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ORIGINAL ARTICLE

Myricetin exhibits anti-glioma potential by inducing mitochondrial-mediated apoptosis, cell cycle arrest, inhibition of cell migration and ROS generation

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Summary

Purpose: *To study the antiproliferative effects of myricetin in human glioma U251 cells together with assessing its effects on cell cycle, apoptosis, apoptosis-related proteins, reactive oxygen species (ROS) generation and cell migration.*

Methods: *Cell viability of human glioma cells after myricetin treatment was assessed by MTT assay. Phase-contrast and confocal fluorescence microscopies were used to assess the morphological changes that occured in these cells following myricetin treatment. Flow cytometry using propidium iodide (PI) and Annexin-V FITC as probes was employed to evaluate the effects on cell cycle arrest and apoptosis induction, respectively. The effect of myricetin on intracellular ROS production was measured by flow cytometry with a fluorescent probe CM-DCFH2-DA.*

Results: *Myricetin induced a dose-dependent as well as time-dependent growth inhibitory effect in U251 human glioma cells. Myricetin treatment resulted in U251 cells detachment from adjacent cells making clusters of cells floating in the medium. Detached cells had irregular shape and incapable to maintain their membranes intact. Apoptotic cell death was induced by myricetin treatment as witnessed by fluorescence microscopy. The percentage of early and late apoptotic cells increased from 0.41% and 8.2% to 23.1% and 10.2%, 25.2% and 19.4%, to finally 36.2% and 28.4% after treatment with 15 μM, 60 μM and 120 μM of myricetin, respectively. We also observed a dose-dependent increase in Bax and Bad levels and a dose-dependent decrease in Bcl-2 and Bcl-xl expression levels following myricetin treatment. Cell cycle arrest in G2/M phase of the cell cycle was also induced by the drug treatment. A concentration-dependent ROS generation was also witnessed and a 3-fold increase of ROS production was seen after 60 μM myricetin treatment.*

Conclusion: *Myricetin exerts anticancer effects in U251 human glioma cells by inducing mitochondrial-mediated apoptosis, G2/M phase cell cycle arrest, ROS generation and inhibition of cell migration.*

Key words: *apoptosis, cell cycle, glioma, flavonoids, flow cytometry, myricetin*

Introduction

Cancer has become one of the deadly health problems worldwide because of its high incidence and mortality. Despite the latest developments in therapeutic and diagnostic tools, cancer remains a serious health threat globally, creating an immediate need to design and develop novel therapeutic approaches. Glioblastoma multiforme (GBM) is the most common cancer of the central nervous system (CNS) in adults characterized by enormously aggressive behavior and bad prognosis. Despite recent advances in treatment options such as surgery, chemotherapy and radiotherapy, glioblastoma patients show very low median survival of less than 2 years [1,2]. In the past two decades, the incidence of primary brain cancers has seen a sharp upsurge and as the people get

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older, this trend is expected to rise further. GBM is the most malignant form of brain tumor in adults and is classified as a grade IV astrocytoma by the World Health Organization (WHO). The 2007 WHO classification of CNS tumors classifies glioma into grades I–IV, whereby grade I and II are classified as low grade and grade III and IV are defined as high grade (also known as malignant glioma) [3]. GBM is the most common type of primary malignant brain tumor in the People's Republic of China also, where the incidence of GBM is 1-5/100,000 [4,5]. Despite advances in surgery, radiotherapy and chemotherapy, malignant brain tumors continue to be a therapeutic challenge mainly because of their characteristic resistance to apoptosis [6]. Doxorubicin, a familiar chemotherapeutic anticancer drug used in treating gliomas has many severe side effects that contribute to congestive heart failure. Additionally, this is further compounded by the growing menace of resistance by cancer cells to radiation and chemotherapy [7]. Thus, there is an urgent need for the development of novel and efficient treatment options which should be able to overcome resistance and should have low toxicity to normal cells.

Natural products isolated from plants have acknowledged much consideration as potential chemopreventive and chemotherapeutic agents. Natural products are very promising sources for cancer prevention and treatment in preclinical and clinical trials. Recently published reports have revealed the antitumor effects of natural products and plant extracts against a wide range of human cancers [8-10]. Furthermore, there has been a specific rising interest to explore the anticancer properties of natural products for the treatment of glioblastoma [11,12]. To the best of our knowledge, the anticancer potential of myricetin against human glioblastoma cells has not been reported so far.

The aim of the present investigation was to determine the anticancer properties of myricetin in human glioblastoma U251 cells along with studying its effect on apoptosis induction, cell cycle arrest, cell migration and invasion and caspase activation.

Methods

Chemicals and other reagents

Myricetin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide (MTT) and Annexin V-fluorescein isothiocyanate (FITC) PI apoptosis detection kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Myricetin was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to get a 100 mM stock solution, which was diluted in the medium to yield the desired myricetin concentration. An equivalent volume of DMSO in complete culture medium was used as the vehicle control. To exclude the cytotoxicity of DMSO, the ultimate concentration of DMSO for all experiments was kept at less than 0.2%. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin, phosphate-buffered saline (PBS) with calcium chloride and magnesium chloride were purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). PI and acridine orange (AO) were purchased from Biotium Inc., Canada. Rabbit polyclonal antihuman Bcl-2, Bax, Bcl-xl, Bad, caspase-3, cleaved-caspase-3, PARP, and cleaved-PARP antibodies were all purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). All other chemicals and solvents used were of the highest purity grade.

Cell line, culture conditions and cell proliferation assay

The U251 human glioblastoma cell line was purchased from the Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). The cells were cultured in DMEM supplemented with 10% (v/v) FBS in humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced every 3 days. Cells were subcultured every 4 days. The cells were seeded on a 96-well plate at 4×10^3 cells per well. After 24 hrs, myricetin was dissolved in DMSO at various concentrations (0, 2.5, 5, 15, 30, 60, 120 and 240 μM) before treating the cells with it. After incubation times of 24, 48 and 72 hrs, MTT solution was added.

Phase contrast microscopy

U251 cells were plated in six-well plates at a density of $2x10^6$ cells/ml and then cultured for 24 hrs. Subsequently, the cells were exposed to treatment with various concentrations of myricetin (0, 15, 60 or 120 μM) for 48 hrs. Following drug treatment, culture plates were examined using an inverted light microscope (Nikon Corp., Tokyo, Japan) and images were captured.

Confocal fluorescence microscopy- cellular morphological evaluation

U251 cells were seeded on a chamber slide (Thermo Scientific Nunc Lab Tek II, Suzhou, P.R.China) at a cell density of 2×10^2 cells per chamber. AO (5 μg/mL) and PI (5 μg/mL) were added to each chamber and the cells were then observed under Fluoview 1000 laser scanning confocal microscope (Olympus IX 81 Motorized Inverted Microsope, Tokyo, Japan).

Annexin V-FITC assay

Cell apoptosis was evaluated by flow cytometry using double staining with FITC-conjugated. Cells

treated with or without myricetin (0, 15, 60 and 120 μM) were stained with PI and Annexin V-FITC, according to the manufacturer's instructions. Subsequent to incuba¬tion with myricetin for 48 hrs, cells were harvested and incubated in a binding buffer comprising Annexin V-FITC and PI at room temperature for 20 min. Analysis was performed using a flow cytometer (Becton-Dickinson FACS Calibur flow cytometry, Franklin Lakes, NJ, USA) and the data was analyzed using Cell Quest software.

Western blot analysis

U251 cells were treated with myricetin (15, 60, or 120 μM) for 48 hrs or left untreated (control). Cells were harvested by trypsinization, washed with cold PBS, lysed with ice-cold lysis buffer for 30 min, and centrifuged at 15000×g for 10 min at 4°C. The protein concentration of the clear supernatant was quantified using a BCA protein assay kit (Pierce Life Science, Rockford, IL, USA). An equivalent amount of each protein lysate (30 μl) was separated by electrophoresis on a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Pall, Radnor, PA, USA). The membranes were soaked in blocking buffer (5% skimmed milk) for 2 hrs at room temperature and washed three times in Tris-buffered saline containing Tween 20 (TBST). To probe for Bcl-2, Bax, Bcl-xl, Bad, Cytochrome C, cleaved caspase-3, cleaved-PARP, and β-actin, membranes were incubated overnight at 4 °C with the appropriate antibodies. After washing twice with TBST, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibodies at room temperature for 1 h. Signals were detected using an ECL Plus Chemiluminescence kit.

Cell cycle analysis by flow cytometry

The cell cycle analysis was carried out by flow cytometry (Becton-Dickinson FACS Calibur flow cytometry). Following incuba¬tion with myricetin for 48 hrs, the cells were collected from the different treatments, fixed with 70% ice-cold ethanol for 24 hrs, treated with 10 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA), stained with 10 μg/ml PI, and then analyzed by the flow cytometer.

Wound healing assay

Cells ($2x10^5$ cells/ml) were seeded in a 6-well plate and incubated at 37°C until 100% confluency of cells was achieved. Then, the confluent cells were scratched with a 100 ml pipet tip, followed by washing with PBS, and then treated with myricetin (15, 60, or 120 μM) in a medium. After incubating for 48 hrs, the glioma cells were fixed and then stained with 3% ethanol containing 0.3% crystal violet powder for 20 min. After that, randomly selected fields were photographed using a light microscope. The number of cells migrated into the scratched area was calculated.

Measurement of intracellular ROS generation

Intracellular ROS generation was evaluated using fluorescent CM-DCFH2-DA. U251 cells were planted in 6-well plates and after adhesion the cells were pretreated with 10 μM CM-DCFH2-DA for 20 min followed by co-incubation with various concentrations of myricetin for another 2 hrs and washed with ice-cold PBS twice. The cells were collected and analyzed using flow cytometry (Becton Dickinson FACS CantoTM, Franklin Lakes, NJ) with wavelength of excitation and emission at 488 nm and 525 nm respectively.

Statistics

The values shown were the result of three independent experiments with the data expressed as the means±SD. Differences between the control and treatment groups were examined using the Student's t-test SPSS 17.0 software was used for data analyses. A p val-

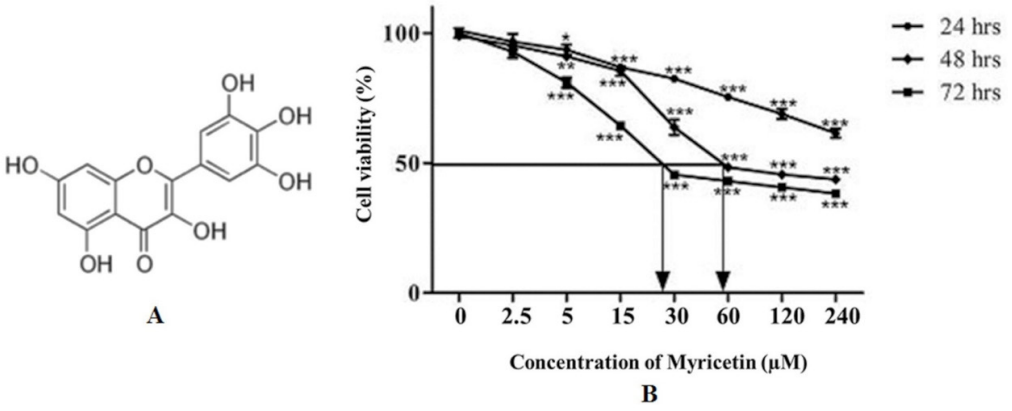


Figure 1. A: Chemical structure of myricetin. **B:** Cytotoxic effect of various concentrations of myricetin on U251 human glioblastoma cell viability at 24, 48 and 72 hrs. Each point represents the mean ± standard deviation (SD) from three independent experiments (n=3). ***p<0.001, **p<0.05 and *p<0.01 indicate the statistical significant difference with respect to untreated control group.

ue <0.05 was considered statistically significant.

Results

Myricetin induced cell death and reduced cell viability in human glioma cells

The chemical structure of myricetin is given in Figure 1 A. Cell viability of the U251 cells was evaluated by MTT assay and the results revealed that increasing doses of myricetin led to a significant loss of cell viability and increased cell death (Figure 1 B). The cell death induced by myricetin exhibited both dose-dependence as well as time-dependence. After 72 hrs, it was observed that 50% of the cell death occurred only at 27.1 µM (IC_{50} value of myricetin after 72 hrs) confirming the effectiveness of the drug. The IC50 value for myricetin after 48 hrs was 58.3 µM, while as after 24 hrs the IC_{50} value was more than 240 µM.

Myricetin induced morphological changes in U251 human glioma cells

U251 cells were treated with increasing doses of myricetin at 48 hrs to investigate the induced morphological alterations using phase contrast microscope (×100) (Figure 2). As the concentration of myricetin was increased from 0 to 15 µM and from 15 µM to 60 µM, U251 cells detached from adjacent cells making clusters of cells floating in the medium. Also, detached cells had irregular shape and became incapable to maintain their intact membranes (indicated by arrows). Similar morphological changes were observed in U251 cells after concentration was further increased from 60 µM to 120 µM.

Similar changes in cellular morphology were detected in confocal fluorescence microscopy using AO and PI as staining agents. AO is able to pass through plasma membrane of viable cells and cells that are undergoing early apoptosis and stain DNA. From our results, U251 cells showed robust emission of green fluorescence in untreated cells (Figure 3 A), but when exposed to different doses of myricetin, cells started to emit orange red fluorescence more intensely at the centre of the cells, representing late apoptosis (Figure 3 B-D). More dead cells were stained with PI after 48 hrs of myricetin treatment emitting red fluorescence.

Myricetin induced cell apoptosis in U251 human glioma cells

The flow cytometric analysis of the different groups of myricetin-treated cells showed that the proportion of apop¬totic cells was considerably higher in the myricetin-treated groups than in the control group (untreated cells, Figure 4 A), and the pro-apoptotic effect of myricetin improved with increasing concentration (Figure 4 B-D). Myricetin induced both early and late apoptosis in these

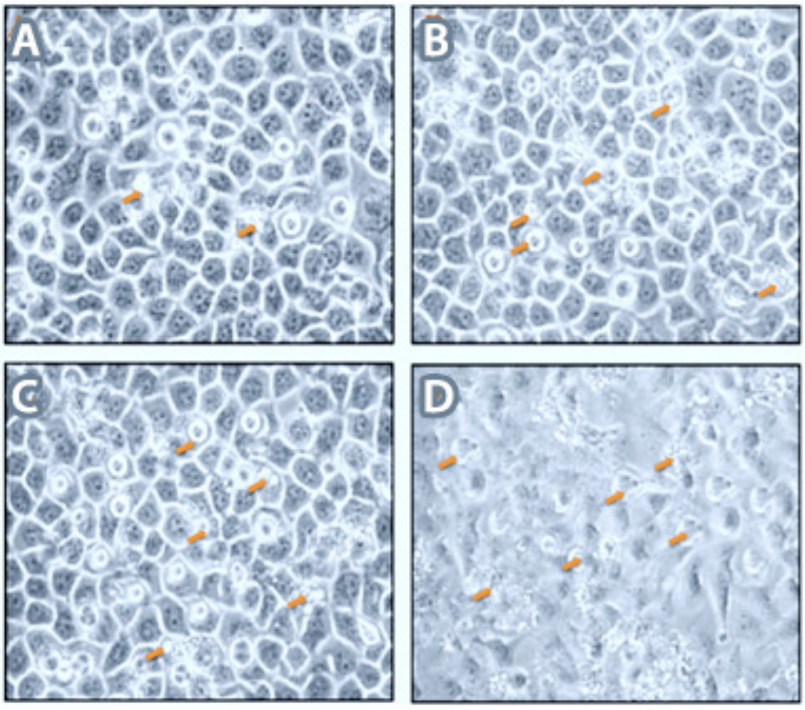


Figure 2. Phase contrast microscopy evaluation of the cell death induced by myricetin in U251 human glioma cells. The cells were treated without (**A**) and with 15 µM (**B**), 60 µM (**C**) and 120 µM (**D**) of myricetin for 48 hrs. Arrows show the dead cells or cells with altered cellular morphology.

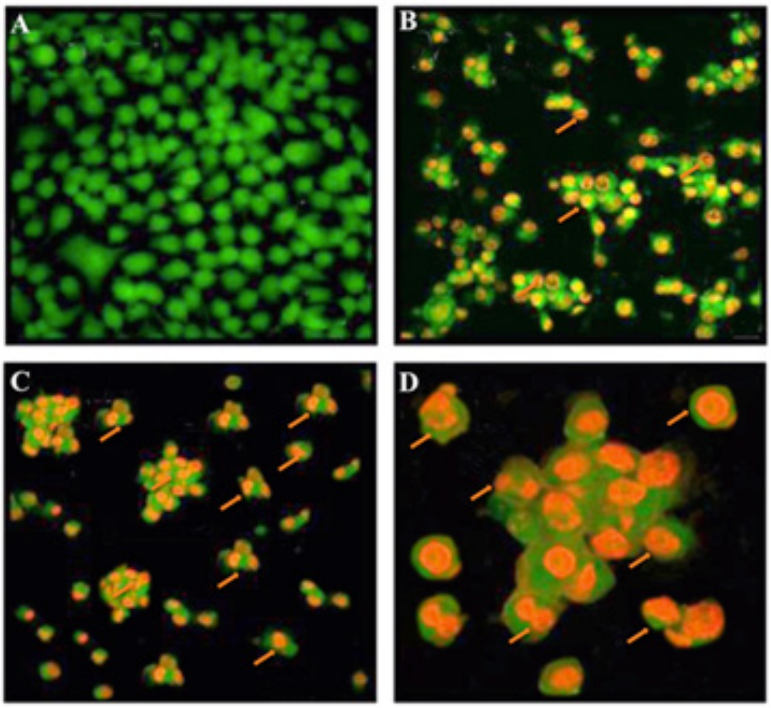
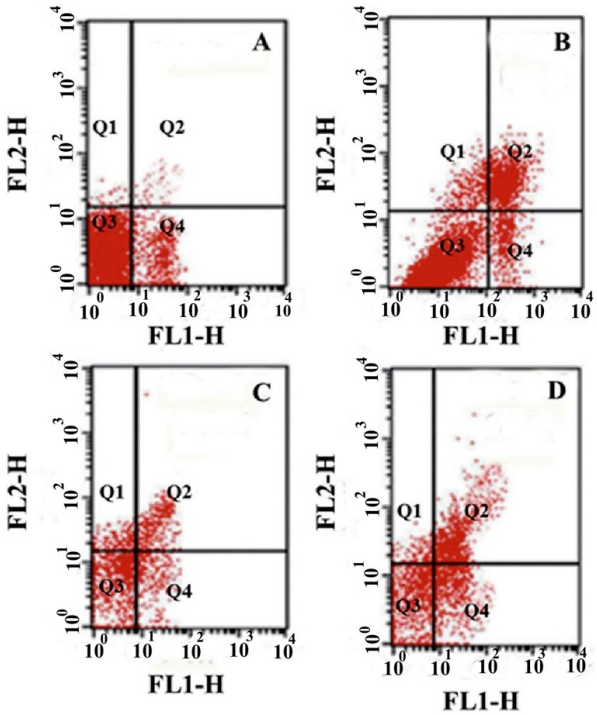


Figure 3. Confocal fluorescence microscopic evaluation of the apoptotic cell death induced by myricetin in U251 human glioma cells. The cells were stained with AO and PI and observed using laser confocal microscope (x400). The cells were treated without (**A**) and with 15 μM (**B**), 60 μM (**C**) and 120 μM (**D**) of myricetin for 48 hrs. Arrows depict apoptotic cells. .



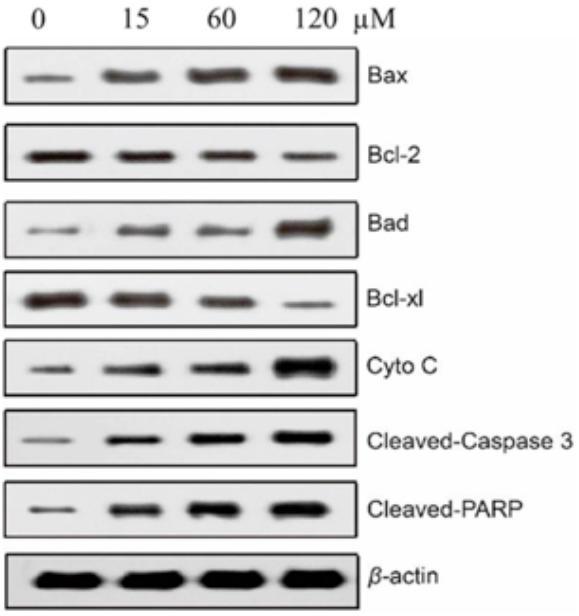


Figure 4. Effect of myricetin on the apoptosis induction in human glioma U251 cells. U251 cells were treated with (**B**) 15 μM, (**C**) 60 μM or (**D**) 120 μM for 48 hrs. (**A**) shows the control (untreated) group. Normal healthy, early apoptotic, late apoptotic and dead/necrotic cell populations are shown as percentage of total cells in the quadrants Q3, Q4, Q2 and Q1, respectively.

Figure 5. Effects of myricetin on expression levels of apoptosis-related proteins in U251 human glioma cells. U251 cells were exposed to 0, 15, 60, 120 μM of myricetin for 48 hrs, after which cell lysates were extracted and subjected to Western blot analysis to monitor expression of Bax, Bcl-2, Bad, Bcl-xl, Cytochrome C, cleaved-Caspase 3, and cleaved-PARP.

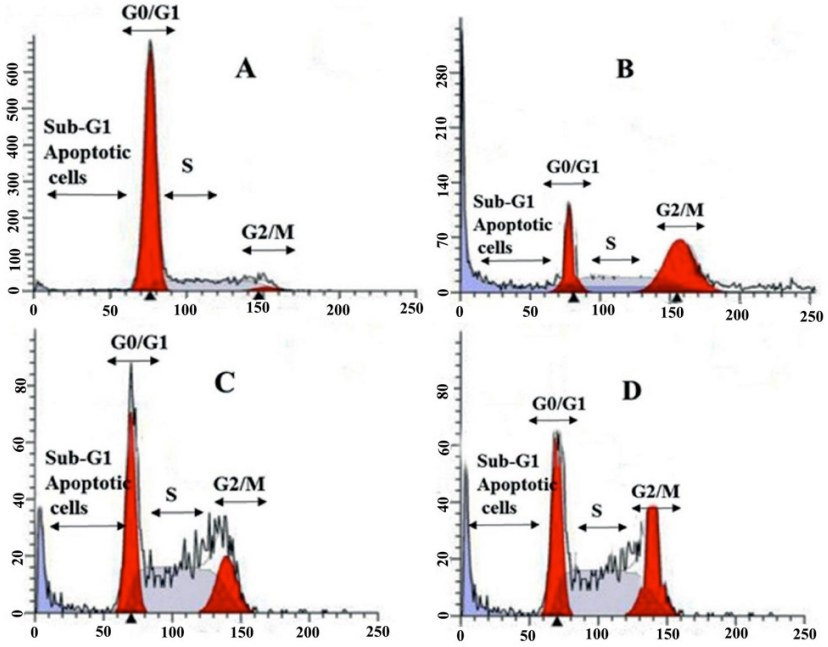


Figure 6. Cell cycle analysis of U251 cancer cells treated with myricetin. Myricetin induced G2/M and sub-G1 phase cell cycle arrest. The distribution of cells in various phases of the cell cycle was determined following treatment with myricetin at various concentrations. (**A**), (**B**), (**C**) and (**D**) represent treatment with 0, 15, 60 and 120 μM, respectively. Values are presented as the mean ± standard error of the mean of three determinations.

cells along with a slight necrosis. The percentage of early and late apoptotic cells increased from 0.41% and 8.2% to 23.1% and 10.2%, 25.2% and 19.4%, to finally 36.2% and 28.4% after treatment with 15 μM, 60 μM and 120 μM of myricetin, respectively.

Myricetin affected the ratios of Bcl-2/Bax and Bcl-xl/Bad protein pattern in U251 cells

We investigated the effect of myricetin on the expression levels of pro-apoptotic proteins (Bax and Bad) as well as anti-apoptotic proteins (Bcl-2 and Bcl-xl), both of which are from the Bcl-2 family and play a key role in the apoptotic process involving release of cytochrome C. Following treatment with increasing doses of myricetin (0, 15, 60 and 120 μM), we observed a dose-dependent increase in Bax and Bad levels and a dose-dependent decrease in Bcl-2 and Bcl-xl expression levels (Figure 5). This is indicative of the fact that myricetin may induce apoptosis via mitochondrial dependent pathway.

Myricetin induced sub-G1 and G2/M phase cell cycle arrest in U251 cells

The results revealed that myricetin induced cell cycle arrest at sub-G1 phase and G2/M phase (Figure 6). As the concentration of myricetin increased from 0 to 15 μM, 60 μM and finally to 120 μM, the number of cells in G2/M phase increased in comparison to the control cells. Concomitantly, we also found a sharp decrease in the G0/G1 cell population as the myricetin concentration increased. At higher doses of myricetin, we also noticed an increase in the sub-G1 cell population (apoptotic cells).

Myricetin inhibited cell migration in U251 human glioma cells

We also assessed the effect of myricetin on cell migration of U251 cells. Confluent cells were scratched and then treated with myricetin in a complete medium for 48 hrs. The number of cells migrated into the scratched area was photographed (x40) and calculated as a percentage (%) of migration. As can be seen in Figure 7, myricetin prominently reduced U251 cell migration in a concentration-dependent manner.

Myricetin induced ROS formation in U251 cells

The effect of myricetin on intracellular ROS production was measured by flow cytometry with a fluorescent probe CM-DCFH2-DA. As shown in Figure 8, treatment of U251 cells with myricetin for 2 hrs induced strong ROS formation. A concentration-dependent ROS generation was witnessed and 3-fold increase of ROS production was seen

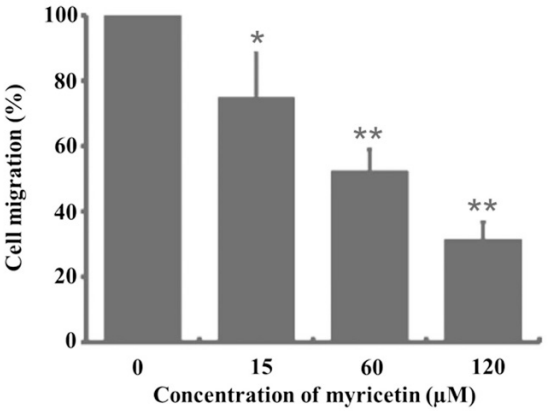


Figure 7. Myricetin inhibited cell migration of human glioma U251 cancer cells in a dose-dependent manner. Data are shown as the mean±SD of three independent experiments after analysis with Student's t test. * p< 0.05, and ** p<0.01, vs 0 μM.

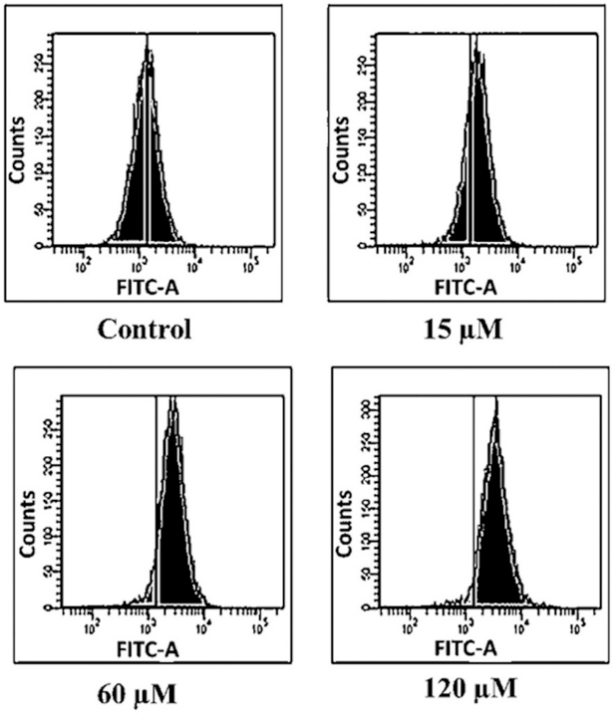


Figure 8. Myricetin induced ROS generation in U251 human glioma cells. The cells were treated with myricetin (0, 15, 60 and 120 mM) for 2 hrs, and the ROS generation was evaluated using CM-DCFH2-DA staining by flow cytometry.

after 60 μM myricetin treatment.

Discussion

Myricetin belongs to the flavonoid class of natural products which contain polyphenolic structural moieties that furnish them excellent antioxidant compounds. It occurs in vegetables, berries, red wine, nuts, tea etc and is structurally very identical with quercetin, luteolin, fisetin etc [13-15]. Flavonoids including myricetin are capable of scavenging ROS and can chelate intracellular transition metal ions that eventually produce ROS. Myricetin has been reported to potentiate the antioxidant tendency of other antioxidants along by inducing the enzyme glutathione S-transferase (GST) [14]. Being an antioxidant, myricetin has the tendency to protect living cells from different malignancies. It has been reported that myricetin attenuated the risk of skin cancer in mice models after cancer initiating and promoting agents were applied to the skin [13]. Myricetin has also been shown to induce cell death in human colon cancer cells via BAX/BCL2-dependent pathway [16]. Myricetin has also been reported to exert anticancer effects in human T24 bladder cancer cells both *in vitro* and *in vivo*. Many previous studies revealed that myricetin exerts anticancer effects on a large variety of cancers including esophageal, lung, leukemia and prostate cancer cells [17-20]. This compound has also been shown to affect MMP-2 protein expression and enzyme activity in colon carcinoma cells [21]. However, to the best of our knowledge, the anticancer activity of myricetin in U251 human glioma cells has not been reported so far. As such, the objective of the present investigation was to describe the anticancer and apoptotic effects of myricetin in U251 human glioma cells along with evaluating its effects on caspases, cell cycle phase distribution, ROS generation and cell migration.

Caspases play a vital role in mediating nuclear apoptosis. The protease enzyme caspase-3 is mainly associated with formation of apoptotic bodies which occurs from a series of biological events that triggers cell death [22]. Caspase-3-defective organisms are less expected to induce apoptosis although ultimately typical signs of cell death do occur. Delay in cell death under deficiency of caspases provides strong evidence that caspases are important in the event to trigger apoptosis [23]. Therefore, in the present study, we hypothesized that myricetin induced cell death through up-regulation of caspases which subsequently induced apoptosis, thereby potentially demonstrating anticancer effect against glioma cells. Following treatment with increasing doses of myricetin (0, 15, 60 and 120 μM), we observed

a dose-dependent increase in Bax and Bad levels and a dose-dependent decrease in Bcl-2 and Bcl-xl expression levels. This is suggestive of the fact that myricetin might induce apoptosis via mitochondrial-dependent pathway. The percentage of early and late apoptotic cells increased from 0.41% and 8.2% to 23.1% and 10.2%, 25.2% and 19.4%, to finally 36.2% and 28.4% after treatment with 15 μM, 60 μM and 120 μM of myricetin, respectively.

In addition, myricetin induced G2/M and sub-G1 phase cell cycle arrest in U251 human glioma cells. As the concentration of myricetin increased from 0 to 15 μM, 60 μM and finally to 120 μM, the number of cells in G2/M phase increased in comparison to the control cells. Concurrently, a sharp decrease in the G0/G1 cell population was seen as the myricetin concentration increased. Myricetin also led to ROS formation in these cells and a 3-fold increase of ROS production was observed after 60 μM myricetin treatment. Cell migration of U 251 cells was also significantly inhibited after myricetin treatment.

In conclusion, myricetin induces antiproliferative effects in U251 human glioma cells through induction of mitochondrial-mediated apoptosis, G2/M phase cell cycle arrest, ROS generation and inhibition of cell migration.

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