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Erianin, a promising agent in the treatment of glioblastoma multiforme triggers apoptosis in U373 and A172 glioblastoma cells

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Abstract: Glioblastoma is an aggressive, common and deadly primary intracranial brain tumor in adults. The antitumor activity of erianin, a dibenzyl compound found in *Dendrobium chrysotoxum* Lindl. extract, has not been previously demonstrated in glioblastoma. We investigated the anticancer activity and underlying mechanisms of erianin in human U373 and A172 glioma cells. The effects of erianin on cell viability, apoptosis, migration and invasion were estimated by the XTT test, the reverse transcription-polymerase chain reaction (RT-PCR), annexin V staining assay protocol for apoptosis, wound healing assay, and Matrigel® invasion chamber, respectively. The effective amounts of erianin in U373 and A172 cells were 16 and 64 μM at 48 h, respectively. Erianin also significantly induced apoptosis by inhibiting B-cell lymphoma 2 (Bcl-2), caspase-8, caspase-9 and tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), and activation of caspase-3 and BH3 interacting domain death agonist (*BID*) gene expression. In addition, erianin significantly increased the number of apoptotic cells in U373 and A172 cells and significantly decreased invasion and migration in U373 and A172 cells. Taken together, our results suggest that erianin may be a new therapeutic anticancer drug component with a potent apoptotic effect and a potential for treating glioblastoma.

Keywords: glioblastoma; erianin; apoptosis; anticancer; *Dendrobium chrysotoxum* Lindl

INTRODUCTION

Glioblastoma accounts for about 81% of intracranial tumors and is the most common primary intracranial tumor [1]. Glioblastoma multiforme (GBM), which accounts for about 45% of all gliomas, is the most aggressive and common primary brain tumor; astrocyte-derived glioblastoma is the most invasive type and spreads very rapidly [2]. The incidence of GBM is low (4.67-5.73 cases per 100,000 individuals). However, the tumor grows rapidly and spreads around the tumor, invading life-regulating centers. Even in patients receiving chemotherapy and radiotherapy, the survival time is between 7-15 months and the survival rate is only between 0.05% and 4.7% [1]. The poor prognosis of GBM as well as its low survival rate make diagnosis, treatment and monitoring very difficult [3]. Neurosurgery, radiotherapy and temozolomide (TMZ) chemotherapy are currently accepted and recommended treatment options for GBM. TMZ is the most effective chemotherapy agent for GBM treatment, designed to kill glioblastoma cancer cells and slow their growth. TMZ can improve a patient's life quality and prolong survival; however, it has many negative effects such as drug resistance, as well as side effects [1,4], and it is necessary to develop new treatment approaches that will trigger apoptosis and decrease cell proliferation.

Herbs have been used in medicine and been accepted by many human societies and cultures. Phytochemical agents have been used for cancer treatment in human clinical trials because of their safety, low toxicity and easy accessibility [5]. In pharmacological studies, it was established that plants of the *Dendrobium* genus contain many chemical components with pharmacological effects [6].

Erianin, a dibenzyl compound derived from *Dendrobium chrysotoxum Lindl*. has been used as an herbal drug due to its antioxidative and antitumor activities [7]. The anticarcinogenic effects and underlying molecular mechanisms of erianin have been demonstrated in a limited number of cancer cell types such as human cervical cancer [8], bladder cancer [6], liver cancer [9], osteosarcoma [10], lung cancer [11], nasopharyngeal carcinoma [12], breast cancer [13], oral squamous cell carcinoma [14] and hepatocellular carcinoma [7]. However, the role of erianin in GBM cancer cells remains unclear. In this study, we investigated, for the first time, the anticancer effect of erianin on U373 and A172 GBM cells and revealed its underlying mechanism of action, including its effects on apoptosis, migration and invasion.

MATERIALS AND METHODS

Cell culture and reagents

U373 and A172 glioblastoma cancer cell lines obtained from ATCC, USA, were used. Human umbilical vein endothelial cell (HUVEC) lines were supplied by Pamukkale University, Turkey. Glioblastoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (20 U/mL), streptomycin (20 µg/mL) and 2 mM L-glutamine and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Erianin (≥98% purity) was purchased from ChemFaces (Wuhan, China). The stock was dissolved in dimethyl sulfoxide (DMSO). U373, A172 and HUVEC cells were treated with 2, 4, 8, 16, 32, 64 and 128 µM of erianin for 24, 48 and 72 h [15].

Cell viability assay

The XTT [2,3-bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] test was used according to the manufacturer's instructions (Biotium, USA) to evaluate the effects of erianin on the viability of U373, A172 and HUVEC cells. The cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated overnight. The next day, the cells were treated with different concentrations of erianin and incubated for 24, 48 and 72 h. All applications were carried out in three replicates. Formazan formation was spectrophotometrically quantified at 450 nM (reference wavelength 630 nM) using a microplate reader (BioTek, USA). Cell viability was calculated using the formula:

$$\text{Cell Viability (\%)} = \text{A of experimental well} / \text{A of control well} \times 100$$

Wound healing assay

To determine the effects of erianin on cell migration, the wound healing migration assay was used. Control and erianin cells were plated at a density of 10^6 cells/well and incubated overnight at 37°C with 5% CO_2. When confluence was about 90%, the mediums were removed. Six-well dishes were scratched with a 200-µL sterile pipette tip. The dose group cells were treated with erianin; DMEM supplemented with 10% fetal bovine serum was used for the control group. Images were taken at 0 and 24 h for after-scratch comparison of cell proliferation in the control and erianin groups, with the assay performed in triplicate. The scratch assay was used to measure changes in wound width (µm) with the ImageJ software program.

Cell invasion assay

To evaluate the invasion capacity of U373 and A172 cells, a Matrigel® transwell invasion chamber (BD Biosciences, USA) was used. Cells were seeded by adding serum-free DMEM/F-12 to the upper part of the Matrigel membrane with 2 x 10^5 cells in a well and DMEM/F-12 with serum was added to the lower part of the Matrigel membrane. Different doses of erianin were administered in serum-free DMEM/F-12 and incubated for 48 h at 37°C. After incubation, the medium in the invasion chamber was removed and the non-invasive cells remaining on the upper part of the membrane were cleaned with a soft-tipped cotton swab. Invasive cells were fixed with methanol and stained with crystal violet (Merck Millipore, USA). The cells invading the lower surface of the filter were viewed under an inverted microscope (Olympus CKX41, Japan). The method of quantifying invasion was used to observe the stained invaded cells at 20×magnification.

Real-time PCR analysis

In the RT-PCR method, which was used to determine the expression of genes associated with apoptosis in U373 and A172 cells, 3×10^5 cells/well were seeded into 6-well plates. Control and dose groups were formed and incubated for 48 h. Total RNA was isolated from the dose and control groups using TRIzol (Invitrogen, USA) reagent according to the manufacturer's instructions. cDNA synthesis from total RNA was performed using the cDNA synthesis kit (Roche Diagnostics, Germany), according to the manufacturer's instructions. Quantitative expression analysis of genes associated with apoptosis (*BCL-2*, *CASP-3*, *CASP-8*, *CASP-9*, *CASP-10*, *BAX*, *FADD*, *BID*, *TRADD*, *DR4*) was performed based on real-time RT-PCR according to the SYBR Green qPCR Master Mix (Applied Biosystems, Thermo Scientific, USA) protocol. The RT-PCR assay was performed using gene-specific validated primers. Normalization was realized using housekeeping gene β-actin (ACTB), and fold-changes were calculated by comparison with the untreated control group sample using the $2^{-\Delta\Delta CT}$ method in the GeneGlobe RT² Profiler ™ PCR Array Data Analysis platform.

Determination of apoptosis by annexin V/PI staining

To evaluate the effect of erianin on apoptosis of U373 and A172 cells, the cells were seeded in six-well plates (2×10^5/per well), and incubated with erianin for 48 h. The cells were harvested and washed with cold PBS and stained with the Annexin V-FITC Apoptosis Detection Kit (ABP Biosciences, USA) according to the manufacturer's instructions. The cells were analyzed using an Arthur Image-Based Cytometer (NanoEntek, USA). The percentage of apoptotic and necrotic cells was calculated in the total cell population. Hydrogen peroxide was used as a positive control for apoptosis. The apoptotic cell percentage was represented as the mean of independent experimental sets ± SD.

Statistical analysis

SPSS 23 analysis was performed. Statistical comparison of two data sets was evaluated with the Student's t-test, and multiple data set comparisons were evaluated with one-way ANOVA, Tukey's or Tamhane's post-hoc comparison tests, and $P<0.05$ was considered significant.

RESULTS

Erianin inhibits cell proliferation in glioblastoma cells

The effects of erianin on the proliferation of U373, A172 and HUVEC cells were analyzed by the XTT test. Erianin decreased the proliferation of U373 and A172 cells depending on dose and time ($P<0.05$). Effective doses of erianin on U373 and A172 glioblastoma cells were determined at 24, 48 and 72 h (Supplementary Table S1), and no dose of erianin was found to reduce A172 cell viability below 50% at 24 h. At 48 h, 64 μM of erianin significantly decreased the viability of A172 cells below 50% ($P=0.000$); at 72 h, 16 and 32 μM of erianin decreased the viability of A172 cells below 50%, but this was not significant; at 24 h, 64 μM of erianin killed 53% of U373 cells ($P=0.000$). At 48 h, at the dose range of 2 to 128 μM, erianin decreased the viability of U373 cells by 36% and 83%, respectively; 16 μM of erianin at 48 h decreased U373 cell viability by up to 17% ($P=0.018$). The most effective dose of erianin that reduced glioblastoma cell viability below 50% and did not show a significant effect on normal cells (HUVEC), was 16 μM in U373 cells (Fig. 1A) at 48 h, while it was 64 μM in A172 cells (Fig. 1B), which was the preferred dose in other experiments. No significant decrease in HUVEC cell viability was observed after administration of increasing concentrations of erianin at 24 and 48 h (Fig. 1C).

Gene expression analysis with RT-PCR

The effects of erianin on the changes in the expression of genes associated with apoptosis in U373 and A172 GBM cells were examined by RT-PCR. The genes analyzed are shown in Table 1. The expression analysis of *BCL-2, CASP-3, CASP-8, CASP-9, CASP-10, BAX, FADD, BID, TRADD, DR4* and *DR5* was determined by real-time PCR. *CASPASE-3* ($P=0.013$) and *BID* ($P=0.008$) gene expression increased, and *BCL-2* ($P=0.0002$), *CASPASE-8* ($P=0.0001$), *CASPASE-9* ($P=0.00001$) and *TRADD* ($P=0.039$) expression was reduced significantly in the U373 cell line when

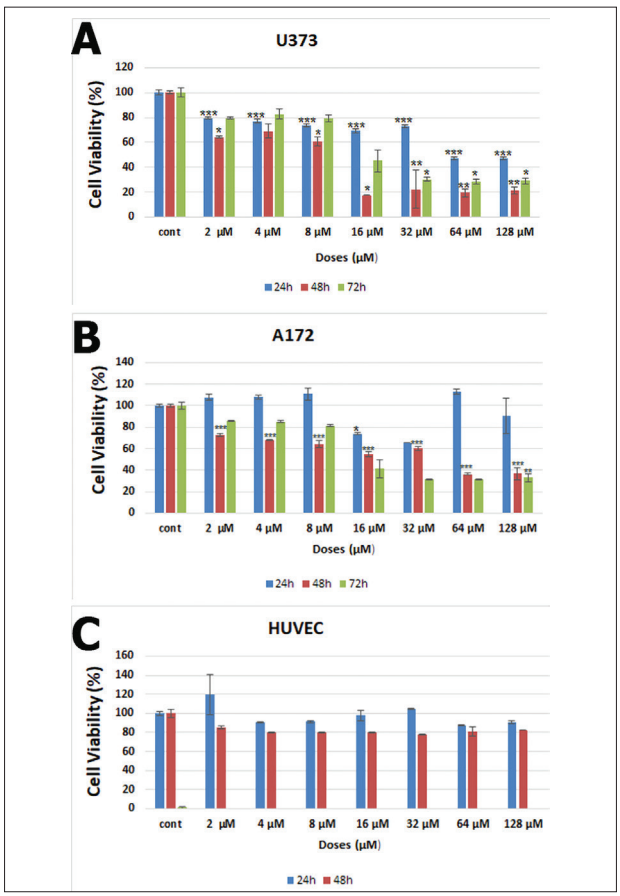

Fig. 1. Erianin inhibits cell proliferation in glioblastoma cancer cells. Percentage cell viability of U373 and A172 cells induced by erianin at different concentrations calculated by the XTT assay. Effective doses of erianin in U373 cells were detected as 16 μM at 48 h (a); effective doses of erianin in A172 cells were detected as 64 μM at 48 h (b); when concentrations of erianin were increased, at 24 and 48 h a significant decrease in the viable number of HUVEC was not observed (c). Data are the mean±SD, *P<0.05, **P<0.01, ***P<0.001. HUVEC – human umbilical vein endothelial cell.

compared with the control group cells (Table 1, P<0.05). No significant change was observed for other genes and the A172 cell line cells (Table 1, P>0.05).

Erianin exhibits anti-invasive potential in glioblastoma cells

To investigate the effects of erianin on cell migration in U373 and A172 GBM cells, the Matrigel invasion chamber assay was used. Cell invasion was decreased in the erianin-treated group when compared with the control group (Fig. 2). The invaded cells were purple in appearance and the pores in the membrane appeared as small and regular circles.

Erianin decreases migration of glioblastoma cells

To investigate the effects of erianin on cell migration, the wound healing assay was used. Erianin significantly decreased glioblastoma cell migration compared to the control group (Fig. 3A-C). The distances of migrating cells in U373 and A172 cells, respectively, were as follows: control group (71±4.58 vs 77±9.64), erianin dose group (185±26.57 (P=0.002) vs 136±7.54 (P=0.001) (Fig. 3B, C). The distances of migrating cells were quantified using ImageJ software.

Erianin increases apoptosis of glioblastoma cells

Annexin V results showed increased apoptosis in U373 and A172 cells after erianin treatment (Fig. 4). The percentages of apoptotic, living and dead cells in U373 cells after 48 h of incubation with erianin were

Table 1. The mRNA expression changes of apoptosis genes in U373 and A172 cell lines.

U373			A172		
Gene	**Fold-change**	**P-value**	**Gene**	**Fold-change**	**P-value**
BCL-2	-25.22	0.000271	*BCL-2*	-1.49	0.083789
CASPASE-3	1.44	0.013434	*CASPASE-3*	1.33	0.301101
CASPASE-8	-10.55	0.000128	*CASPASE-8*	1.24	0.214760
CASPASE-9	-4.09	0.000012	*CASPASE-9*	-1.29	0.124212
CASPASE10	-1.07	0.996342	*CASPASE10*	1.65	0.294446
BAX	-4.45	0.677156	*BAX*	-1.25	0.307624
FADD	-1.03	0.910380	*FADD*	-1.32	0.374573
BID	1.60	0.008644	*TRADD*	-1.68	0.082146
TRADD	-1.78	0.039353	*DR4*	-1.04	0.838473
DR4	1.01	0.817577	*DR5*	1.24	0.516358
BETA-ACTIN	1.00		*BETA-ACTIN*	1.00	

Bcl-2 (B-cell lymphoma 2), *BAX* (Bcl-2-associated X protein), *FADD* (Fas-associated protein with death domain), *BID* (BH3 interacting domain death agonist), *TRADD* (TNFRSF1A associated via death domain), *DR4* (death receptor 4), *DR5* (death receptor 5); P<0.05 statistically significant.

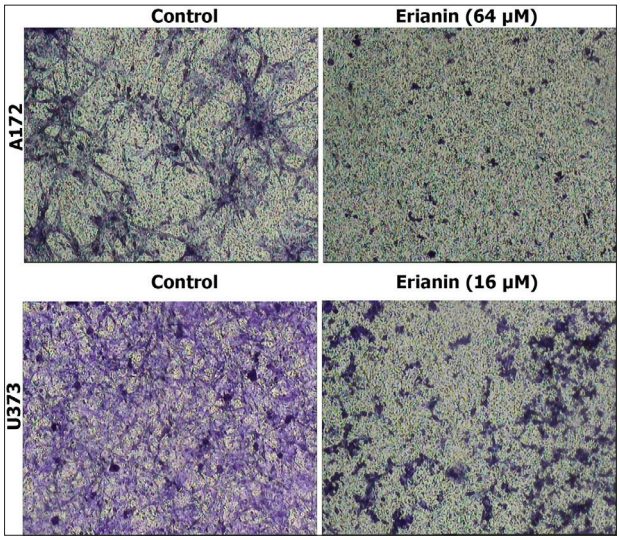

Fig. 2. Erianin provides an anti-invasive potential to glioblastoma cancer cells. Descriptive micrograph images of U373 and A172 cell invasion.

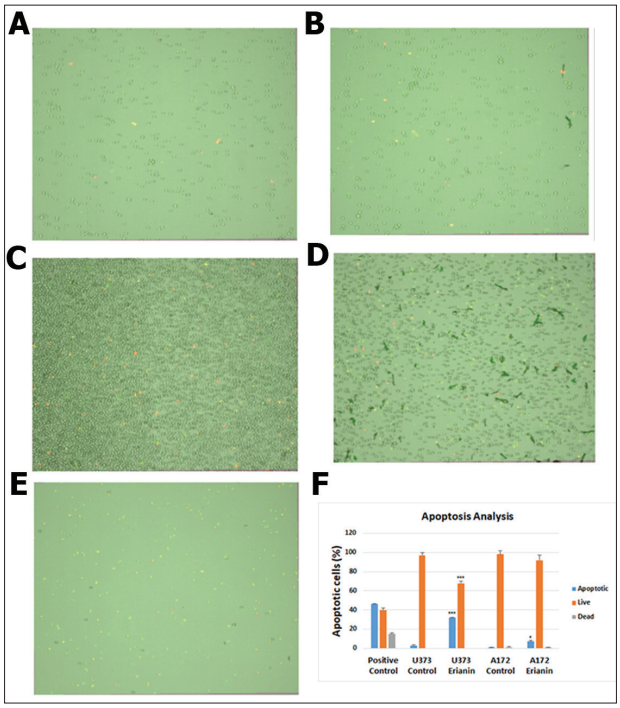

Fig. 4. Apoptosis assay obtained using an image-based cytometer. Following 48 h incubation with erianin, cells were evaluated for apoptosis: U373 control cells (a); erianin-treated U373 cells (b); A172 control cells (c); erianin-treated A172 cells (d); H_2O_2 (200 μM) was used as a positive control (e); apoptosis analysis (f), data are the mean±SD, *P<0.05, ***P<0.001 vs control cells.

32%, 68% and 0%, respectively (Fig. 4B). In the matching control group, these values were respectively 3%, 97%, and 0% (Fig. 4A). As a result of 48 h of incubation with erianin, in U373 cells the number of apoptotic cells increased significantly (P=0.000) compared to the control group, while the number of viable cells significantly decreased (P=0.000). In A172 cells, the percentages of apoptotic cells were 7%, 92% of living cells, and 1% of dead cells (Fig. 4D). In the matching control group, these values were respectively 1%, 98% and 1% (Fig. 4C). In A172 cells, 48 h of incubation with erianin caused a significant increase in the number of apoptotic cells (P=0.01) and caused a decrease in the number of viable cells.

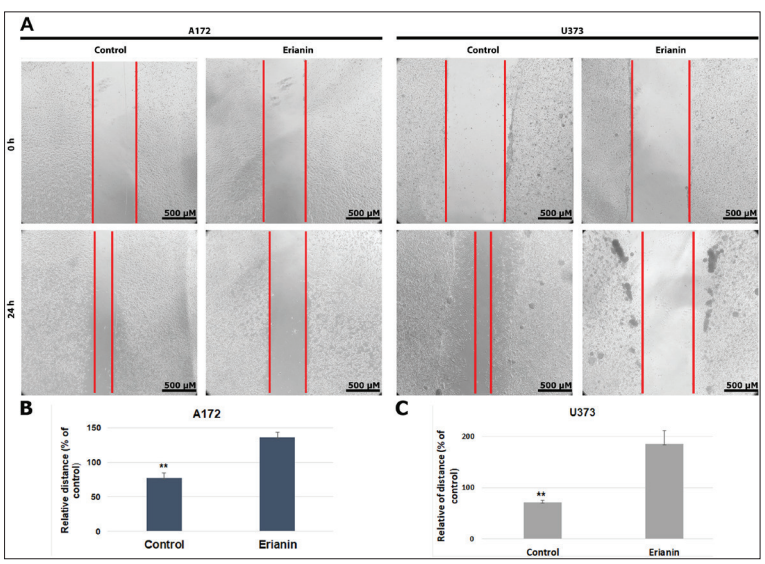

Fig. 3. Erianin suppresses the migration of glioblastoma cancer cells. Representative results of wound healing assays. Cells were treated with different doses of erianin (16 and 64 μM erianin, U373 and A172 cells, respectively) for 0 or 24 h, and the migration was analyzed using the wound healing assays. Data are expressed as percentages relative to the corresponding control cells as the mean±SD, n=3, **P<0.01 vs control cells.

DISCUSSION

GBM, which spreads rapidly and aggressively in patients, is defined as a grade 4 glioma by the WHO [1,2]. Even when combined treatments with surgery, radiotherapy and TMZ chemotherapy are applied, the survival time is not more than 15 months [1,4], and therefore, newer and complementary treatment options need to be developed. In recent

years, traditional Chinese medicine has made great progress in terms of alternative medicine and has produced effective results in the treatment of different types of cancer [6,12]. Elucidation of the mechanisms underlying the anticancer activity of active compounds obtained from natural products in various types of cancer will enable the production of safe and effective anticancer drugs. The anticancer activity of erianin has been demonstrated in some types of cancer, and it was shown that erianin may be an effective and promising drug that acts by triggering autophagy, apoptosis and cell cycle G2/M arrest in human osteosarcoma cells via the reactive oxygen species (ROS)/c-Jun N-terminal kinase (JNK) signaling pathway [10]. Erianin is also thought to be a promising natural agent that triggers apoptosis through the extracellular signal-regulated kinase (ERK) signaling pathway in human nasopharyngeal cancer [12]. Moreover, erianin has been shown to reduce the growth and migration of lung cancer cells via calcium/calmodulin-dependent ferroptosis [11]. However, the role of erianin in glioblastoma cancer remains unclear. In this research, we observed the effects of erianin on cell proliferation, migration, invasion and apoptotic mechanisms in glioblastoma cancer cells. Although different studies of erianin and its anticancer effects were conducted for various cancer types, in this study we examined for the first time the effects of erianin on glioblastoma cells.

Using the XTT assay we showed that effective doses of erianin in U373 and A172 cell lines were 16 and 64 μM at 48 h, respectively. Sun et al. [13] showed that erianin could inhibit the viability of T47D epithelial cells isolated from a pleural effusion obtained from a patient with an infiltrating ductal carcinoma of the breast in a dose-dependent manner. Antiproliferative effects of erianin in human cervical cancer were also reported [8]. Another study showed that erianin suppressed human bladder cancer cell proliferation [6]. We examined the effect of erianin on normal cell (HUVEC) viability and found that erianin did not show a significant cytotoxic effect on HUVEC cell viability. Human umbilical vein endothelial cells (HUVECs) are an *in vitro* model of endothelial cells [16]. LYP, a bestatin dimethylaminoethyl ester, did not have a significant effect on HUVEC cell proliferation in a study investigating its effects on cancer angiogenesis [17]. The antiproliferative activities of the lichen substances usnic acid and vulpinic acid on the viability of hepatocarcinoma cells, neuroblastoma cells and HUVEC endothelial cells were examined by the MTT assay, and it was shown that both substances were more cytotoxic to cancer cells than to normal cells [18]. In this study, we used HUVEC cells to evaluate the effect of erianin on normal cells and we also examined whether erianin had a cytotoxic effect on HUVEC cells. Erianin doses that reduced U373 and A172 cell viability below 50% did not cause a significant change in HUVEC cell proliferation.

Apoptosis is an important regulator of tumor development and response to therapy. Therapy methods used in the killing of cancer cells are associated with the activation of intrinsic and/or extrinsic apoptosis signal transduction pathways [19]. Caspases, which have an important role in the regulation of apoptosis, belong to the cysteine-aspartic protease family [20]. While caspases-2, -8, -9 and -10 are involved in the initiation of apoptosis, caspases-3, -6, and -7 have important roles in the maintenance of apoptosis. The evasion of apoptosis is one of the hallmarks of cancer, therefore, most current therapy methods target the caspase pathways to kill cancer cells and activate apoptosis signaling pathways [21].

In this study, the effects of erianin on the apoptosis of glioblastoma cancer cells were determined by RT-PCR analysis of genes associated with apoptosis, which have a significant role in cancer. According to our results, a significant increase in mRNA expression of *CASPASE-3* and *BID* and a reduction in the expression of *BCL-2*, *CASPASE-8*, *CASPASE-9* and *TRADD* genes were detected in the erianin-treatment group compared to the control group in the U373 cell line. Erianin triggers apoptosis in human nasopharyngeal cancer by reducing the expression of Bcl-2, an anti-apoptotic protein, in a dose- and time-dependent manner [21]. In other studies, it was shown that erianin triggers apoptosis by reducing the level of Bcl-2 expression in human breast cancer cells and leukemia [13,22]. The BH3 domain-only death agonist (BID) protein is a pro-apoptotic gene that can induce apoptosis by binding Bcl-2. This suggests that BID serves as a 'death ligand' that inactivates Bcl-2 or activates Bax in the cytosol and mitochondrial membrane [23]. Apoptosis occurs through intrinsic (mitochondrial) and extrinsic pathways, and after

receiving apoptotic stimulation by intracellular signals in the intrinsic pathway, BID, one of the proapoptotic proteins, inactivates the antiapoptotic protein, Bcl-2, and activates Bax and Bak, which induce pore formation in the mitochondrial membrane and change the membrane potential and activate caspase-3 [24]. Our results suggest that BID expressed in the outer membrane of mitochondria may trigger apoptosis via the intrinsic pathway by decreasing Bcl-2 expression and activating caspase-3 rather than caspase-8 and caspase-9. Our findings show that erianin activates cell apoptosis by inducing the intrinsic pathway. In addition, the results of annexin V show that erianin increases apoptosis in glioblastoma cells, and after 48 h of erianin treatment, apoptosis was increased 10-fold in U373 cells and 7-fold in A172 cells when compared to the control group.

Cell migration plays a key role in many processes including embryological development, immunity, tissue formation and cancer progression [25]. In previous studies, the effects of erianin on cell migration and invasion were shown in different cancer cells [6,8,11,13,26], and using wound healing and transwell assays, erianin suppresses lung cancer cell migration [11], while erianin suppressed H1975 lung cancer cell metastasis [27]. In another study, Ecust004, a drug candidate optimized from the structure-activity relationship studies of sulfamate derivatives of erianin and CA4, was shown to suppress breast cancer cell invasion and migration [22]. In our study, the wound healing protocol showed that erianin suppressed U373 and A172 cell migration after 48 h; additionally, this data was supported by the transwell assay. In addition, the decrease in cell invasion in the treatment group may be due to the cytotoxic effect of erianin. The limitations of this study are that specific markers were not used to show the effects of erianin on invasion and migration.

In conclusion, this study demonstrates that erianin decreases cell proliferation by activating apoptotic pathways and inhibiting migration and invasion in glioblastoma cancer cells. We demonstrated for the first time the anticarcinogenic effect of erianin on apoptosis, cell invasion and migration in glioblastoma cells. Our results suggest that erianin could be an effective and safe natural compound in glioblastoma cancer therapy. For the clinical evaluation of erianin, larger and multi-centered collaborative clinical studies are needed.

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Conflict of interest disclosure: The authors declare no potential conflicts of interest with respect to the research, authorship and publication of this article.

Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Serter%20Kocoglu%20et%20al_7505_Data%20Report.pdf

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Supplementary Data

The Supplementary Material is available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Serter%20Kocoglu%20et%20al_7505_Supplementary%20Material.pdf