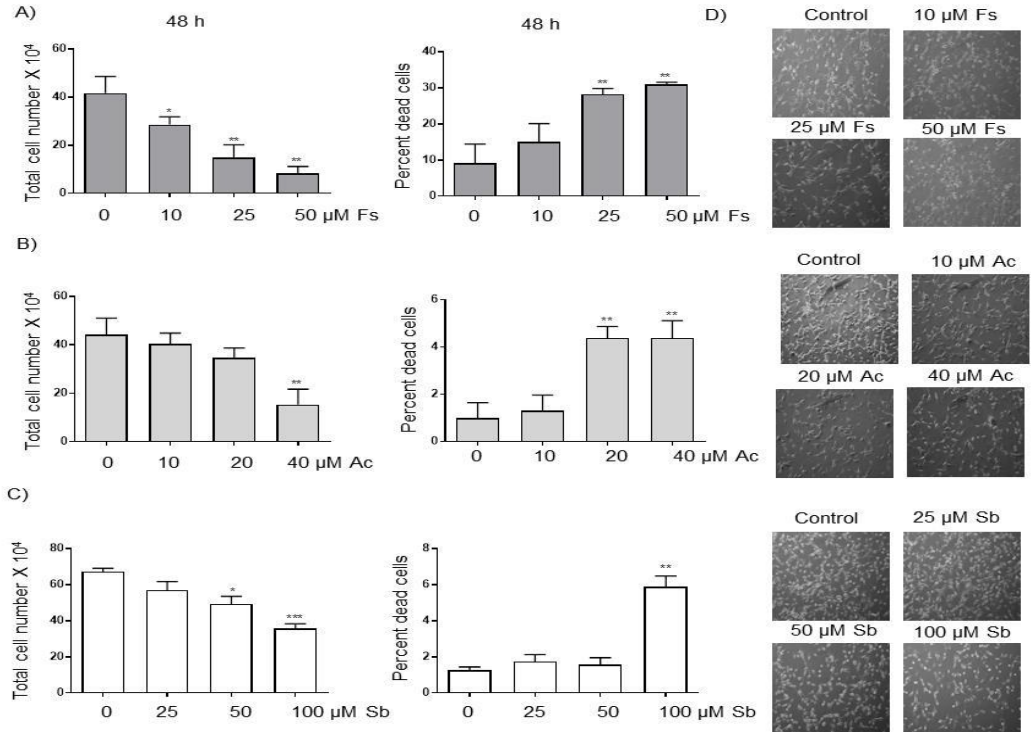

Figure 2: Effect of phytochemical on cell growth and death in U87MG cells. Briefly, 1X10^5. Cells were seeded and treated next day with indicated doses of phytochemical in minimum essential medium without glutamine. After 48 h the cells were scored on the basis of trypan blue dye exclusion test. (A) Total cell number and Percent dead cells counted in Fs (B) Total cell number and Percent dead cells in Ac and (C) Total

cell number and Percent dead cell in Sb (D) Photographs of phytochemical treated cells (Fs, Ac and Sb) at 100x. Student's t-test was done. Bars, SE; P *<0.05, **<0.01, *<0.001 as compared to control.**

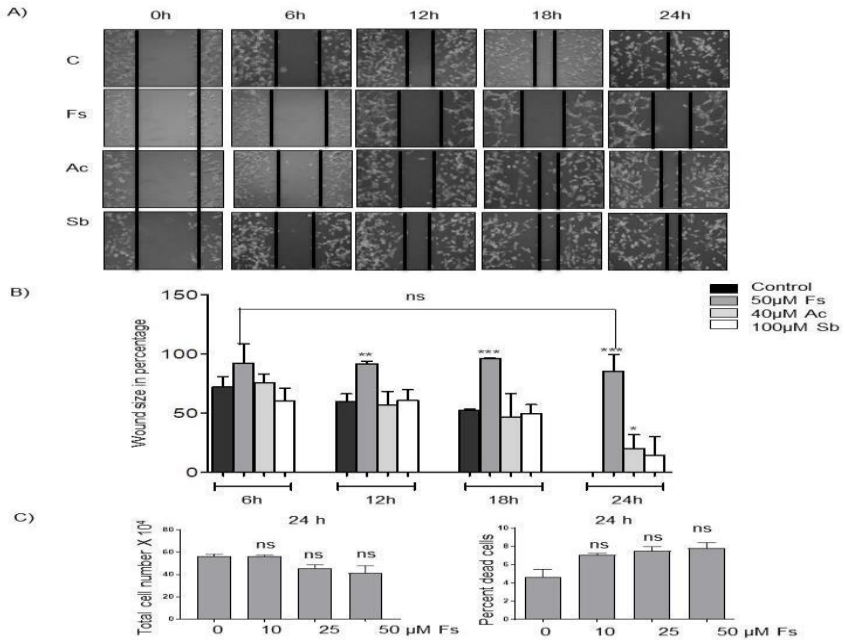

Figure 3: Effect of phytochemicals on cell migration in U87MG cells. Cells were cultured to 90% confluency, then a wound was created on the cells monolayer surface using a micropipette tip, and then Ac (40 µM), Fs (50 µM) and Sb (100 µM) was added. The migration of cells was assessed by wound healing depicting effect of various phytochemicals analysed with time and images were captured with a microscope. (A) Representative images at different time intervals 0, 6, 12, 18 and 24h (magnification, 100x). (B) Graphical representation in terms of wound filled calculated by counting six random fields across the black lines from each treatment. (C) Cell viability evaluated by trypan blue for Fs at 24h. The experiments were performed in duplicates, Student's t-test was done. **P<0.01, ***P<0.001 vs. the control.

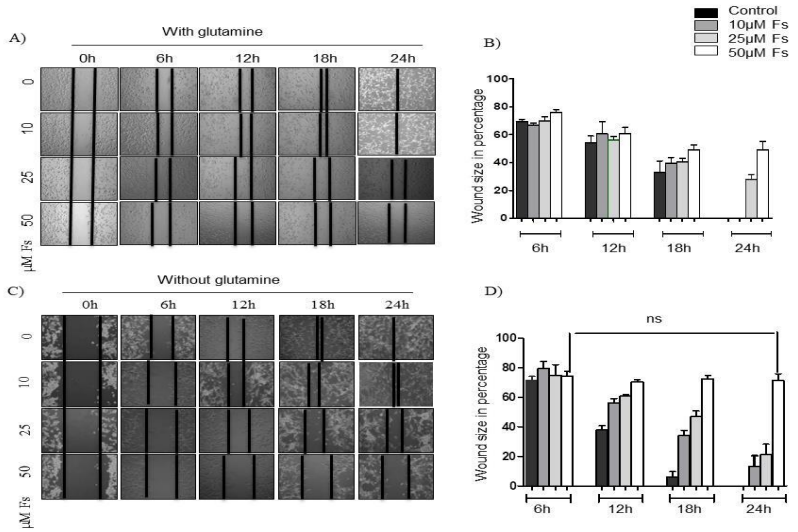

Figure 4: Effects of Fs on U87MG cell migration. Migration of U87MG cells was assayed by wound-healing at different time intervals 0, 6, 12, 18 and 24h (A) Representative images at different time intervals 0, 6, 12, 18 and 24h, with glutamine conditions (magnification, 100x). (B) Graphical representation in terms of wound filled calculated, with glutamine conditions (C) Representative images at different time intervals 0, 6, 12, 18 and 24h, without glutamine conditions (magnification, 100x) (D) Graphical representation in terms of wound filled calculated, without glutamine conditions. Data was analyzed using student's t-test. * P<0.05, **P<0.01, ***P<0.001 vs. the control.

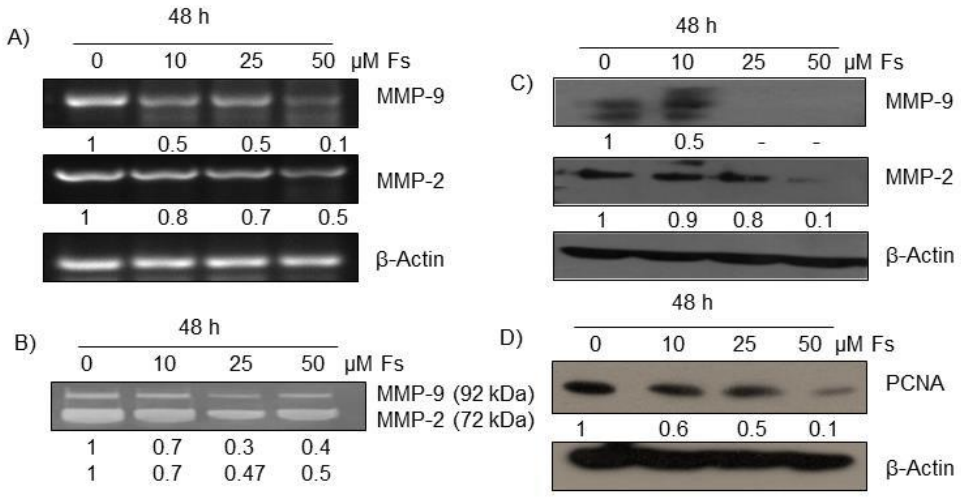

Figure 5: Effect of Fs on proliferation and invasion pathways in U87MG cells. (A) Semi quantitative PCR to show the mRNA expression of MMP-9 and MMP-2 in absence of glutamine (B) Gelatin zymography showing enzyme activity of MMP-2 and MMP-9 in without glutamine conditions (C, D) Western blotting of proliferation, invasion and migration related proteins to show expression of MMP-9, MMP-2 and PCNA in without glutamine conditions. β actin is the loading control. After normalization with β actin a fold induction calculated by image J software is shown below the blots.

Fisetin suppresses migration of U87MG cells more effectively than acacetin and silibinin

A comparative study was done by taking the most effective concentrations of flavonoids, fisetin (50 µM), acacetin (40 µM), and silibinin (100 µM) decreased the ability of U87MG cells migration, and the highest gap width calculated was 85% with fisetin **(p<0.001)** whereas in control plate, the wound gap was filled completely after 24 hours **(Fig. 3A, B)**. The effects with acacetin and silibinin were moderate showing only 20% and 14% gap width, respectively **(Fig. 3B)**. Fisetin did not show any considerable increase in dead cells in the same treatment **(Fig. 3C)**. All the results indicate that fisetin strongly suppress the migration of U87MG cells when compared with acacetin and silibinin.

Fisetin inhibits glutamate-mediated migration in U87MG cells

The glutamate-glutamine cycle is fundamental in the understanding of brain glutamate metabolism [22]. The glutamate which is manufactured from the L-glutamine is the crucial factor for migration and survival in the normal development of brain [23]. The effect of fisetin on U87MG cell migration was determined in both with and without glutamine conditions using scratch-wound assay. After fisetin treatment in with glutamine conditions at 24 hours we observed the gap in 25 and 50 µM **(**28% and 49% respectively**; p<0.001) (Fig. 4A, B)** and similarly in without glutamine conditions the gap width observed after fisetin treatment gap in 25 and 50 µM **(**21% and 70% respectively**; p<0.001) (Fig. 4C, D)** as compared to control. Again, fisetin showed better effect in without glutamine conditions as compared to the presence of glutamine.

Fisetin inhibits expression and enzymatic activity of MMP-2 and MMP-9

Fisetin (50 µM for 48 hours) treatment showed a strong reduction of 90% in MMP-9 and 50% in MMP-2 mRNA expression levels **(Fig. 5A)**. Gelatin zymography was performed to check the effect of fisetin on the enzymatic activity of MMPs. White unstained bands against a blue background were observed for the proteolytic activity. Fisetin could significantly decrease the enzymatic activity of MMP-9 and MMP-2 at 48 hours at all the doses. Fisetin (50 µM) decreased the enzymatic activity of MMP-9 (60%) and MMP-2 (50%) at 48 hours **(Fig. 5B)**. Further, immunoblot analysis was consistent with RT-PCR results and at 50 µM, 90% reduction in MMP-2 and no expression in MMP-9 was also observed **(Fig. 5C)**.

Fisetin suppresses the DNA replication marker

Proliferating cell nuclear antigen is essential for DNA replication. Hence, we assessed the expression of PCNA protein by western blotting in without glutamine conditions. The cells treated with fisetin significantly decreased the expression of PCNA in dose dependent manner. Fisetin treated cells showed significant dose dependent decrease in PCNA in without glutamine conditions up to 40%, 50% and 90% in 10, 25 and 50 µM respectively as

compared to control **(Fig. 5D)**. The loading control used was β-actin for normalizations

DISCUSSION

Glioblastoma (GBMs) is the most malignant and dangerous tumors among all the brain cancers. The distinguishing features which make these tumors aggressive are uncontrolled cell proliferation, invasion into the nearby healthy brain tissues [24].

In present study, we have chosen three flavonoids (acacetin, fisetin and silibinin) for initial screening studies. Acacetin is a flavonoid has shown to have antioxidant, anti-inflammatory and anticancer activities [10, 25]. Fisetin studies have shown that fisetin is very effective anticancer therapeutic against many cancer like colon cancer [26], pancreatic cancer [27], cervical cancer [28], prostate cancer [29] and many others. Silibinin since ages has been used as hepatoprotective agents an effect which has been attributed to antioxidant properties [15]. There was no report demonstrating the effect of these phytochemicals on glutamate associated growth and proliferation of gliomas. So in our study we explored the effects of these phytochemicals on U87MG and their effects on glutamate mediated growth and survival of glioma cells.

We screened out all the three phytochemicals (Ac, Fs and Sb) on U87MG cell viability in both with and without glutamine conditions. Since glutamate is shown to augment the growth and survival of glioma cells, we first analyzed the effect of fisetin on viability of glioma cells in both with and without glutamine conditions. Our findings suggested that fisetin has better inhibitory effect on growth and survival of glioma cells in without glutamine conditions. The anticancer efficiency of any compound can be assessed by checking its effect on cell death and proliferation. Fisetin were effective against decreasing the total cell count and increasing the percent dead cells. Hence, fisetin showed relatively better effect as compared to other two flavonoids, acacetin and silibinin. Fisetin was also observed to have stronger inhibitory effect on migration of glioma cells as compared to other two flavonoids.

PCNA is a useful marker for the estimation of tumour cell growth, proliferation and development. Recent studies proposed that PCNA occupies the significant position in defining the tumor progress and the effect of anticancer treatment [30]. Fisetin has showed to decrease the expression of PCNA in rat bladder carcinogenesis model [31]. Consistent with the previous results our study showed that fisetin decrease in glioma cell proliferation was accompanied by the significant inhibition in the expression of PCNA, a DNA replication marker.

L-glutamine which is precursor for many proteins and nucleic acids but immediate product of glutamine metabolism in the majority of the cells is L-glutamate [32, 33]. These data propose that U87MG proliferation and migration is regulated by glutamate. Our findings clearly show that glutamate is important factors for migration as in the presence of glutamine there was more migration and the gap width calculated was less as compared to without glutamine conditions. But, fisetin decreases migratory behavior of U87MG cells in presence and absence of glutamine showing it has a very potent anti-migratory capacity.

Similar to other tumor metastasis, glioblastoma invasion also involves complicated succession of integrated events which provides a favorable environment for the invasion and metastasis [34]. The proteases which play a significant role contributing to ECM turnover are matrix metalloproteinase (MMPs) [35]. MMPs are synthesized and secreted into the extracellular spaces where their activation requires proteolytic activation which involves enzymatic cleavage of the propeptide domains [36]. In glioblastoma substantial verification has pointed out major MMPs especially MMP-2, MMP-9 required for invasion [35]. Fisetin inhibits migration and invasion in melanoma and nasopharyngeal carcinoma [37]. Fisetin is also shown to decrease MMP activities in cervical cancer cell [38]. Migration and tumor cell invasion involving matrix metalloproteinases are hallmarks of aggressive gliomas. As better effect in wound healing was observed in absence of glutamine so, the molecular mechanisms were explored in without glutamine conditions. Fisetin was found to decrease the migration of U87MG cells which was accompanied by reduced expression of MMP-2 and MMP-9 at both mRNA and protein levels with a concomitant decrease in their enzymatic activity as observed in gelatin zymography. So, we conclude that fisetin is capable to modulate the migratory and invasion related proteins and can be potent anti-migratory therapeutic molecule.

CONCLUSIONS

The essential conclusions of the present study are that fisetin (a) inhibited cell viability growth and migration of U87MG cells effectively as compared to acacetin and silibinin (b) inhibit glutamate induced migration in U87MG cells (c) suppress the expression of PCNA, DNA replicating marker in U87MG cells (d) inhibit MMP-2 and MMP-9 expression at gene,

protein and enzymatic level thus, inhibiting migration and invasion of glioma cells.

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