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ORIGINAL ARTICLE

Anticancer effects of catechin flavonoid in human glioma cells are mediated via autophagy induction, cell cycle arrest, inhibition of cell migration and invasion and targeting MAPK/ERK signalling pathway

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Summary

Purpose: *Despite the emergence of innovative cancer treatment strategies, the global burden imposed by malignant glioma is expected to increase. Therefore there is an immediate need to find novel and better approaches for its treatment. The main aim of the current research work was to evaluate the anticancer effects of naturally occurring catechin flavonoid along with examining its effects on cell autophagy, cell cycle phase distribution, cell migration and invasion and MAPK/ERK signalling pathway.*

Methods: *MTT cell viability assay was used to assess the effects on cell proliferation and clonogenic assay was used to assess the effects on cell colony formation. Transmission electron microscopy (TEM) and western blot assay were used to examine the effects on autophagy. Flow cytometry was used to assess the effects of catechin on cell cycle, while the effects on cell migration and cell invasion were examined by wound healing assay and transwell chambers assay. Effects on MAPK/ERK signalling pathway were assessed by western blot assay.*

Results: *The results indicated that catechin molecule led to significant and dose-dependent growth inhibitory effects on U87MG human glioma cells with lower cytotoxicity in normal astrocytes. Clonogenic assay indicated strong decrease in glioma cell colonies. TEM revealed that catechin induced autophagy in U87MG cells by creating autophagosomes and autophagic vacuoles. The catechin-triggered autophagy was also linked with increase in the expression of LC3II and decrease in p62 expression. However, moderate effects were observed on the LC3 I expression. Catechin also triggered G2/M phase cell cycle arrest along with inhibiting cell migration and invasion in a dose-dependent manner. This molecule also led to blocking of MAPK/ERK signalling pathway.*

Conclusions: *In conclusion, the results indicate that the naturally occurring catechin showed strong anticancer effects in U87MG human glioma cells by targeting autophagy, cell cycle, cell migration and invasion and targeting MAPK/ERK signalling pathway.*

Key words: *glioma, catechin, flow cytometry, autophagy, cell invasion*

Introduction

Glioma is a frequent and lethal type of primary malignancy associated with human nervous system (mainly brain and spine) [1]. This malignant disease is characterized by quick progression, maximum infiltration, radiotherapy resistance, resistance to many chemotherapeutic agents and ineffectiveness of traditional surgery [2,3]. Despite recent comprehensive advancements made in the treatment of cancer, treating glioma still remains a major challenge for researchers [4]. Chinese glioma genome atlas statistics has revealed that the overall survival rate of patients with glioma remains very short [5]. Mortality of glioma is mainly due to its deceptive invasion, high potential of frequent proliferation, poor prognosis, disease relapse and lower overall survival rate [6,7]. The main cause of recurring hu-

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man glioma is infiltration of cancer cells to brain parenchyma. Throughout the course of invaded cell growth, migration of neoplastic cells takes place far beyond to neoplastic node, making conventional surgical procedure ineffective [8]. Thus, the hunt for novel treatment methodologies, strategies and chemotherapeutic agents to curb human glioma continues. Past studies have revealed significant role of natural products in suppressing human glioma cell proliferation via different mechanisms with lower or no side-effects and minimum cytotoxicity in normal cells [9,10]. Flavonoids are the naturally occurring vast class of natural products with diverse pharmacological applications including cancer treatment. Recent epidemiological studies regarding flavonoids revealed their high intake lowers cancer incidence and unveiled its protective cover against cancer [11,12]. *In vitro* examination of flavonoids indicate that their induced anticancer effects are mediated through inhibition of cell viability, invasion, apoptosis, cell cycle arrest, adhesion and inducing cell differentiation [13,14]. Tea extracts, mainly comprising of catechins known as tea catechins, have demonstrated anticancer properties in various animal models including oesophagus, oral, stomach, colon, small intestine, pancreas, liver, bladder, lung, prostate, skin and breast [15-18]. Herein, the main aim of the current research work was to evaluate the anticancer effects of naturally occurring catechin flavonoid along with examining its effects on cell autophagy, cell cycle phase distribution, cell migration and invasion and MAPK/ERK signalling pathway.

Methods

Cell culture conditions

Human glioma U87MG cells and normal astrocytes were provided from American type culture collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) and RPMI1640 with 10% fetal bovine serum (FBS) were used as culture media under experimental conditions of 37°C with antibiotics and 5% CO_2 humidified air.

Cell viability determination via MTT assay

In 96-well plates, both U87MG glioma and astrocyte cells were cultured at a concentration of 5×10^4 cells/well under experimental conditions. Cultured cells were treated with varying catechin concentrations i.e. 0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM for 24 h. Prior to incubation at 37°C for 5 h cells, 8 µl MTT solution (5 mg/ml) was added to all wells of 96-well plates. Blue formazan crystals formed were dissolved using dimethyl sulfoxide (DMSO). Finally, absorbance was measured using a microplate reader at 630 and 540 nm wavelengths.

Clonogenic assay

In 6-well plates, human U87MG glioma cells (500 cells/well) were seeded and then subjected to catechin exposure for 48 h at changing concentrations of control, 5 10 and 20 µM. Controls received only 0.1% DMSO. After catechin exposure, the media were totally drawn off and fresh media were inserted. Cells were then incubated for 10 days followed by fixation in ethanol (70%) and crystal violet staining. The experiments were performed in triplicate and the cells were counted using light microscope.

Transmission electron microscopy (TEM)

In 6-well plates, human U87MG glioma cell line was grown with varying catechin doses (control, 5, 10 and 20 µM) for 48 h followed by trypsinization. Around 4×10^6 cells were centrifuged till pellet formation (nearly 6 min). Catechin-treated cells were washed three times with phosphate buffer saline (PBS) and then fixed in ice-cold glutaraldehyde (2.5%) in sodium cacodylate/sucrose buffer (0.1M/1%, respectively) for 24 h. After fixation, cells were further washed three times with PBS followed by post-fixation with osmium tetroxide (1%) for 60 min. Next, cells were encapsulated in agar (1%) and stained with phosphotungstic acid and uranyl acetate, dehydrated in an array of graded ethanol and incubated with propylene oxide. Afterwards, cells were embedded in Epon-812-araldite mixture and thin sections were made with LKL-208 ultramicrotome. Finally, thin sections were placed in 200 mesh standard copper grids and subjected to TEM analysis (H-7500, TEM).

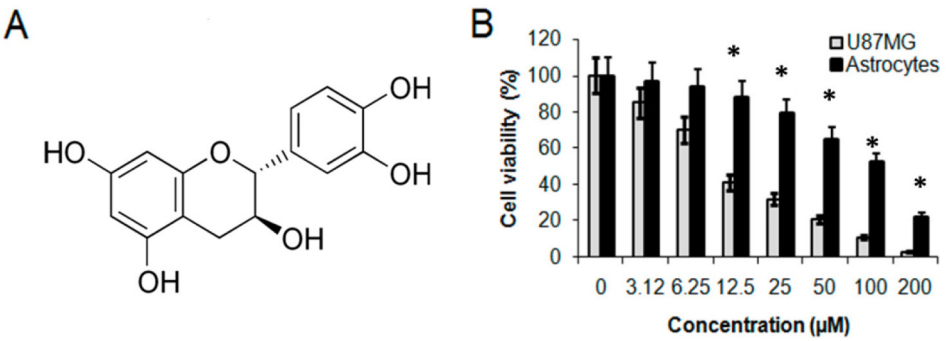

Figure 1. A: Chemical structure of catechin flavonoid. **B:** Anti-proliferative determination of catechin flavonoid on U87MG glioma cell line via MTT assay after treatment with indicated doses. Experimental data is shown as mean ± SD and experiments were performed in triplicate. $p<0.05$.

Cell cycle analysis through flow cytometry

U87MG glioma cells were treated with varying doses of catechin (control, 5 10 and 20 μM) and washed three times with PBS. Washing was followed by staining with Triton X-100 (0.1%), sodium citrate (3.8 mM) and 7 kU/ml of RNase B (nuclei staining buffer) for 3 h. Finally, cell cycle phase-distribution was evaluated via Becton-Dickinson FACScan cytofluorometer (Mansfield, MA, United States).

Cell migration analysis through wound healing assay

Human U87MG glioma cells were harvested at 80% confluence in 6-well plates with complete medium. A wound was scratched in each well using 1mm plastic tip followed by washing 3-4 times using PBS for debris removal. Afterwards, cells were treated with 10 μM concentration of catechin for 24 h. Inverted microscope was used to study the cell migration and distance of migrated cells from wound surface.

Cell invasion analysis

Transwell chambers coated with Matrigel (Corning Costar, Cambridge, Massachusetts) were used for cell invasion analysis with polycarbonate membrane of 5 mm radius and 8 mm pore size. Human U87MG glioma cells were first trypsinized and then suspended with a final density of 4×10^5 cells/ml in serum free medium (L15 medium). Cells were subjected to varying catechin concentrations for 24 h (control, 5, 10 and 20 μM). The upper chambers of the transwell were filled with cell suspension and the bottom chambers only with medium and 5% FBS. Cells were incubated under experimental conditions of 5% CO_2 incubator for 24 h and 37°C. Non-invaded cells were scrapped using cotton swab and invaded cells were fixed in methanol. After fixation, cells were stained with hematoxylin and eosin (Nanjing Sunshine Biotechnology Ltd, China). Ten fields were counted for each assay and the number of invaded cells was counted microscopically.

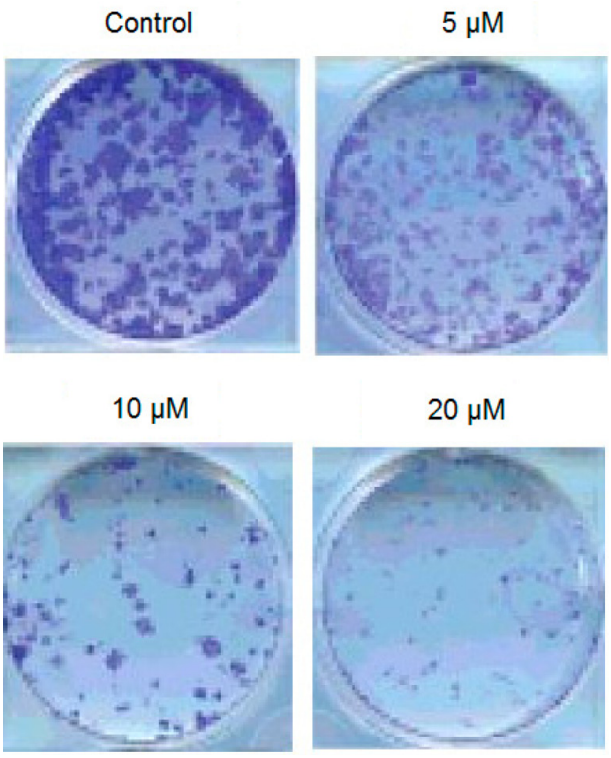

Figure 2. Effect of catechin flavonoid on colony formation of U87MG glioma cell line after treatment with different doses as indicated. Experimental data are shown as mean ± SD and experiments were performed in triplicate.

Protein level estimation via western blotting analysis

Cells were treated with changing concentrations of catechin (control, 5, 10 and 20 μM) followed by preparation of lysates. Protein content was measured using bicinchoninic acid (BCA) assay, 30 μM of protein from each sample were separated through electrophoresis via SDS-PAGE gels followed by transference to Hybond-C super membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Following manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ) the protein bands were visualized under enhanced chemiluminescence.

Statistics

All the experimental data is presented as mean ± SD. Data analysis was performed by one-way analysis of variance (ANOVA) and for multiple comparisons Student-Newman-Keuls test was used. $P<0.05$ was considereds show statistically significant differences.

Results

Suppression of cell viability of human U87MG glioma cells by catechin flavonoid

Cell viability, determined via MTT cell viability assay, after treatment with varying doses of catechin (Figure 1A) (0, 3.12, 6.24, 12.5, 25, 50, 100 and 200 μM) revealed remarkable suppression in cell viability of U87MG cancer cells in a concentration-dependent manner. Viability decreased from 100 to nearly 0% after catechin exposure of U87MG cells in contrast with normal astrocytes whose viability wasn't altered much by catechin exposure (Figure 1B). This shows the selective cytotoxicity potential induced by catechin in U87MG human glioma cells.

Inhibition of colony formation in U87MG cells by catechin

Clonogenic assay was performed to determine the effect of catechin on colony formation. After treatment (control, 5, 10 and 20 μM) the results revealed significant inhibition of colony formation in a dose-dependent manner (Figure 2). Thus it may be concluded that catechin is a potential anti-colony formation agent in human U87MG glioma cells and the cytotoxicity induced by catechin can be attributed to inhibition of cell forming colonies.

Morphology determination of catechin-treated U87MG glioma cells through TEM

TEM was performed to check whether the antiproliferative effects are autophagy-mediated. The results revealed significant morphological changes in catechin (5 μM) treated U87MG glioma cells, indicating autophagic cell death. Formation of autophagic vesicles can be clearly seen after catechin treatment (Figure 3). Further, autophagic cell death was validated via determining protein expressions of autophagy-associated proteins through western blotting analysis. The results revealed that after treatment with varying catechin concentrations (control, 5, 10 and 20 μM), the levels of LC3-II increased remarkably in contrast with p-62 which was suppressed (Figure 4), while as in the case of LC3-I it increased moderately, thus revealing the capability of catechin as a potential autophagy inducer.

Catechin flavonoid induces cell cycle arrest in human U87MG glioma cells

Cell cycle-phase distribution was carried out by flow cytometry. After U87MG cells were sub-

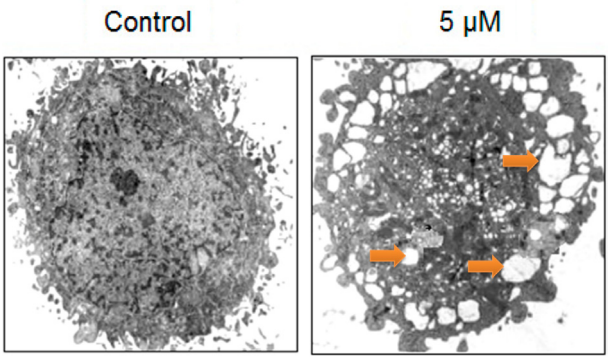

Figure 3. Transmission electron microscopy (TEM) revealing formation of autophagic vesicles after catechin exposure at indicating doses. The experiments were repeated thrice. Arrows indicate autophagosomes which are a hall mark of autophagy induced by catechin.

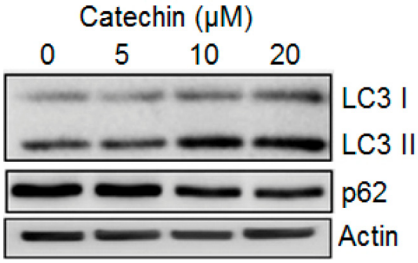

Figure 4. Autophagy-associated protein expressions via western blotting analysis. Actin was used as normalization control. The Figure shows that catechin led to increase in the expression LC3-I, LC3-II and decrease in p62 expression. The experiment was repeated thrice.

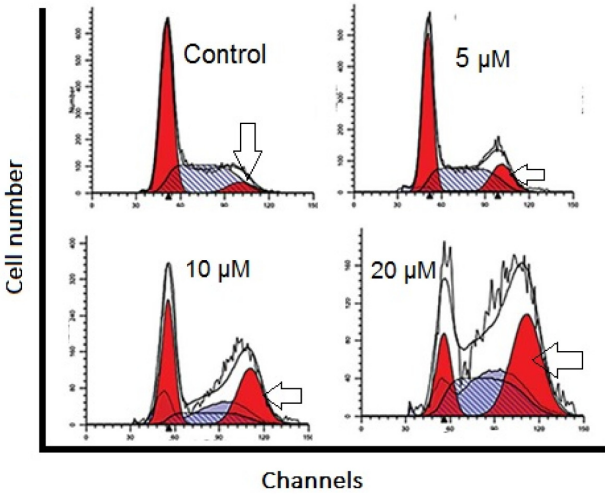

Figure 5. Cell cycle phase distribution analysis through flow cytometry after treatment with catechin at indicated doses. Arrows indicate increase in the G2/M phase cells with increase in the concentration of catechin, indicating G2/M phase cycle arrest. The experiments were performed in triplicate.

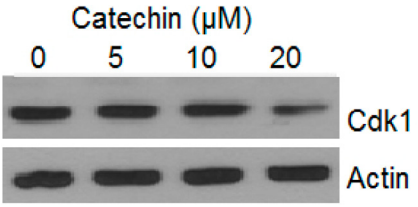

Figure 6. Western blotting analysis for determination of cell cycle related protein expression (Cdk1) after catechin treatment. Actin was used as normalization control. Catechin treatment led to increase in the expression of Cdk1 protein.

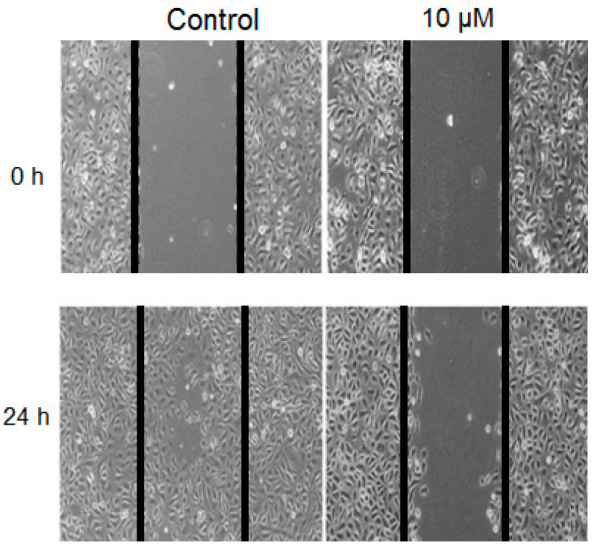

Figure 7. *In vitro* wound healing assay for determination of the effects of catechin on cell migration ability of U87MG glioma cell line. Catechin treatment led to decrease in the migration of U87MG cells. The experiments were performed in triplicate.

jected to catechin treatment at different doses (control, 5, 10 and 20 μM) the results revealed that the number of cells distributed in S- and G0/G1-phase reduced tremendously in a dose-dependent manner. Simultaneously, it was observed that the number of G2/M-phase cells increased remarkably with increased catechin doses, revealing the cell cycle arrest at this phase of the cell cycle (Figure 5). In addition, this was confirmed by western blotting analysis, which revealed significant alteration in the levels of Cdk1 protein which is related to cell cycle. It revealed potential inhibition of Cdk1 expression by catechin and further confirmed that the effect of G2/M-phase cell cycle arrest was dose-dependent (Figure 6).

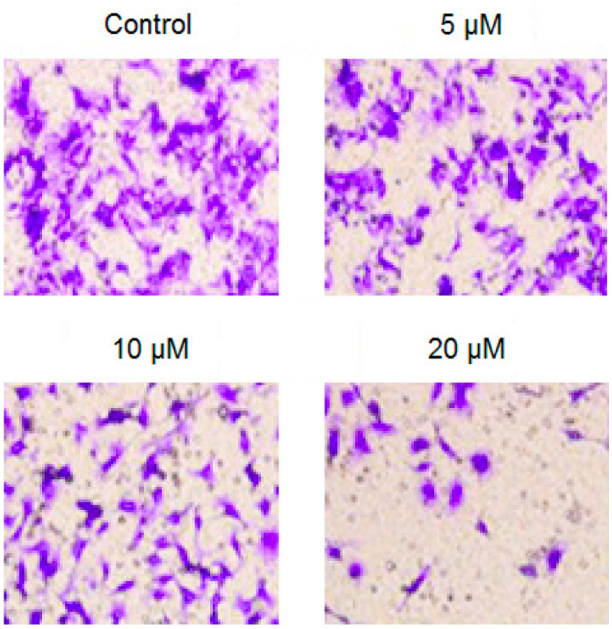

Figure 8. Cell invasion inhibition by catechin flavonoid on U87MG glioma cell line at indicated doses. Catechin led to dose-dependent decrease in the invasion of U87MG cells. The experiments were performed in triplicate.

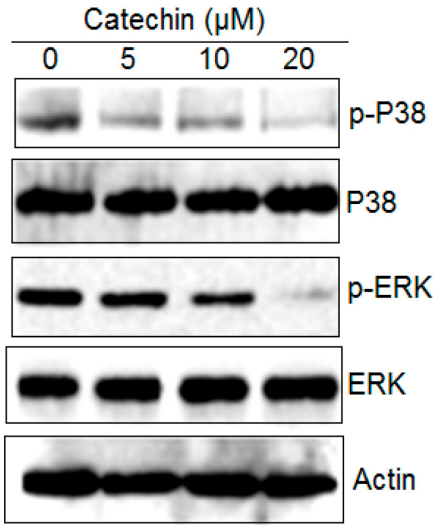

Figure 9. Determination of effect of catechin flavonoid on MAPK/ERK signalling pathway through western blotting analysis. Actin was used as normalization control. Catechin treatment led to decrease in p-p38 and p-ERK, while the expression of p38 and ERK remained unchanged. The experiments were executed in triplicate.

Inhibitory effects of catechin on cell migration and invasion

Cell migration and invasion were determined by *in vitro* wound healing assay using 6-well plates and transwell chambers with Matrigel, respectively. Wound healing assay revealed that the cell migration was inhibited significantly by catechin exposure for 24 h. The migratory effects were assessed by wound closure method comparing to that of controls (Figure 7). Further, cell invasion assay showed remarkable suppression of cell invasion ability in U87MG cells by catechin treatment. This impact on cell invasion was observed to be dose-dependent, i.e. upon increasing the doses of catechin in control, 5, 10 and 20 μM cell invasion decreased significantly (Figure 8).

Catechin flavonoid targets MAPK/ERK signalling pathway

Western blotting analysis was performed to unveil the effects of catechin flavonoid on MAPK/ERK signalling pathway. After exposure to varying catechin doses (control, 5, 10 and 20 μM) protein samples were collected and studied through western blotting. The results revealed almost no change in the levels of P-38 and p-ERK (Figure 9), thus revealing the dose-dependent blocking effect of catechin on MAPK/ERK signalling pathway.

Discussion

Glioma is a lethal malignancy accounting for a large number of deaths worldwide. The major hurdles in glioma treatment are chemotherapeutic resistance, ineffectiveness of conventional surgical methods, deceptive invasion, frequent proliferation, poor prognosis, disease recurrence and lower overall survival rate. Hence, the search for new efficient methodologies and chemotherapeutic agents is the need of the hour. Apoptosis and autophagy are the two major forms of programmed cell death (PCD) and serve as major therapeutic targets in cancer treatment. Apoptosis and autophagy are classified by Clarke as type I and type II morphological forms of PCD, respectively [19]. Autophagy is a cell regulatory mechanism resulting in degradation of organelles and intracellular

proteins [20,21]. Autophagy results in encasing of organelles and cytosol in a vacuole known as autophagosome which subsequently fuses lysosomes and are degraded through lysosomal hydrolases. Herein, the current study of catechin flavonoid was designed to unveil its anticancer properties against human glioma cells mediated via autophagy induction, cell cycle arrest, inhibition of cell migration and invasion and targeting MAPK-ERK signalling pathway. MTT assay was performed for cell viability determination and revealed significant inhibitory effects of catechin on the viability of U87MG cells in a concentration-dependent manner without hampering the viability of normal astrocytes. Clonogenic assay was performed to unveil the effects of catechin on colony formation and revealed tremendous inhibitory effects on colony formation. Further, TEM was performed to check whether the anti-proliferative effects of catechin are autophagy-mediated and the results revealed the formation of autophagic vesicles, clearly indicating autophagic cell death. It was also validated via western blotting analysis which revealed increased expression of LC3-I and LC3-II in contrast with p-62 which decreased remarkably. Next, cell cycle phase analysis revealed dose-dependent decrease in the number of S- and G0/G1-phase cells as compared to G2/M phase cells, which showed tremendous increase, indicating G2/M-phase cell cycle arrest. Furthermore, western blotting analysis revealed decreased CDK1 levels, supporting the results of cell cycle analysis through flow cytometry. Thereafter, the effect of catechin on cell migration and invasion was evaluated through wound healing and transwell chambers with Matrigel assays, revealing dose-dependent inhibition of both. Finally, the impact of catechin on MAPK/ERK signalling pathway was observed using western blotting analysis, revealing inhibitory effects on p-P38 and p-ERK levels and tremendously enhancing the levels of P38 and ERK, which clearly confirms that catechin treatment of U87MG glioma cells resulted in blocking of MAPK/ERK signalling pathway.

Conclusions

In conclusion, it is clear that catechin flavonoid is a potential anticancer agent against human glioma cells. Furthermore, its anticancer effects were found to be mediated via autophagy induction, cell cycle arrest, inhibition of cell migration and invasion and targeting MAPK/ERK signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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