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Shilajit decreases viability of C6 cell line: a possible role of several cytokines involved in the inflammatory pathway

Mehrnaz Mehrabani[1], Zeinab Kordestani[2*], Hossein Parsa[3], Mojdeh Esmaeili Tarzi[1], Samaneh Fathizadeh[4], Sara Yaghoubi[5]

[1]Physiology Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran
[2]Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran
[3]Endocrinology and Metabolism Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran
[4]Cardiovascular Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran
[5]Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran

*Corresponding Author: Zeinab Kordestani, Email: zeinabkordestani@yahoo.com

Abstract

Background: Glioma is the most malignant type of brain tumor. Since glioma cells secrete various inflammatory mediators that contribute to tumor development and expansion, anti-inflammatory agents may ameliorate glioma invasion and progression. Shilajit (SH) or mumie is a natural compound with antioxidant, anti-inflammatory, and anticancer activity, which is expected to reduce tumor invasion and spread, thereby increasing the survival rate in patients suffering from glioblastoma. The present research was conducted to examine the impact of SH on some inflammatory cytokines secreted by the C6 cell line, as glioblastoma cells.

Methods: SH was used at different concentrations to treat C6 and fibroblast cells. Then, the half-maximal inhibitory concentration (IC_{50}) of SH was assessed after 1, 2, and 3 days through the 3-(4, 5-dimethylthiazol 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test. By measuring interleukin (IL)-6, transforming growth factor-1(TGF-1β), tumor necrosis factor-alpha (TNF-α), and IL-10 gene expression levels through the quantitative real-time PCR technique, the impact of SH on these genes was assessed in C6 and fibroblast cell lines.

Results: The IC_{50} values of SH after 72 h were 325±28 µg/mL in C6. At the IC_{50} concentration, SH decreased the levels of gene expression of TGF-β1, TNF-α, and IL-6 only in the C6 cell line. However, for both cell lines, this concentration raised IL-10 gene expression.

Conclusion: Our findings showed that SH may exert inhibitory effects on C6 cells through the up-regulation of IL-10 and the down-regulation of IL-6, TNF-α, and TGF-1β gene expression. It might also negatively affect the invasion and progression of glioma in patients.

Keywords: Inflammation, Glioblastoma, Mumie, Cancer

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Introduction

Glioblastoma multiforme (GBM) is among the most aggressive forms of tumors affecting the central nervous system. The World Health Organization (WHO) has categorized gliomas into 4 grades (I, II, III, and IV). Grade I is noninvasive, and the next three are invasive and malignant gliomas. Grade IV is identified as GBM with a very poor prognosis (1). Cytological examination of GBM smears reveals large, spindle-shaped to oval nuclei that are hyperchromatic and exhibit pleomorphism. Additionally, a fibrillary or necrotic background can be observed among some of the tumor cells in GBM (2).

In Iran, glioblastoma ranks among the most prevalent malignant brain tumors. Over 9 years, GBM demonstrated the highest incidence rates (1.36 per 100,000 individuals) compared to other histological types. Additionally, the overall occurrence rates of malignant gliomas were notably higher in men (2.19/100,000) compared to women (1.47/100,000) (3).

Inflammation exerts a crucial and complex role in the progression and metastasis of GBM. Inflammatory signaling induces genetic/epigenetic changes of GBM cells, activating oncogenic pathways and causing DNA damage, which drives tumor progression. Inflammation also activates brain immune cells (e.g., microglia and macrophages), promoting tumor growth,

invasion, and angiogenesis, and finally developing an immunosuppressive tumor microenvironment. As a result, it seems that the use of anti-inflammatory drugs can be considered a promising approach for the management of GBM (4). The tumor microenvironment is crucial in cancer progression, including the secretion of cytokines that promote tumor growth. TGF-β is one of the secreted cytokines. It was previously shown that in malignant glioma tissues, TGF-β is overexpressed and induces cancer cell proliferation, migration, and angiogenesis. Furthermore, it has shown to have an immunosuppressive effect by negative modulations of T or NK cell activity. T and NK cells are vital immune cells for eradicating glioma cells. Research indicates that strategies aimed at inhibiting TGF-β signaling may offer promising prospects for enhancing the prognosis of patients with malignant gliomas (5).

Furthermore, A direct relationship has been demonstrated between the malignancy of glioma and increased inflammatory cytokines of the tumor cells, including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (6). After binding IL-6 to its receptor and the phosphorylation of STAT3, it activates certain oncogenic pathways that promote tumor survival, facilitate angiogenesis, increase resistance to cell death, and drive cell cycle progression. It is well known that the IL-6-STAT3 signaling pathway, besides other cytokines such as TNFα, has been implicated in cancer progression, including glioblastoma (7).

TNF-α is another cytokine secreted by tumor cells and activated macrophages. TNF-α promotes the development and progression of the tumor inflammatory microenvironment (8). IL-10 is an immunosuppressive cytokine with a paradoxical role in cancer progression. It may enhance tumor cell survival (9), and by contrast, it also may reduce angiogenesis, tumor growth, and metastasis (10).

Natural compounds have been used for thousands of years to improve and treat many diseases due to their beneficial effects (11). Numerous previous studies have shown the therapeutic effects of natural compounds on different diseases, including diabetes, skin problems, kidney diseases, CNS disorders, and cancers (12-20). Shilajit (SH) or mumie or mumio has been recognized as a tar-like substance that varies in color from pale brown to dark brown, created over centuries from the decomposition of mineral rock and plant matter (21). The anti-cancer activity of SH has been described in some previous reports. SH can be useful for bladder cancer improvement because, through cell cycle arrest and induction of apoptosis, it disrupts cancer cell growth (22). SH also decreases MCF-7 breast cancer cell line viability at the IC50 of 280 μg/mL, probably through inhibition of the inhibitory -κB kinase Nuclear Factor-kappa B (IKK/NF-κB) signaling pathway (23). To the best of our knowledge, there was no research assessing the impact of SH on the C6 glioma cell line and its anti-inflammatory effect on this type of cell. Hence, this study aimed to assess the expression levels of several inflammatory and anti-inflammatory cytokine genes (TNF-α, IL-10, IL-6, and TGF-1β) involved in the progression of GBM to evaluate the effects of SH on the glioblastoma cell line.

Methods

Preparation of Shilajit

SH was obtained from a local store in Kerman Province, and its quality was validated by the Department of Pharmacognosy at the School of Pharmacy, Kerman University of Medical Sciences in Kerman, Iran (voucher number: 1732). It was washed (three times), dried, and then dissolved in the culture medium (Dulbecco's Modified Eagle Medium: DMEM).

Design and Group

To obtain IC50 values of SH, C6 and fibroblast cells were exposed to different concentrations of SH (0, 50, 100, 200, and 400 μg/mL) for 24, 48, and 72 h. Then, cellular viability was evaluated using the MTT assay. Afterwards, to assess cytokine gene expression, both cell lines were treated with an IC50 concentration of SH of the C6 cell line after 72 h, and the quantitative real-time PCR technique was performed subsequently.

Cell Culture

All cell culture material was obtained from Gibco (Invitrogen). The Pasture Institute (Tehran, Iran) provided the C6 cell line (NCBI code: C575). The cells were grown in Dulbecco's modified Eagle medium (DMEM)-high glucose containing 10% fetal bovine serum (FBS) and 1% antibiotic in an incubator (humidified atmosphere, 5% CO_2) (24).

Assessment of Cell Viability

Firstly, 1×10^4 cells/well of each cell line were transferred into 96-well plates. Twenty-four hours after seeding, SH was added at various concentrations (0, 50, 100, 200, and 400 μg/mL). At the next step, after 24, 48, and 72 h, the cells received the MTT for 4 h. After medium removal, dimethyl sulfoxide (DMSO) was added. Finally, the optical density was analyzed at 570 and *630 nm* (reference wavelength). Then, IC50 values for each cell line were calculated, and the best effective IC50 value on the C6 cell line was chosen for the next step (25).

Real-Time RT-PCR

RNA extraction from cells was carried out using RNX-Plus (Cinnagen, Tehran, Iran) after the 72-hour treatment with SH. The RNA concentration was calculated to be 500 ng/μL. Complementary DNA (cDNA) was made using the cDNA Synthesis Kit (Thermo Fisher Germany) (K1621).

The Real Q Plus *2x Master Mix Green* with *high* ROX™ (Amplicon, Denmark) (A325402) (6.5 µL) was used for the real-time polymerase chain reaction (PCR) technique, 1.2 µL of the cDNA product (50 ng), 0.8 µL of the primers forward and reverse, and 3.1 µL of DEPC water (total volume of 12.5 µL). Table 1 reports the primer sequences (24). After denaturation at 95°C for 15 min, a Step One Plus real-time PCR system (Applied Biosystem, USA) was employed to perform the reaction in 45 cycles at 95°C for 45 s and at 60°C for 60 s. Beta-actin (housekeeping gene) was used as an internal control. Data analysis was performed using comparative $2^{-\Delta\Delta CT}$ methods (26).

Statistical Analysis

The statistics are obtainable as mean ± standard deviation (SD). Normality was evaluated through the Shapiro-Wilk test. Statistical analysis was performed using one-way ANOVA accompanied by Dunnett's post-hoc test. A t-test was used to assess the difference in means between two groups. IC50 was analyzed with a nonlinear regression model using GraphPad Prism 8.0. P-values below 0.05 were deemed statistically significant.

Table 1. Rat primers used for qPCR

Gene	Sequence (5'->3')	
	Forward	Reverse
TNF-α	AGTCCGGGCAGGTCTACTTT	TGAGCCACAATTCCCTTTCT
TGF-1β	TGCTAATGGTGGACCGCAA	CACTGCTTCCCGAATGTCTGA
IL-10	GAGAGAAGCTGAAGACCCTCTG	TCATTCATGGCCTTGTAGACAC
IL-6	GTCAACTCCATCTGCCCTTC	TGTGGGTGGTATCCTCTGTG
Beta-actin	CTCTGTGTGGATTGGTGGCT	CGCAGCTCAGTAACAGTCCG

TGF-1β: Transforming growth factor-1β; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IL-10: Interleukin-10.

Results

The Cytotoxic Effect of SH *on C6 and Fibroblast Cells*

SH showed cytotoxicity against C6 and fibroblast cells in a concentration- and time-dependent manner. However, SH exhibited lower toxicity against normal fibroblast cells (the toxic concentrations were (200 µg/mL; $P<0.001$ and 400 µg/mL; $P<0.01$ after 72 h) than the C6 cell line (the toxic concentrations were 100 µg/mL; $P<0.001$, 200 µg/mL; $P<0.001$, and 400 µg/mL; $P<0.001$ after 72 h). The IC_{50} value of SH was 325 ± 28 and 406 ± 27 µg/mL on the C6 cell line and the fibroblast cell line, respectively, after 72 h ($P<0.05$; Figure 1).

SH Reduced Inflammatory Gene Expression at the mRNA Level

As shown in Figure 2, TGF-β ($P<0.01$), TNF-α ($P<0.01$), and IL-6 ($P<0.01$) are significantly more expressed in the C6 cancerous cells than in fibroblast cells (Figure 2).

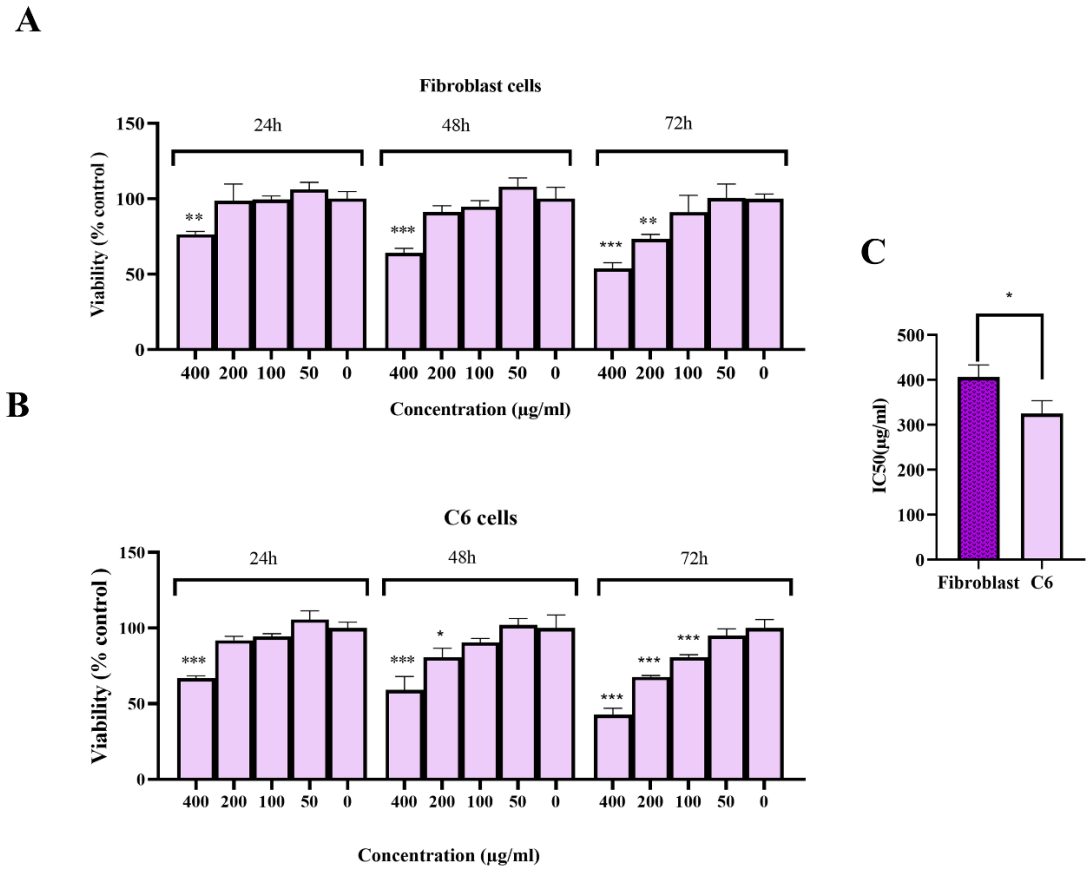

Figure 1. The effect of Shilajit (SH) on the viability of (A) fibroblast cells and (B) C6. (C) IC50 value of SH after 72 h in C6 and fibroblast cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. control. Values are expressed as mean ± SD. $n=3$

Furthermore, C6 and fibroblast cells were treated with SH at the IC50 value for the C6 cell line (325 µg/mL). SH significantly decreased TGF-β (P<0.05), TNF-α (P<0.001), and IL-6 (P<0.01) expression levels in the C6 cell line when it was compared to the control. However, their expression levels were not significantly decreased by SH at the mentioned concentration in fibroblast cells. In addition, SH (325 µg/mL) significantly increased IL-10 gene expression levels (P<0.01) compared with the control (Figure 3). As shown in Figure 3, SH significantly decreases TGF-β (P<0.01), TNF-α (P<0.001), and IL-6 (P<0.01) expression levels in the C6 cell line compared to the fibroblast cell line.

Discussion

One of the aggressive types of brain tumors with a poor prognosis is GBM. It was found that a higher degree of malignancy is correlated with higher levels of inflammatory cytokines in tumor cells (27).

Therefore, considering the low cost and negligible toxicity, medicinal plants that possess anti-inflammatory effects may present a new way to treat glioma. Previous data have confirmed that SH exerts antioxidant (28), anti-inflammatory (29, 30), and anti-cancer (31) effects, but the possible molecular mechanisms of SH are not fully understood. Kloskowski *et al.* have shown that SH is significantly effective in preventing the development or recurrence of bladder cancer (22).

To date, limited studies have assessed the mechanisms of action of SH on cancer cells. A probable mechanism may be the anti-inflammatory effect of SH (31). Previous studies have shown that SH influences the expression of various cytokines engaged in the inflammatory pathway. SH is composed of humic substances, including humic acid and fulvic acid, which are responsible for the main activities of SH (32, 33). A previous study on humic substances has shown their potent inflammatory effect (34). Van Rensburg *et al.* have found that cytokine levels (TNF-α, IL-6, IL-10, and IL-1β) are reduced by potassium humate (35).

However, the results of the present study showed that *IL-10* gene expression is significantly amplified at the mRNA level by SH at the IC50 value for the C6 cell line compared to the control. This difference may be attributed to the utilization of a total extract instead of

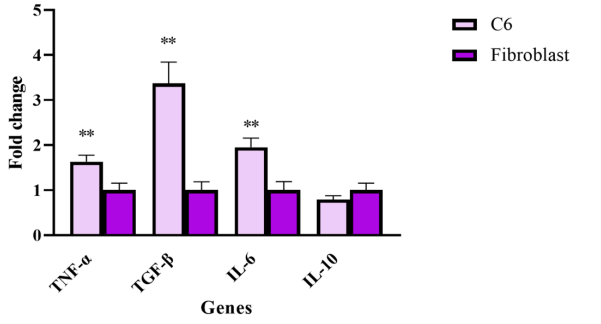

Figure 2. *IL-10, TNF-α, IL-6, and TGF-*β1 expression levels in fibroblast and C6 cells. **P<0.01 vs. fibroblast cells. Values are expressed as mean±SD. n=3

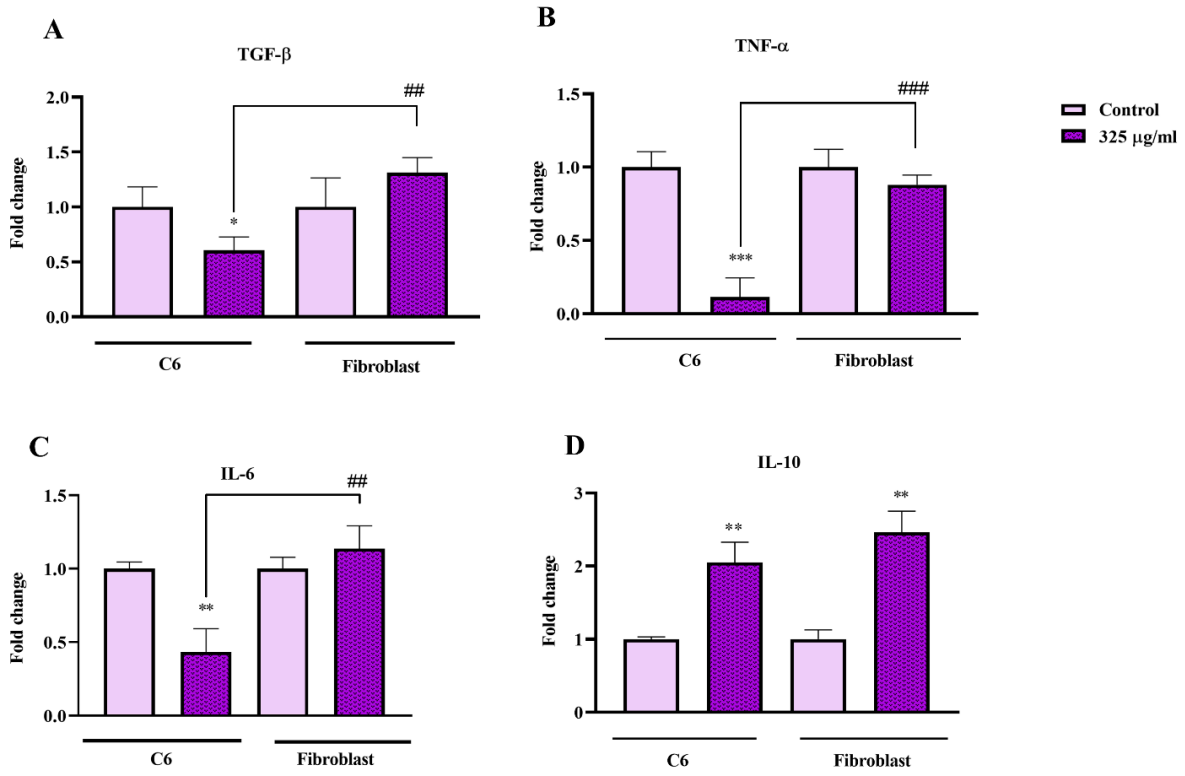

Figure 3. The effect of Shilajit (SH) on the expression levels of (A) *TGF-β*, (B) *TNF-α*, (C) *IL-6*, and (D) *IL-10*. *P<0.05, **P<0.01, and ***P<0.001 compared to relevant control. ##P<0.01, and ###P<0.001 compared treated C6 cells with treated fibroblast cells. Values are expressed as mean±SD. n=3

a specific compound. It was observed that following the induction of non-alcoholic fatty liver disease in rats, levels of IL-10 increased, while levels of IL-1β and TNF-α were notably decreased as a result of treatment with SH and pioglitazone (36).

Also, in previous studies, it has been suggested that SH was effective in treating ulcerative colitis. They have reported that SH can treat ulcerative colitis by decreasing IL-1β and TNF-α, also increasing IL-10 and IL-4 (37).

In the present study, we investigated the effects of SH on the TGF-β1 gene expression level in C6 glioma cells. In normal epithelial cells, TGF-β is a substance that restricts cell growth. However, during the development of malignancy in cancer cells, the inhibitory properties of TGF decrease, ultimately leading to tumor metastasis (38). TGF-β plays a key role in tumor invasion and metastasis. TGF-β is produced by microglia/macrophages that promotes glioma invasion both in vivo and in vitro. TGF-β can elevate MMP expression and lower levels of tissue inhibitors of metalloproteinase (TIMP), resulting in the degradation of the extracellular matrix and thereby promoting tumor cell growth and invasion (38, 39).

Similarly, the results of the present study revealed that the TGF-β gene expression level increased in C6 glioma cells compared to the fibroblast cell line. Treatment with SH decreased TGF-β mRNA expression level in C6 glioma cells. Kordestani et al. showed that SH significantly inhibits the NF-κB signaling pathway and has anti-inflammatory activity. The NF-κB signaling pathway may indeed be an essential factor in tumor progression driven by inflammation (40).

A study on oral cancer shows that TNF-α is essential in regulating the inflammatory process in tumor growth and increases oral cancer's aggressive metastatic ability through the NF-kB signaling pathway. They reported an important rise in the expression levels of NF-kB (IKKb and p65) in oral cells after TNF-α stimulation (41). Similarly, our results showed a decrease in gene expression levels of TNF-α in C6 glioblastoma after SH treatment. This study assessed the effects of SH on the C6 cell line compared to the fibroblast cells by measuring the expression levels of several cytokine genes (TNF-α, IL-10, IL-6, and TGF-β1) involved in the inflammatory pathway. However, numerous limitations should be addressed in upcoming research. For example, an in vivo experiment is needed to clarify the possible effect of SH on cancerous cells in the body environment. Moreover, measuring protein levels of these mediators provides more accurate results. Another limitation of the present work study is the use of a non-human cell line. The application of the human cell lines is suggested to make the results more consistent. Also, in the present study, a limited number of genes were examined. It is proposed that a greater number of genes involved in inflammatory pathways, such as genes involved in the NF-kB signaling pathway, should be examined in the future. Furthermore, to determine the exact role of these genes in C6 cells, specific inhibitors may be useful for the interpretation of results.

Conclusion

This study showed that SH might exert an anti-cancer effect on C6 cells, probably through decreasing TGF-1β gene expression, and also IL-6 and TNF-α. Hence, it may be useful in reducing invasion and improving the condition of glioma patients.

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Authors' Contribution

Conceptualization: Mehrnaz Mehrabani, Zeinab Kordestani.
Data curation: Mehrnaz Mehrabani, Zeinab Kordestani.
Formal analysis: Hossein Parsa, Samaneh Fathizadeh.
Funding acquisition: Zeinab Kordestani.
Investigation: Samaneh Fathizadeh, Mojdeh Esmaeili Tarzi, Sara Yaghoubi.
Methodology: Mehrnaz Mehrabani, Zeinab Kordestani.
Project administration: Zeinab Kordestani.
Resources: Mehrnaz Mehrabani, Zeinab Kordestani.
Software: Mehrnaz Mehrabani.
Supervision: Mehrnaz Mehrabani, Zeinab Kordestani.
Validation: Mehrnaz Mehrabani, Zeinab Kordestani.
Visualization: Zeinab Kordestani.
Writing–original draft: Mehrnaz Mehrabani, Zeinab Kordestani, Hossein Parsa, Samaneh Fathizadeh.

Competing Interests

The authors declare that they have no conflict of interest.

Data Availability Statement

Data are available by corresponding author on a reasonable request.

Ethical Approval

The study was approved by the Ethics Committee of Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences (Ethical approval code: EC/KNRC 99-17). Given that our study is an in vitro experiment, informed consent was not applicable.

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