Sigma J Eng Nat Sci, Vol. 42, No. 4, pp. 965–972, August, 2024

Sigma Journal of Engineering and Natural Sciences
Web page info: https://sigma.yildiz.edu.tr
DOI: 10.14744/sigma.2023.00146

Research Article

Investigation of the cytotoxic effects of juglone on C6 glioma cell line

Ekin BEKTAŞ[1,*], Mine KUÇAK[1], Firuze ÜNLÜ BEKTAŞ[1], Tolga ÖNTÜRK[1], Muhammed Hamza MÜSLÜMANOĞLU[1]

[1]Department of Molecular Biology and Genetics, Yıldız Technical University, İstanbul, 34220, Türkiye

ARTICLE INFO

Article history
Received: 19 October 2022
Revised: 17 December 2022
Accepted: 12 March 2023

Keywords:
Cancer; Cytotoxicity; Glioma; Juglone; RT-qPCR

ABSTRACT

Juglone is an organic compound found naturally in the leaves, roots, bark, and fruits of plants belonging to the Juglandaceae family. It has toxic and growth inhibiting features in many plant species. Glioma is a type of tumorigenesis that occurs when glial cells begin to proliferate uncontrollably because of various reasons. C6 glioma cell line is a tumour line derived from brain glial cells of *Rattus norvegicus*. In this study, after juglone application to C6 glioma cell line, MTT and WST-1 tests were performed and IC_{50} values were found 6.666 µM and 5.646 µM, respectively. The data obtained showed that the results of two tests were closely related and the cytotoxic effects of juglone were present in this cell line. Furthermore, colony formation assay showed a decrease in colony formation abilities of the cells after juglone treatment. These observations were reinforced by wound healing assay and similarly, the migration ability of the cells decreased with juglone application. Morphological examinations of the cell groups were performed under fluorescent microscopy by DAPI staining, which showed a reduction in cell number. As a final step, the expression levels on the cancer related genes were determined by RT-qPCR. While a significant increase was observed in the expression levels of pro-apoptotic genes compared to the control group, a significant decrease was observed in the expression of the anti-apoptotic gene and the gene involved in proliferation. In line with the data presented, the cytotoxic effects of juglone were determined in the C6 glioma cell line.

Cite this article as: Bektaş E, Kuçak M, Ünlü Bektaş F, Öntürk T, Müslümanoğlu MH. Investigation of the cytotoxic effects of juglone on C6 glioma cell line. Sigma J Eng Nat Sci 2024;42(4):965–972.

INTRODUCTION

Juglone, also called 5-hydroxy-1,4-naphthaledione, is an organic compound with the molecular formula $C_{10}H_6O_3$. It is found naturally in the leaves, roots, bark, fruit, and bark of plants in the Juglandaceae family, particularly the black walnut, and is toxic or growth retarding for many plant species [1]. Juglone is not a substance found or newly discovered in the 21st century, however; research on the effects of juglone on cancer has recently gained momentum. Juglone has cytotoxic effects in a variety of human cancer lines such as prostate cancer, breast cancer, skin, and lung cancer [2,3]. In addition, there are effects of juglone not only on human cancer lines but also on rat cancer cell

*Corresponding author.
*E-mail address: ekinbektas@hotmail.com

This paper was recommended for publication in revised form by Editor in-Chief Ahmet Selim Dalkilic

lines. The action mechanism of juglone and how it works are tried to be clarified [4]. Juglone has cytotoxic and genotoxic effects on rat melanoma cells [5].

Gliomas are tumours originating from glial cells and are common tumours of the central nervous system [6]. Many model cells are used to understand glioma cell biology. One of the most well-known and widely used among these is C6 glioblastoma cells. C6 glioblastoma cells were first obtained from Wistar furth rat brains by Benda et al. (1968) by forming glioblastoma with N-nitroso methyl urea [7]. C6 glioblastoma cells exhibit fibroblast-like morphology, are in polygonal form and multiply well on the surface they attach to [8]. C6 glioma cells are frequently used for glioblastoma research since they have high mitotic activity as well as various malignant glioblastoma features such as intratumor haemorrhage, nuclear polymorphism, and tumour necrosis foci [9]. C6 cell line was determined to be the cell line most similar to the mechanism of human brain tumours [9].

In this study, it was aimed to demonstrate and observe the cytotoxic effects of juglone, a secondary metabolite, in C6 glioma cancer cell line. Juglone is advantageous agent because it is a substance that can be obtained naturally and has been used in various industries for a long time [10,11]. This study was carried out to observe its effect on brain cancer, which is one of the biggest problems of today. Therefore, it was aimed to use juglone in further studies and to show whether it is a potential therapeutic agent.

MATERIALS AND METHODS

Cell Culture

The C6 glioma cells were obtained from Yildiz Technical University (Istanbul, Turkey). The C6 glioma cells were cultured in Dulbecco's Modified Eagle Medium-High glucose (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher) and 1% penicillin/streptomycin (Capricorn). The cells were grown in a 5% CO_2 incubator at 37°C in a humidified atmosphere. All cell culture was limited to twenty-five passages (P25).

Cell Viability Assays

The viability of C6 glioma cells was first determined using the MTT test. For 24 hours, the cells (10^4) were plated in 96-well culture plates. For another 24 hours, the experimental cells were exposed to juglone (Across) doses of 5, 10, 15, 20, 40, and 80 µM. After 24 hours of treatment, 10 µL MTT (Goldbio) was applied to the cell and incubated for 2 hours at 37°C. Then, 100 µL dimethyl sulfoxide was added to dissolve the formazan crystals and reveal the cells. The absorbance at 570 nm was measured, and the IC_{50} of juglone was computed using GraphPad Prism 9.3.0.

The viability of the cells was also tested with the WST-1 method using given procedure. The cells (10^4) were seeded in 96-well culture plates overnight. The experimental cells were treated with 5, 10, 20, and 40 µM concentrations of juglone for 24 hours. These doses were determined according IC_{50} value of juglone in MTT test. After treatment, 10 µL WST-1 solution (Cayman) was added into cells and incubated at 37°C for 2 hours. Optical density was measured at 450 nm absorbance and the IC_{50} of juglone was calculated via GraphPad Prism 9.3.0.

Considering the IC_{50} value of both cell viability assays, two promising doses were chosen for the next tests. Doses one above and one below of the IC_{50} value were selected to observe whether there was a steady dose-dependent decrease when juglone was applied to the cells.

Colony Formation Assay

In this method, (2×10^5) cells were seeded in each well of a 6-well plate and incubated for 24 hours. The first well was determined as the control group and no treatment was applied. 5 µM and 10 µM juglone doses were added to the other wells, respectively, and incubated for 24 hours. After 24 hours of incubation, untreated and juglone treated cells were replated in a 6-well plate at a density of 250 cells per well. They were incubated for 10 days at 37°C and 5% CO_2. After 10 days, the images of the colonies formed after the cells were stained with crystal violet were taken. The colony numbers of control and experimental groups determined with image processing program ImageJ.

DAPI Staining

The cells were plated in T25 flasks and cultured to 70-80% confluency. They were subsequently cultured for 24 hours in the presence of 5 µM, 10 µM juglone and in the absence of juglone. The cells were rinsed with 1X PBS (Wisent) and fixed for 10 minutes in ice cold methanol (Merck). Following fixation, the nuclei were stained with 850 nM DAPI staining solution (Cayman) and kept in dark. Finally, the DAPI solution was withdrawn from the cells, and they were rinsed with 1X PBS. A fluorescent microscope was used to image the cells (Zeiss). Image processing program ImageJ was used to count the cells.

Wound-Healing Assay

The cells (5×10^4) were seeded onto 24-well culture plates and cultured to 80% confluency. Using sterile 100 µL pipette tips, a wound was scraped. The cells were subsequently treated with 5 µM and 10 µM juglone. Images of the cells were obtained under a light microscope at 0 and 24 hours (Zeiss). Microscopy image analysis program Wimasis was used to compute the scratch areas.

Reverse Transcription-Quantitative PCR Assay

Total RNA of cells was isolated with RiboEx™ solution (Geneall) using the manufacturer's instructions and the concentration of total RNA was determined via 260/280 nm absorbance via a NanoDrop spectrophotometer (Thermo Scientific). Reverse transcription reactions were performed with a smART First Strand cDNA Synthesis kit (EUR_X) by

Table 1. Primer sequences

Genes	Forward sequence (5'3')	Reverse sequence (5'3')
β-actin	CTCTGTGTGGATTGGTGGCT	GCAGCTCAGTAACAGTCCGC
MMP8	TGGAGTGTGCCATCAACCCTGAC	CACCATGGTCTCTTGAGACGAAAGC
BAX	GAGGACTCCAGCCACAAAGA	CGAGCTGATCAGAACCATCA
BCL-2	TATATGGCCCCAGCATGCGA	GGGCAGGTTTGTCGACCTCA
Caspase3	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCAGGGT
PARP1	TCTACTTTGCTGATATGGTGTCC	TGGGTAACTTGCTGATGTGAG

Table 2. qPCR protocol

Step	Cycles	Temperature	Time
Polymerase activation	1	95 °C	2 min
Denaturation	40	95 °C	5 s
Annealing		60 °C	10 s
Extension		72 °C	30 s

given protocol. The quantitative PCR reaction performed on Applied Biosystem 7500 with the total volume of 20 µL. The sequences of specific PCR primers are shown in Table 1 and PCR reaction are set as in Table 2. β-actin primer was used as a housekeeping gene. The relative quantification of the mRNA was calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

The tests were repeated three times. Comparisons among groups were performed using one-way ANOVA by GraphPad Prism 9.3.0. The significant level was set at $p < 0.05$.

RESULTS AND DISCUSSION

Juglone Inhibits Cell Viability of C6 Glioma Cells

By MTT assay, the effects of various juglone doses on cell viability were measured for 24 hours. After treatment, juglone inhibited proliferation of C6 glioma cells in a dose-dependent manner (Figure 1A). The IC_{50} value was calculated as 6.666 µM (Figure 1B). Additionally, R^2 value was found as 0.9953 (Figure 1A). In addition, IC_{50} value was measured with the WST-1 test. This value was calculated as 5.646 µM and the R^2 value of the result was determined as 0.9504 (Figure 1C&D). The calculated IC_{50} values were found to be statistically significant. The results of two cytotoxicity tests show that, juglone remarkably reduces the survival rate of C6 glioma cells. According to the IC_{50} value determined by MTT Assay and WST-1 test, the doses to be used in the continuation of the study were determined as 5 µM and 10 µM. Choosing two doses, above and below the average of the IC_{50} values, will be beneficial for observing the dose-dependent effect of juglone in the later steps.

Juglone Decreases Colony Formation Capability of C6 Glioma Cells

Colony formation experiment was performed to validate the cytotoxic effects of juglone on C6 glioma cells as well as to observe the long-term effects of juglone. The test was done using 5 µM and 10 µM doses to compare with the control group so as to determine the effects of juglone on cells gradually. In treated C6 glioma cells, the number of colonies decreased compared to the control group. (Figure 2A). The colony formation test shows that juglone significantly reduces the colony forming ability of the cells, depending on the dose.

Juglone Causes Apoptotic Bodies in C6 Glioma Cells

The number of DAPI-stained nuclei was counted, and the number of nuclei was decreased as shown in Figure 2B&2C for juglone-treated cells. In addition to a dose-dependent decrease in the number, it was observed that apoptotic structures were formed, especially when 10 µM dose was applied because of the cytotoxic effects of juglone. Furthermore, when compared with the control group, it was observed that nuclei of the cells treated with juglone were smaller and their structure was deteriorated.

Juglone Inhibits Migration of C6 Glioma Cells

Cell migration is an important part of metastasis. Thus, the ability of C6 glioma cells to migrate after being treated with juglone was assessed using a wound-healing test. In the absence or presence of juglone, 80% confluent cells were scratched using a sterile pipette tip. Scratch areas were larger in juglone-treated cells than in the untreated group after 24 hours of treatment (Figure 3A), showing that juglone caused a decrease in cell-cell communication. Juglone application for 24 hours inhibited the ability of C6 glioma cells to migrate, according to the findings. The efficiency of migration was reduced by 31,94% and 75,694%, respectively (Figure 3B).

Juglone Alters the Expression Levels of MMP8, BAX, BCL2, CASPASE3 and PARP1

β-actin has been used as a housekeeping gene. A significant increase was observed in the expression level of BAX gene, a gene with a pro-apoptotic role in the apoptosis mechanism [12], at a dose of 5 µM compared to the control group. A significant decrease was observed in the expression

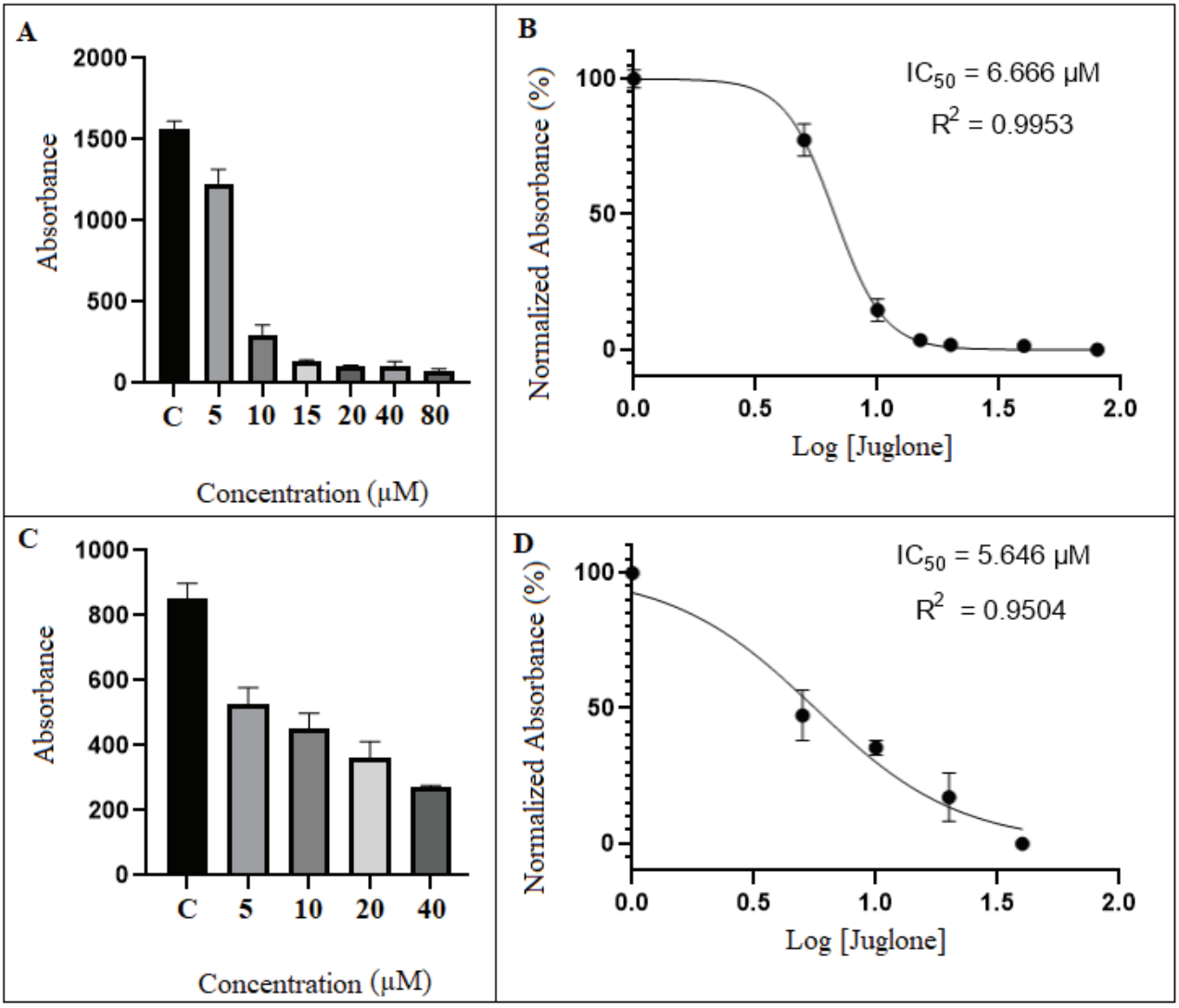

Figure 1. Juglone inhibits cell viability of C6 glioma cells. **(A)** MTT assay, C6 glioma cells were treated with juglone for 24 hours. The cell viability reduced in a dose-dependent manner ($P < 0.05$). **(B)** IC_{50} value was found with MTT assay as 6.666 µM via GraphPad Prism 9.3.0. **(C)** WST-1 assay, C6 glioma cells were treated with juglone for 24 hours. The cell viability reduced in a dose-dependent manner ($P < 0.05$). **(D)** IC_{50} value was found with WST-1 assay as 5.646 µM via GraphPad Prism 9.3.0.

level of the BCL2 gene, a gene with an anti-apoptotic role in the apoptosis mechanism [13], at a dose of 5 µM compared to the control group. A significant decrease was observed in the expression level of the MMP8 gene, a gene that has a role in the proliferation mechanism [14], at a dose of 5 µM compared to the control group. A significant increase was observed in the expression level of Caspase3, a gene with a proapoptotic role in the mechanism of apoptosis [15], at a dose of 5 µM compared to the control group. An increase in the expression level of the PARP1 gene, a gene that has a role in the DNA repair mechanism [16], was observed at a dose of 5 µM compared to the control group, but it was not found significant (Figure 3C).

There are findings showing that juglone has an effect on prostate, colon, lung, breast and skin cancer cell lines [3]. Juglone has been shown to have an inhibitory activity on tumour growth as the dose increases in the mouse model [17]. Based on these findings, this study was carried out to show that juglone also affects glioma cells and finally to suggest the use of juglone as an effective agent. Based on Zhang et al.'s[3] and our findings, the IC_{50} value differs depending on the cell line and the cytotoxicity method used. Similarly, Karki et al. [18] showed that cytotoxic effect of juglone changes according to the type of cancer cell line used and they also found that the IC_{50} value of juglone in MIA Paca-2 cells was 5.05 µM after

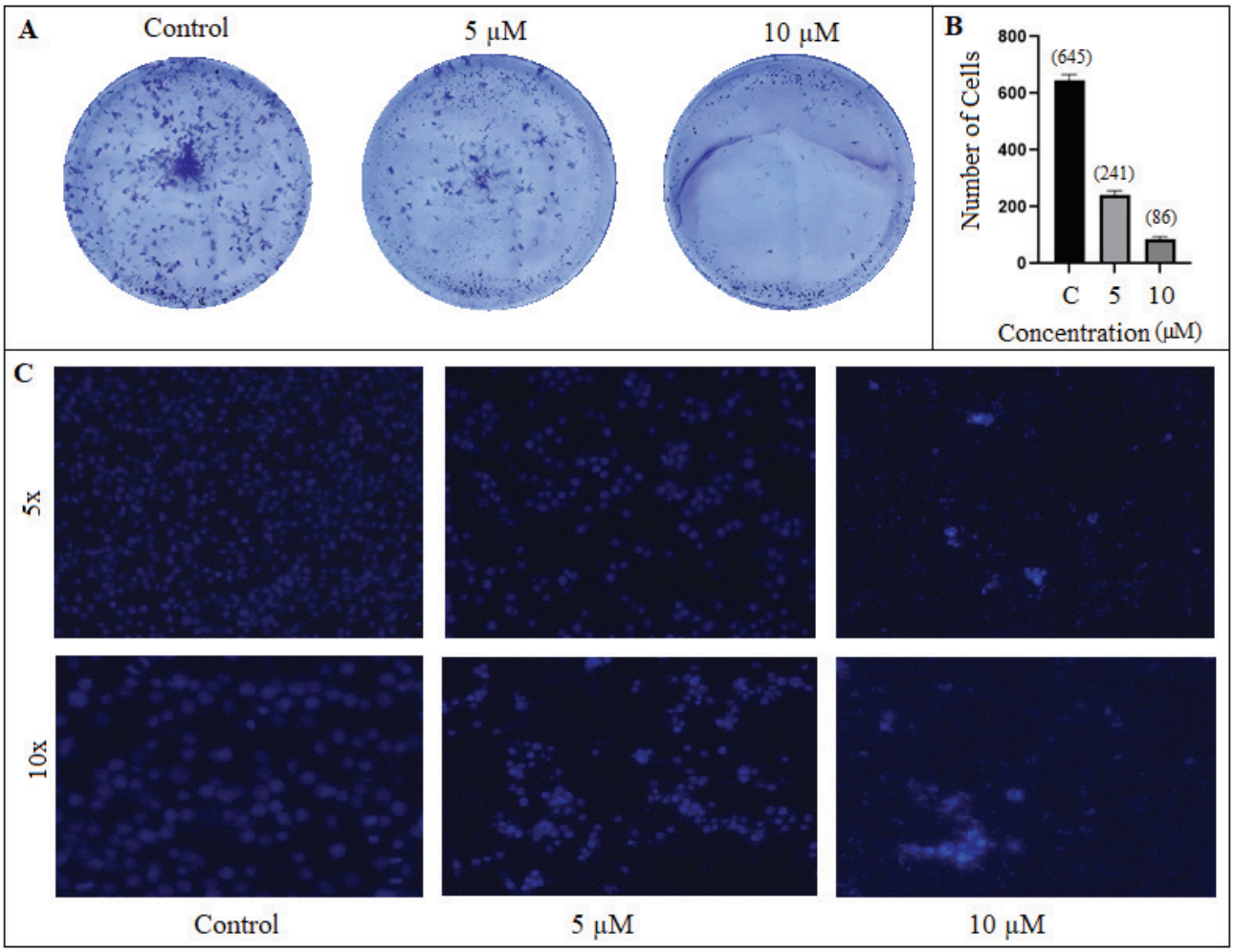

Figure 2. Juglone reduces colony formation capability of C6 glioma cells and the number of cells **(A)** Juglone significantly reduced the number of colonies in both treated groups, 5 μM and 10 μM ($P < 0.05$). **(B)** The number of nuclei reduced in 5 μM and 10 μM juglone treated cells ($P < 0.05$). **(C)** The image of DAPI stained nucleus at 5x and 10x.

24 hours application. Furthermore, another study was demonstrated that IC_{50} value for juglone was 9 μM on the HaCat cell line [1]. Mahdavi et al. [19] demonstrated that juglone has cytotoxic and apoptotic effects on PC3 and DU145 cell lines at a dose of 10 μg/mL. In addition, they showed that the cell death rate increased as the exposure time increased, although there was no increase in the dose amount depending on the dosing time [19]. Accordingly, the IC_{50} value of juglone decreases as the dosing time increases. Based on our findings in this study, it is possible to suggest that there is a decrease in cell number due to the increase in the amount of juglone.

As Aithal et al. [20] demonstrated in B16F1 cell line, juglone treatment is the reason of decrease in number of colonies, which is in line with our findings. Therefore, juglone shows anti-proliferative effects in cell lines [20]. Inbaraj et al. [1] demonstrated that on the HaCat cell line, juglone and another quinone, plumbagin, had cytotoxic effects and therefore they detected a significant decrease in the number of colonies. Karki et al. [18] used the same amount of juglone, which are 5 and 10 μM, on MIA Paca-2 cells and showed that juglone treatment causes to loss of colony forming ability after 6 hours. The similar effects of juglone in different cell lines make it possible to suggest that juglone is an effective cytotoxic agent. The decrease in colony forming ability is due to the decreased ability of cells to divide and migrate after administration of juglone.

It has been reported by Wang et al. [21] that on U-251 cells, apoptotic effects of juglone lead to a decrease in cell migration and a decrease in wound healing [21]. Wound healing assay's and MTT assay's results and changes in Caspase3 expression, which are the experimental methods used in this study, are found to be similar. Obtaining similar results in our study supports that juglone causes decrease on wound healing, reduces cell migration and creates changes in gene expression levels. Shi et al. [22] showed that juglone has apoptotic effects on OVCAR-3 cell line via DAPI

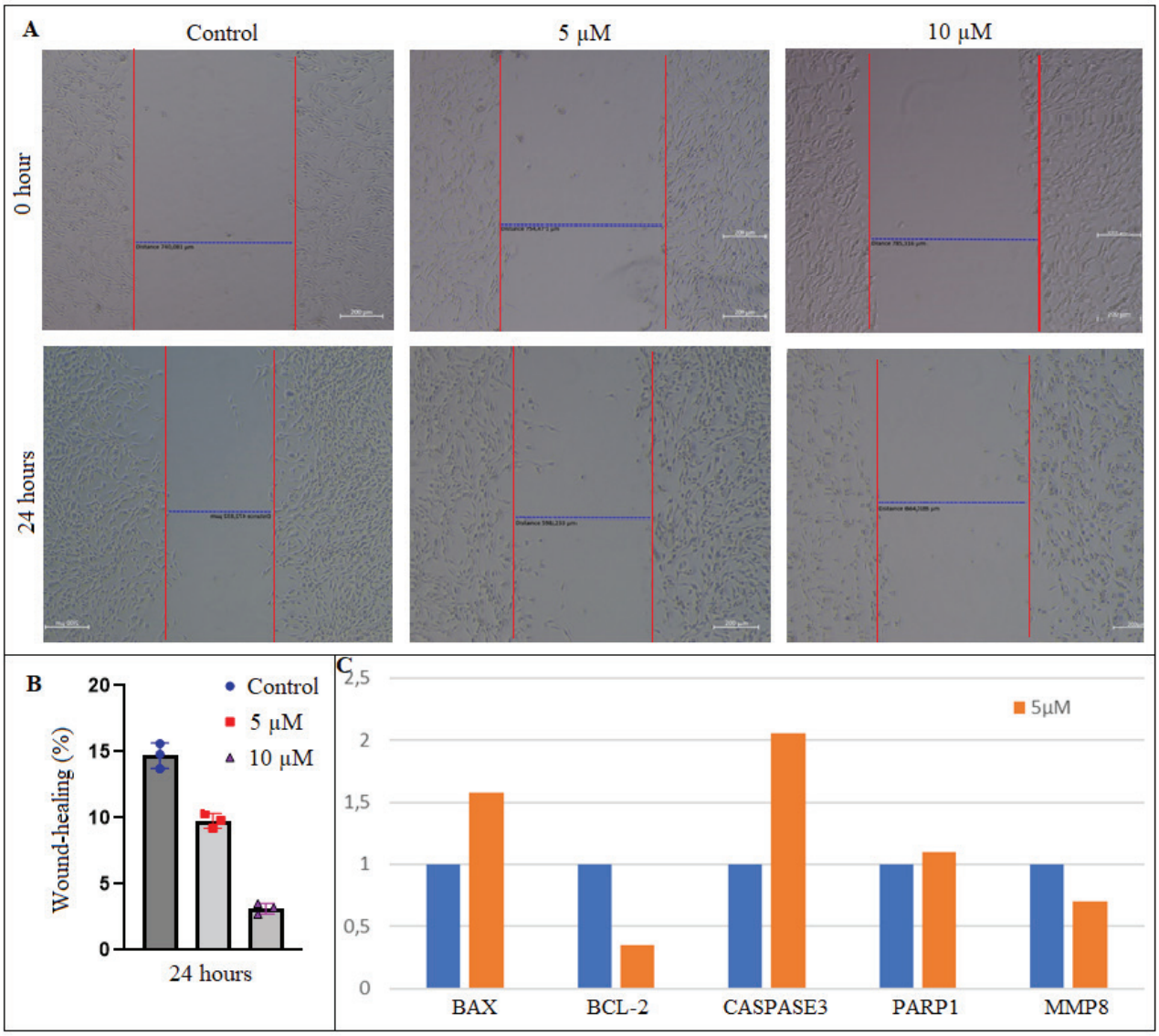

Figure 3. Juglone inhibits migration of rat glioma cells. Juglone alters the expression levels of MMP8, BAX, BCL2, Caspase3 and PARP1. **(A)** Wound areas of 5 µM and 10 µM juglone treated cells captured after 24 hours. **(B)** After 24-hour treatment with juglone migration efficiencies were reduced by 31,94% and 75,694%. **(C)** Expression differences of MMP8, BAX, BCL-2, Caspase-3 and PARP1 between untreated and 5 µM treated C6 glioma cells.

staining method [22]. Staining the cells with this method allowed to observe the change in the treated groups to be clearly understood. Another study showed that juglone application for 24 hours causes small, condensed and bright nuclei and apoptotic bodies in MIA Paca-2 cells, which was observed by Hoechst staining method [18]. Similarly, in the current study, juglone causes deformation in cell morphology and formation of apoptotic bodies.

It was stated in the study of Lu et al. [23] that the apoptotic and anti-proliferative effects created by juglone were determined by using the c-Jun N-terminal kinase/c-Jun pathway. Furthermore, there was an increase in BAX and Caspase3 gene expressions after juglone treatment [23]. These results show that the expression of BAX and Caspase3, which are pro-apoptotic genes, underwent a change compared to the control group after juglone administration, and the decrease in the cell number may have been caused by apoptosis due to the change in these gene expression levels. In parallel with our study, Xu et al. showed that juglone application increased Caspase3 gene expression, increased BAX gene expression and decreased BCL-2 gene expression of HL-60 cells compared to the control group [24]. The observed changes in gene expression levels provide information on how juglone alters the expression of certain genes in C6 glioma cells.

CONCLUSION

The experiments of juglone performed on many different cell lines in many studies show slight differences depending on the experimental and the methods used. However, the common point observed in all studies is that juglone has cytotoxic effects on many cell lines, as we observed in our study. We applied cytotoxicity tests to determine the cytotoxicity of juglone on C6 glioma cells and supported these results with the colony formation assay and the wound-healing assay. Similarly, we examined cells under fluorescence microscope to visualize these effects. Finally, we observed how juglone affects expression levels in certain genes. Considering the cytotoxic effects of juglone on the C6 glioma cell line, it is possible to suggest juglone as an effective agent. It can be suggested that juglone affects C6 glioma cells, especially based on our results of the RT-qPCR experiment performed with 5μM juglone application.

Further studies can be carried out with Western Blot to show how the effects of juglone are related to protein modifications and post-translational modifications. RT-qPCR can be done using more cancer-related genes to observe overall effect of juglone on C6 glioma cell and further, testing different cell lines would help to understand effects of juglone on cancer cells. In order to confirm the determination of apoptosis; TUNEL test, which is used to detect DNA fragments formed during apoptosis, can be applied. After enough *in vitro* studies, this hypothesis can be supported by *in vivo* studies. It is possible to determine whether juglone could potentially be used as a therapeutic agent in the treatment of glial cancer, but further research is required to achieve this.

AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

REFERENCES

[1] Inbaraj JJ, Chignell CF. Cytotoxic action of juglone and plumbagin: A mechanistic study using HaCaT keratinocytes. Chem Res Toxicol 2004;17:55–62. [CrossRef]

[2] Bayram D, Özgöçmen M, Armagan I, Sevimli M, Türel GY, Şenol N. Investigation of apoptotic effect of juglone on CCL-228-SW 480 colon cancer cell line. J Cancer Res Ther 2019;15:68–74. [CrossRef]

[3] Zhang XB, Zou CL, Duan YX, Wu F, Li G. Activity guided isolation and modification of juglone from Juglans regia as potent cytotoxic agent against lung cancer cell lines. BMC Complement Altern Med 2015;15:396. [CrossRef]

[4] Sugie S, Okamoto K, Rahman KM, Tanaka T, Kawai K, Yamahara J, et al. Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats. Cancer Lett 1998;127:177–183. [CrossRef]

[5] Aithal BK, Kumar MR, Rao BN, Udupa N, Rao BS. Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells. Cell Biol Int. 2009 Oct;33(10):1039–1049. [CrossRef]

[6] Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, et al. The 2016 World Health Organization classification of tumors of the central nervous system: A summary. Acta Neuropathol 2016;131:803–820. [CrossRef]

[7] Benda P, Lightbody J, Sato G, Levine L, Sweet W. Differentiated rat glial cell strain in tissue culture. Science 1968;161:370–371. [CrossRef]

[8] Kłopocka W, Korczyński J, Pomorski P. Cytoskeleton and Nucleotide Signaling in Glioma C6 Cells. In: Barańska J, editor. Glioma signaling. Cham, Switzerland: Springer; 2020. p. 109–128. [CrossRef]

[9] Grobben B, De Deyn PP, Slegers H. Rat C6 glioma as experimental model system for the study of glioblastoma growth and invasion. Cell Tissue Res 2002;310:257–270. [CrossRef]

[10] Khalafy J, Bruce JM. Oxidative dehydrogenation of 1-tetralones: Synthesis of juglone, naphthazarin, and α-hydroxyanthraquinones. J Sci IRI 2002;13:131–139.

[11] Cosmulescu S, Gheorghe A, Botu M, Baciu A, Gruia M. Phenolics of green husk in mature walnut fruits. Not Bot Horti Agrobo 2010;38:53–56.

[12] Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J 1998;17:3878–3885. [CrossRef]

[13] Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 1988;335:440–442. [CrossRef]

[14] Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: Regulators of the tumor microenvironment. Cell 2010;141:52–67. [CrossRef]

[15] Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, et al. Human ICE/CED-3 protease nomenclature. Cell 1996;87:171. [CrossRef]

[16] Pascal JM. The comings and goings of PARP-1 in response to DNA damage. DNA Repair (Amst) 2018;71:177–182. [CrossRef]

[17] Wang H, Zou C, Zhao W, Yu Y, Cui Y, Zhang H, et al. Juglone eliminates MDSCs accumulation and enhances antitumor immunity. Int Immunopharmacol 2019;73:118–127. [CrossRef]

[18] Karki N, Aggarwal S, Laine RA, Greenway F, Losso JN. Cytotoxicity of juglone and thymoquinone against pancreatic cancer cells. Chem Biol Interact 2020;327:109142. [CrossRef]

[19] Mahdavi M, Azadbakht M, Vahdati A, Shokrzadeh M, Farhadi A. Cytotoxic effects of juglone and *pterocarya fraxinifolia* on prostate cancer cells. J Pharm Bioallied Sci 2019;11:195–204. [CrossRef]

[20] Aithal KB, Kumar S, Rao BN, Udupa N, Rao SB. Tumor growth inhibitory effect of juglone and its radiation sensitizing potential: In vivo and in vitro studies. Integr Cancer Ther 2012;11:68–80. [CrossRef]

[21] Wang J, Liu K, Wang XF, Sun DJ. Juglone reduces growth and migration of U251 glioblastoma cells and disrupts angiogenesis. Oncol Rep 2017;38:1959–1966. [CrossRef]

[22] Shi JY, Huang ZR, Gao HY, Xu XL. Anticancer effects of juglone in OVCAR-3 human ovarian carcinoma are facilitated through programmed cell death, endogenous ROS production, inhibition of cell migration and invasion and cell cycle arrest. J BUON 2021;26:1188.

[23] Lu Z, Chen H, Zheng XM, Chen ML. Experimental study on the apoptosis of cervical cancer Hela cells induced by juglone through c-Jun N-terminal kinase/c-Jun pathway. Asian Pac J Trop Med 2017;10:572–575. [CrossRef]

[24] Xu HL, Yu XF, Qu SC, Zhang R, Qu XR, Chen YP, et al. Anti-proliferative effect of Juglone from Juglans mandshurica Maxim on human leukemia cell HL-60 by inducing apoptosis through the mitochondria-dependent pathway. Eur J Pharmacol 2010;645:14–22. [CrossRef]