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Original Research Article

Eriodictyol modulates glioma cell autophagy and apoptosis by inhibition of PI3K/Akt/mTOR signaling pathway

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Abstract

Purpose: *To investigate the effects of eriodictyol (ERD) on U251 human glioma cell cycle and viability, autophagy and apoptosis by modulation of PI3/Akt/mTOR signaling cascade.*
Methods: *740 Y-P was used to activate U251 human glioma cells. For exploring ERD effects, the U251 cells were treated with ERD and 740 Y-P together. MTT assay was used to elucidate cell viability and apoptosis. The expression of autophagic proteins (LC3B and Beclin-1), and apoptotic proteins (Bcl-2 and Bax) were quantified using Western blotting. To explore the role of PI3K/Akt/mTOR signaling pathway, their expression was measured in comparison to their respective phosphorylated derivatives by Western blotting.*
Results: *ERD exposure downregulated p-PI3K and p-Akt protein expression. The results also indicate that ERD reduced cell viability and stimulated apoptosis in U251 cells ($p < 0.05$). Consequently, Bax expression was upregulated and the expression of Bcl-2 was downregulated. ERD enhanced the autophagy of glioma cells U251 by enhancing LC3B and Beclin-1 expression ($p < 0.05$). These effects were opposite to that revealed by 740 Y-P exposure alone.*
Conclusion: *ERD reduces U251 human glioma cell viability, and triggers cell autophagy and apoptosis, which is significantly correlated to downregulation of PI3K/Akt/mTOR signalling cascade. Thus, the compound can potentially be used for the treatment of glioma.*

Keywords: *Eriodictyol, Glioma, U251 human glioma cell, Apoptosis, Autophagy*

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INTRODUCTION

Neuroepithelium originated tumors are referred to as gliomas and are one of the most crucial and hostile forms of tumors targeted to the adult nervous system [1]. Among all the brain tumors, gliomas account for 40 – 50 % of them [2]. Similar to all the tumors, gliomas result from an intricate blend of an individual's genetic trait susceptibility, coupled with carcinogenic activators present in the environment. Glioma affects the general health of affected individuals, and may eventually progress to death. Significant mortality in glioma patients is marked by a lower prognosis. The treatment strategies can be varied depending on the infected site, cell

category and stage of malignancy. The current subjective treatments for glioma include surgical intervention and, chemotherapeutic and radiotherapeutic approaches [3]. Even though the glioma treatment approaches have been advanced, the patient revival after a prognosis is still challenging. Individuals with glioma after diagnosis, reveal a mean survival span of around 14 months [4-6]. Therefore, exploring novel and effective treatment strategies for glioma need to be addressed urgently.

The autophagic process influences cellular growth, proliferation, and mortality, and is linked with various pathophysiological mechanisms [5]. The process of autophagy subjects damaged cellular organelles and irregularly deposited proteins and other macromolecules to ultimate destruction and balances the cellular homeostasis [6]. Several investigations have been directed towards investigating the link between autophagy and glioma cells by employing therapeutic molecules. Glioma presence is quite often related to molecular alterations in epidermal growth factor receptor (EGFR) and phosphatidylinositol-3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) [7. Compared to normal individuals, patients with glioma are detected with an activated downstream PI3/Akt/mTOR signaling pathway. Variabilities in the activity of growth factor receptors result in overexpression of the PI3/Akt [8]. Reduction in the PIK3CB gene expression encoding for PI3K catalytic fraction p110b through siRNA in human U251 glioma cells reduces the proliferation of cells, arrests cell cycle, lowers invasion of cells and encourages cell apoptosis [9].

Subsequently, the process of apoptosis is required to maintain the balance of programmed cell death by critical utilization of control of several genes, factors and signaling pathways. Apoptosis is monitored by a series of regulatory proteins; pro-apoptotic proteins, such as Bax and p53, and anti-apoptotic proteins, such as Bcl-2 and Bcl-xL [10]. Investigations suggest that Akt has a capacity to suppress apoptosis via modulation of Bcl-xL/Bcl-2 linked death promoters, such as caspase-3 and caspase-9, together with the family of forkhead transcription factors [7,8]. Overall, at the preliminary, the stimulation of PI3K/Akt/mTOR could effectively reduce autophagy and progress to the tumor.

Eriodictyol (ERD) is a naturally occurring flavonoid, available in citrus fruits such as lemons, oranges, and bitter oranges, tangerines, peanuts, loquat, mugwort, and other food-borne plants [11]. Eriodictyol exhibits a broad spectrum of therapeutic activities, including antioxidant, anti-inflammatory, and antibiotic effects [12]. Earlier investigations indicate that ERD has the potential to protect oxidative stress-induced death of endothelial cells [13]. ERD imparts neuroprotection by reducing b-amyloid peptide-induced cell death, due to oxidative stress [14]. ERD has been recently studied to induce apoptosis in human lung cancer A549 cancer cells.

This anticancer activity of ERD was attributed to the alteration of the mitochondrial membrane potential, downregulation of Bcl-2, stimulation of Bax expression, finally resulting in cell cycle arrest [15]. Eriodictyol has been explored for its anti-inflammatory effect in LPS-induced neuro-inflammation in C57BL/6J mice. The reports revealed that ERD could inhibit the phosphorylation of PI3K/Akt, whereby PI3K/Akt is regarded as a crucial regulator for inflammatory response mediated by NF-kB [16].

In lieu of the investigations made so far on the pharmacological activities of ERD, we investigated the effect of ERD in autophagic and apoptotic responses in glioma cells. The investigation was also directed towards exploring the role of PI3k/Akt/mTOR in glioma cells.

EXPERIMENTAL

ERD, with purity above 98 %, was procured from Chengdu Pufeide Biological Technology Co. Ltd., Chengdu, China. PI3K agonist 740 Y-P was purchased from Selleck Chemical (Texas, US), Human glioma cell line U251 was obtained from Shanghai Institute of Cell Bank, Shanghai, China.

Cell culture

Human glioma cell line U251 was incubated at 37^{o}C, in 5 % carbon dioxide and 95 % oxygen mixture, in a blend of Dulbecco's modified eagle medium (DMEM) (Gibco, USA), fetal bovine serum (10 %) (Sigma, USA), and 1% antibiotic mixture, containing penicillin and streptomycin (100 U/l and 100 mg/l respectively) (Gibco, USA).

The study was by divided into four groups: control group (exposed to equivalent volume of distilled water), ERD group (exposed to 100 mM ERD in dimethyl sulfoxide), 740 Y-P group (exposed to 25 mmol/l 740 Y-P), and test group (exposed to 100 mM/mL ERD in DMSO and 25 mmol/l 740 Y-P). PI3K agonist 740 Y-P is a phosphopeptide that has specific transcellular permeability that eventually activates PI3K.

Cell viability study

MTT assay was used for the estimation of cell survival. The cultured cells (5×10^4) were incubated in a 96-well plate along with 180 mL culture solution for 24 h. The media was then replaced and the cell culture wells were added with increasing concentration of ERD (0, 25, 50, 75 and 100 mM) and cultured at 37ºC for further 24, 48 and 72 h. Thereafter, the culture media was withdrawn and replaced blend of MTT solution (0.5 mg/mL) in phosphate buffered saline and fresh media, followed by incubation for 4 h in carbon dioxide environment (5 % carbon dioxide and 95 % oxygen) at 37ºC. The resulting formazan crystals were dissolved by adding 100 mL in each well. The optical density of each well was measured at 490 nm wavelength using a microplate reader. A similar procedure was performed in triplicate and the readings were recorded.

Determination of cell apoptosis

Human glioma cell line U251 was seeded during their exponential developing phase at the bottom of a shallow welled dish. After cellular adhesion at the bottom, the plates were incubated with respective solutions according to the groups for 24 h. Detection kit for analyzing the stage of cell cycle progression and apoptosis was employed (Beyotime, Shanghai, China). Further, cells were suspended into 1 ml cold phosphate buffered saline. The cells were exposed to propidium iodide solution and RNase-A staining solution. Individual test tubes containing cells were uniformly suspended by the mixing of propidium iodide solution in a dark environment, at 37 ºC for 30 min. The test tubes were further stored at 4 ºC, and the red fluorescence was detected at 488 nm using flow cytometry (Gallios, Beckman Coulter, USA).

Western blot

The cultured cells were collected and lysed using radio immunoprecipitation assay (RIPA) buffer. Protein content was isolated and determined according to the Bradford method [17]. Denaturation of the protein was done by boiling. The denatured protein was electrophoretically processed on a polyvinylidene fluoride membrane using SDS-PAGE. Further, these membranes were blocked for one hour in a mixture of skimmed milk (5 %) and tris buffered saline, followed by incubation at 4 ºC for 24 h with primary antibodies against LC3B (1:1000), Bcl-2 (1:800), Bax (1:800), p-PI3K (1:500), p-Akt (1:800), p-mTOR (1:500) and Beclin-1 (1:1000) (CST Biological Reagents Company, Shanghai, China). The membranes were rinsed, and further incubated with horseradish peroxidase-conjugated rabbit anti-goat secondary antibodies (1:2000) (CST Biological Reagents Company, Shanghai, China). The samples were subjected to intense chemiluminescence and analyzed using LabWorks 4.5 software provided by Mitov Software.

Statistical analysis

The data were statistically treated with GraphPad Prism software v7.0 (CA, USA) software package. Data were presented as mean ± standard deviation. To correlate the data among groups and within the same group, a two-tailed student's t-test or one-way analysis of variance was employed. Values were treated statistically significant when $p < 0.05$.

RESULTS

Cell viability

Variable concentrations of ERD were investigated for the elucidation of U251 cell viability using MTT assay. The results show that with increasing concentration of ERD (25, 50, 75 and 100 mM), the U251 cell count reduced significantly compared with non-ERD exposed cells. Also, a prolonged exposure, up to 72 h, of U251 cells to ERD, revealed a marked reduction in the viable cell count (Figure 1 A and B). These results indicate the cytotoxic effects of ERD on U251 cells.

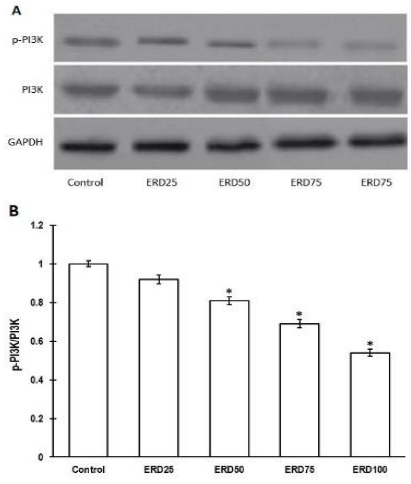

Figure 1: Effect of ERD on the phosphorylation of PI3K. (A) Results of Western blotting for the expressions of p-PI3K and PI3K treated with different concentration of ERD. (B) Relative expression of p-PI3K/PI3K. Data are presented as mean ± SD for 3 independent experiments; *$p < 0.05$ vs. control group

ERD modulates PI3K/Akt/mTOR signaling cascade expression

Activation of PI3K can directly be correlated to its phosphorylation, which is conversion to p-PI3K. The control group revealed a higher activity of p-PI3K, whereas the study group that was treated with ERD, indicated markedly reduced ($p < 0.05$) p-PI3K activity (Figure 1 A). After studying the variable doses of ERD, 100 mM dose was selected for further experiments. To analyze the possible mechanism of ERD on PI3K/Akt/mTOR signaling cascade in U251 cells, the activity of p-PI3K, p-Akt and p-mTOR was quantified using Western blotting. Results revealed that the expression of phosphorylated derivatives of PI3K, Akt and mTOR was significantly enhanced ($p < 0.05$) in the ERD group, whereas they were reduced ($p < 0.05$) in the aliquots from 740 Y-P group, in comparison to the untreated group (Figure 2 A - D).

ERD effectively suppressed cell growth, triggered apoptosis and halted cell cycle

As indicated in Figure 3 A, after 24-, 48- and 72-h treatment, U251 cell viability was significantly enhanced ($p < 0.05$) with 740 Y-P alone, compared to the control group. ERD alone and in combination with 740 Y-P, eventuated in significant lowering ($p < 0.05$) of U251 cell growth related to the control group and 740 Y-P group cells. When correlated with the control group, the extent of apoptosis was significantly elevated ($p < 0.05$) in the ERD group. The rate of apoptosis was markedly lower ($p < 0.05$) in the 740 Y-P groups, compared to the control group (Figure 3 B).

U251 cells that were treated with 740 Y-P for 24 h, not only increased the cell count of the G0/G1 phase but also reduced the cell ratio from the S phase. Consequently, ERD alone and in combination with 740 Y-P had a reverse effect. Cell fraction count arrested during the G2/M phase indicated a significant reduction in ERD group, whereas the 740 Y-P groups and test group exhibited no marked variation against the control group. The outcomes revealed that ERD has the potential to arrest the U251 cell viability during the G0/G1 growth phase (Figure 3 A - C).

ERD modulates expression of proteins Bcl-2 and Bax associated with apoptosis

To elucidate the expression of proteins associated with the apoptotic process, we analyzed the expression of Bcl-2 and Bax by conducting the Western blotting technique.

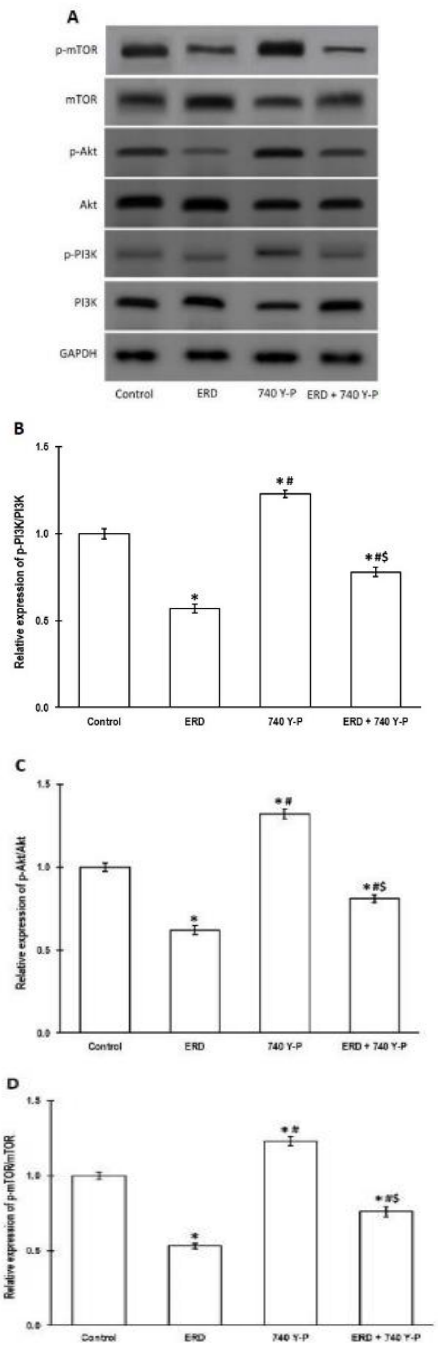

Figure 2: Effect of ERD on the expression of the PI3K/Akt/mTOR signaling pathway. (A) Western blot analysis of p-PI3K, PI3K, p-Akt, Akt, p-mTOR and mTOR in cells with GAPDH as internal control. (B) Relative expression of p-PI3K/PI3K (C) p-Akt/Akt and, (D) p-mTOR/mTOR in U251 cells. 740 Y-P alone upregulated the expression of p-PI3K, p-Akt and p-mTOR significantly, while the phosphorylation of PI3K, Akt and mTOR in ERD group and the mixed group was remarkably higher than that in the control group; *$p < 0.05$ vs. control group; #$p < 0.05$ vs. ERD group; \$$p < 0.05$ vs. 740 Y-P group

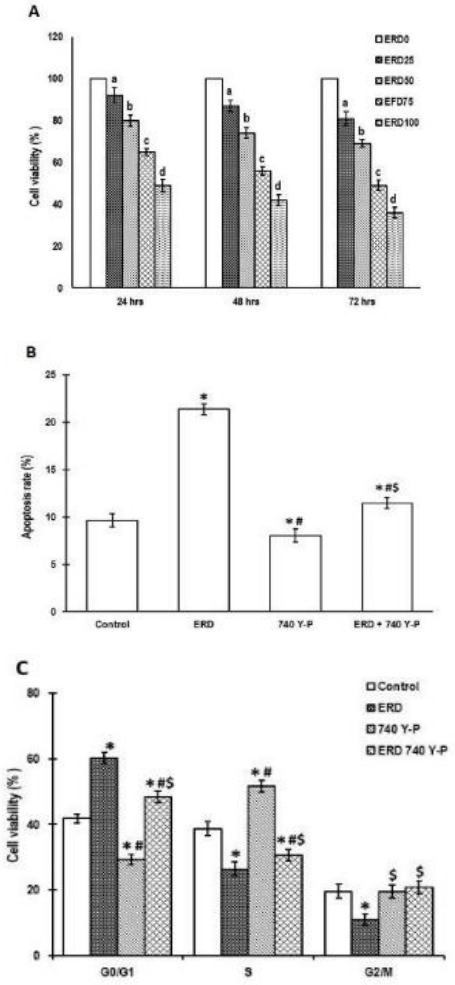

Figure 3: Effect of ERD on viability of U251 cells (A) Viability of U251 cells after 24, 48 and 72 h treatment. ERD alone and a combination of ERD and 740 Y-P downregulated the viability of U251 cells remarkably, though 740 Y-P alone significantly upregulated the viability. ERD induced the apoptosis of U251 cells. [a - $p < 0.05$ vs control group, b –$p < 0.05$ vs ERD25, c - $p < 0.05$ vs ERD50 and d - $p < 0.05$ vs ERD75] (B) Relative apoptosis rate. (C) ERD arrested the cell cycle. Proportion of cells in different phases; *$p < 0.05$ vs. control group; #$p < 0.05$ vs. ERD group; $$p < 0.05$ vs. 740 Y-P group

Alone 740 Y-exhibited significantly enhanced the expression of the anti-apoptotic protein, Bcl-2 (Figure 4 A - C). When correlated with the control group, ERD alone and in combination with 740 Y-P, revealed suppression of Bcl-2 activity. A combination of ERD and 740 Y-P in U251 cells indicated a marked reduction in Bcl-2 expression, compared to ERD exposure alone. Contrary to these findings, the activity of the pro-apoptotic protein, Bax was significantly reduced following exposure to 740 Y-P, compared to the control group. ERD alone indicated marked enhancement in the Bax activity related to the control group. ERD and 740 Y-P together revealed a significant rise in the Bax activity, compared to 740 Y-P alone. Finally, the above results indicate that ERD remarkably attenuated the activity of the anti-apoptotic protein and enhanced the activity of the pro-apoptotic protein, that is Bcl-2 and Bax respectively, and thereby resulted into U251 cell apoptosis.

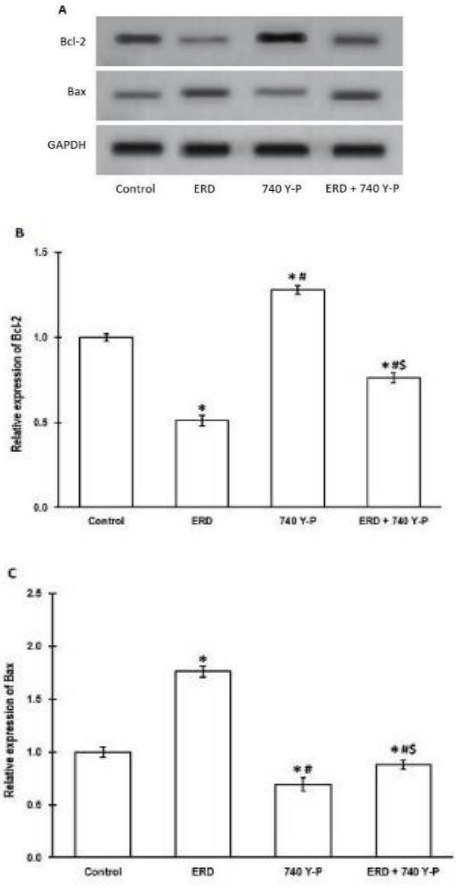

Figure 4: Expression of apoptosis-associated proteins Bcl-2 and Bax in U251 cells. (a) Western blot analysis of Bcl-2 and Bax in cells with GAPDH as internal control. (b) Relative expression levels of Bcl-2 and (c) Relative expression levels of Bax in cells. Data are presented as mean ± SD for 3 independent experiments. *$P < 0.05$ vs. control group; #$p < 0.05$ vs ERD group; $$p < 0.05$ vs. 740 Y-P group

ERD regulates expression of autophagic proteins LC3B and Beclin-1

Western blotting technique was used to quantify the activity of LC3B and Beclin-1, proteins linked with autophagy. In the control group, the activity of both LC3B and Beclin-1 in U251 cells was significantly lower compared to the ERD group. ERD exposure alone significantly elevated the activity of LC3B and Beclin-1. Alone 740 Y-P

exposure to U251 cells indicated marked reduction in the activity of LC3B and Beclin-1, compared to the control group. When U251 cells were exposed to ERD and 740 Y-P combinations, the LC3B and Beclin-1 expression were significantly upregulated compared to the control group and 740 Y-P groups. Results demonstrated that ERD enhanced the activity of autophagy-linked proteins LC3B and Beclin-1 (Figure 5 A - C).

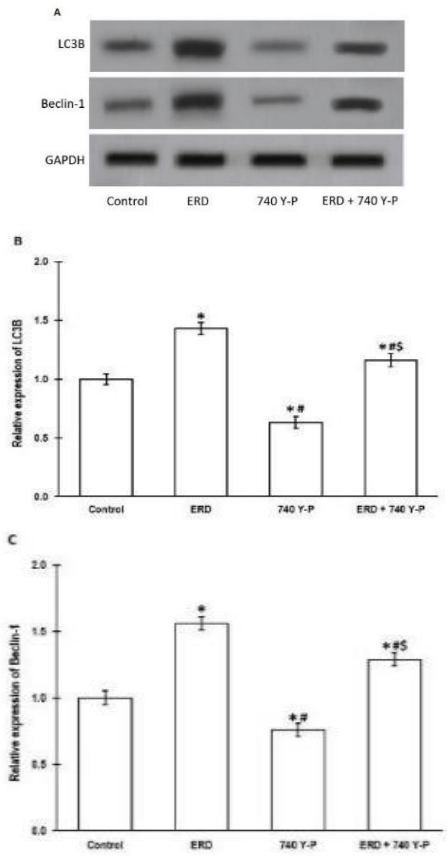

Figure 5: Effect of ERD on expressions of autophagy-associated proteins LC3B and Beclin 1. (a) Results of Western blotting for the expressions of LC3B and Beclin 1 with GAPDH as internal control. (b) Relative expression levels of LC3B (c) Relative expression levels of Beclin 1 in cells. Data is presented as mean ± SD for 3 independent experiments. *P < 0.05 vs. control group; #p < 0.05 vs. ERD group; $^{\$}p$ < 0.05 vs. 740 Y-P group

DISCUSSION

Based on the previous investigations made on ERD, it can be concluded that ERD exerts anticancer activity via modulation of mitochondrial apoptosis, G2/M phase lockdown, and inhibition of PI3K/Akt/mTOR signaling cascade in A549 human lung cancer cell line [15]. Also, ERD has been known for its anti-inflammatory and antioxidant property [16]. In the investigation presented herewith, ERD was evaluated for its activity against glioma cells. ERD has been established to exhibit anticancer activity in Hep-G2 human hepatocellular carcinoma cells. The activity results via induction of apoptosis, stopping G2/M phase of the cell cycle, stimulation of PARP and Bax, and inactivation of Bcl-2 protein [10]. An Nrf2 activator and a derivative of ERD, Eriodictyol-7-O-glucoside has been found to confer against cisplatin-induced toxicity. Oxidative stress-induced endothelial cell death has been found to be attenuated by ERD my modulation of ERK/Nrf2/ARE-dependent activity of HEME oxygenase. However, there has been no investigation made to exhibit the autophagic and apoptotic activity of ERD on U251 human glioma cells.

The PI3K/Akt/mTOR signaling pathway controls normal cellular functions, including those crucial for tumorigenesis, involving cellular proliferation, growth, survival, and death [18]. Curcumin has been found to effective in killing glioblastoma multiforme through inhibition of PI3K/Akt/mTOR pathway. Recent investigations have revealed that PI3K/Akt/mTOR and sonic hedgehog signaling pathways are stimulated in glioblastoma-initiating cells, and their inhibition will suppress the cancer growth [19].

Several molecules have been known to confer anti-proliferative effects via modulation of apoptosis and alteration of the apoptotic signaling pathway. Our results revealed that ERD influenced autophagy and associated apoptosis by downregulating the PI3K/Akt/mTOR signaling cascade. Observations collected from our investigation indicated that the activity of phosphorylated derivates of PI3K, Akt and mTOR was markedly enhanced after ERD and 740Y-P exposure together. The results are in accordance with the study carried out on human lung A549 cancer cells, whereby the target of anticancer activity PI3/Akt/mTOR signaling pathway was modulated by ERD [15].

A study to demonstrate U251 cell viability on ERD exposure revealed a significant reduction in cell sustainability in a dose-relative manner, compared to the cells that were not exposed to ERD. U251 Human glioma cells that were allowed to stay with ERD (100 mM) for 72 h exhibited the least cell viability compared to those exposed to the same dose till 24 h. The results are in accordance with the investigation carried out earlier on A549 human lung cancer cells [15]. The investigation reported earlier revealed that ERD has potential growth-inhibiting activity at IC_{50} of 50 mM [15].

Results from the investigation related to apoptosis and cell cycle status revealed that alone 740Y-P exposure enhanced cell viability of U251 human glioma cells. Upon ERD exposure, the U251 glioma cells indicated a significant reduction in cell viability. Reverse activity trend was observed while carrying out an apoptotic investigation. Exposure of U251 cells to 740 Y-P alone indicated a reduction in apoptosis, whereas ERD and 740 Y-P exposure revealed a significantly enhanced apoptotic effect in U251 glioma cells. Similar results have been found earlier with compounds such as Tanshinone IIA [20].

Cell growth phase pattern investigation indicated that ERD arrested majority of U251 glioma cells during their G0/G1 phase, whereas a prominent decline in the U251 cell count at the G2/M phase was noticed in the case of ERD and 740Y-P exposure. Our results are in parallel with those obtained earlier in similar investigations, wherein several compounds of natural origin have the capacity to inhibit proliferation and freeze cell cycle phase in a dose-incremental manner [21-23].

The family of apoptotic proteins comprising pro-apoptotic and anti-apoptotic proteins, namely Bax and Bcl-2 respectively, are known to be actively involved in the execution of the apoptotic process. We investigated the effect of ERD on the expression of anti- and pro-proteins involved in apoptosis in U251 glioma cells. Results from our investigation revealed that relative to the control group, ERD alone effectively suppressed the expression of Bcl-2. A combination of ERD and 740 Y-P indicated significantly downregulated the Bcl-2 activity. On the other hand, Bax expression was significantly attenuated on exposure to 740 Y-P alone, whereas ERD and 740 Y-P together caused marked elevation of the pro-apoptotic Bax expression.

Western blot analysis related to the quantification of autophagy linked proteins LC3B and Beclin-1 by Western blotting provided an insight into the mechanism of ERD in U251 glioma cells. Results revealed that compared to the control group, 740 Y-P groups indicated a significant reduction in the activity of LC3B and Beclin-1 in U251 human glioma cells. Treatment of U251 glioma cells with ERD and 740 Y-P together, significantly elevated improvised the LC3B and Beclin-1 expression and normalized it compared to 740 Y-P alone.

Assembled together, results revealed that ERD is a potent molecule that could inhibit the progression of glioma. In general, flavonoids are non-toxic and can be extrapolated at higher doses in humans.

CONCLUSION

The findings of this study indicate the modulation of autophagy and apoptotic activity of ERD in U251 human glioma cells, via significant inhibition of PI3K/Akt/mTOR signaling cascade. ERD has the potential to elevate the efficacy of proteins associated with autophagy, LC3B, and Beclin-1. Also, ERD reduces the activity of Bcl-2 and enhances the activity of Bax, wherein the former is known to oppose apoptosis while the latter favours apoptosis in U251 human glioma cells. Thus, ERD is a potential candidate for development of a treatment for glioma.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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