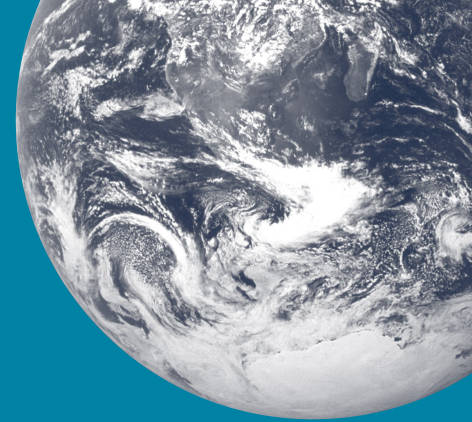

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THE CYTOTOXIC AND MIGRASTATIC POTENTIALS OF ALLIUM JESDIANUM HYDROALCOHOLIC EXTRACT ON GLIOBLASTOMA MULTIFORME CELL LINE MODEL

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Abstract – *Objective:* *Glioblastoma multiforme is one of the most malignant types of central nervous system tumors and temozolomide (TMZ) is currently used as a standard treatment for this type of cancer. However, resistance to temozolomide is a problem in the successful treatment. Plants and herbs are potential sources of cancer therapeutics. This study aimed at evaluating the effect of Allium Jesdianum (AJ) hydroalcoholic extract on glioblastoma multiforme cells.*

Materials and Methods: *The plant material was purchased and extracted. The cell line was treated with extract for 24, 48, and 72 hr. Cell viability was assessed by trypan blue staining, MTT assay, and lactate dehydrogenase activity measurement. Tumor invasion potential was evaluated by cell migration, invasion, and adhesion tests. Real-time PCR was used to assess the changes in the expression pattern of genes involved in cancer invasion.*

Results: *Extract treatment caused a concentration- and time-dependent decrease in cell survival. Also, a decrease in cell migration, invasion and adhesion potential and the expression of metalloproteinases 2 and 9 in cells was observed after treatment.*

Conclusions: *Allium Jesdianum showed promising anti-cancer activity in glioblastoma multiforme cells.*

KEYWORDS: *Glioblastoma multiforme, Allium Jesdianum, Drug resistance, Migration, Invasion, Adhesion.*

INTRODUCTION

Glioma accounts for more than 30% of all primary cancers in the central nervous system and originates from glial cells[1]. Due to the high potential of this tumor to invade, the rapid cell proliferation, and the lack of usual treatment, this cancer causes many problems in the world[2]. Grade IV glioma, also known as glioblastoma multiforme (GBM), is the most malignant and progressive brain cancer, accounting for about 54% of malignant gliomas diagnosed[3]. GBM has an incidence of 3.21 per 100000 population and this number is expected to increase in the coming years[4]. The standard treatments used for GBM, including surgery and tumor resection, chemotherapy, and radiation therapy, lead to a short survival of the patient after diagnosis. Long-term survival is not possible due to the innate and acquired resistance of the tumor cells to therapy[5].

Historically, natural products particularly of

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plant origin have been accepted for the treatment of various human ailments, despite the lack of scientific evidence of their effectiveness and safety[6]. Advances in technology have allowed scientists to identify the active ingredients in plant extracts. Thus, efforts are underway to find new drugs to complement or replace conventional therapies[7]. Allium Jesdianum (AJ) grows at altitudes of 1800-2600 meters in the Zagros mountains (a long mountain range in Iran, northern Iraq, and southeastern Turkey). Native peoples utilize the aerial parts of this plant to cure abdominal pain, rheumatism, and kidney stones. Recently its analgesic effects were reported. The steroids in onions also exhibited cytotoxic and cytostatic effects against malignant tumors[8].

Due to the need to identify new therapies strategies for GBM, this study aimed to investigate the effects of treatment with AJ hydroalcoholic extract on GBM cell viability and invasion potential.

MATERIALS AND METHODS

Cell line and reagents

For this experimental *in vitro* study, GBM cell line (U87MG) were obtained from the Pasteur Institute (Tehran, Iran). Trypsin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), crystal violet, ethanol, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin/streptomycin solution, phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were procured from Gibco (Grand Island, NJ, USA). The cells were seeded in 75 mm^2 cell culture flasks containing RPMI 1640 medium, 10% FBS, and 1% penicillin/streptomycin solution. The cells were maintained at a 37°C and 5% CO^2 in a humidified incubator.

Plant material and extract preparation

AJ was purchased from a local market and its impurities were removed. After approval by a botanist, plant material was washed thoroughly, air-dried, and crushed. The crushed samples were extracted with 95% ethanol (1 g in 10 ml ethanol) for 72 hr at 37°C with occasional shaking. Then the extract was filtered through a Whatman no. 1 filter paper and the filtrate was evaporated to dryness using a rotary evaporator. The dried samples were stored at −20°C until use. For treatments, the plant extract was dissolved in a serum-free culture medium and sterilized with 0.22 μm syringe filters[9].

Viability and cytotoxicity tests

The effect of AJ extract on cell viability was assessed by MTT and trypan blue tests. Briefly, the cells were seeded in 96-well plates (5×103 cells/well) in a 200 μl culture medium. Following attachment, the cells were treated with 1000, 500, 250, 125, and 62.5 μg / ml of extract for 24, 48, and 72 hr. Control cells were treated with a serum-free medium. After treatment, for trypan blue assay, the cells were trypsinized with 0.25% trypsin-EDTA solution, resuspended in PBS, and stained with 0.4% trypan blue dye solution (in PBS). The cells were loaded in a Neubauer chamber, and the number of viable and non-viable cells was counted under phase contrast microscope[10]. The percentage of cell viability was determined as follow:

Number of viable cells / Number of total cells × 100

For the MTT assay, the media was replaced with MTT solution (10 μl of 5 mg / ml per well) prepared in PBS and incubated for 3 hr at 37°C. Then 150 μl of DMSO was added to each well. The plates were gently shaken for 10 min and absorbance was measured at 600 nm, with reference 490 nm, by a microtiter plate reader[10]. Cell survival percentage was calculated according to the following formula:

Absorption rate of control cells /
Absorption rate of treated cells × 100

The half-maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism version 6 software (La Jolla, CA, USA).

For lactate dehydrogenase (LDH) activity assay, each well of 96-well plate was washed to remove FBS content and the cells were lysed using 0.5% Triton-X-100 prepared in PBS. This lysate was mixed with LDH assay reagents of Abcam colorimetric LDH test kit (MA, USA). The absorbance of the orange-red colored formazan product was measured at 490 nm using a microplate reader[10].

Migration assay

In vitro scratch assay was applied to estimate the effect of AJ extract on cell migration capacity. U87MG cells were cultured in 6-well plates. After the cells reached a density of 80%, a scratch was made in the middle of the wells by the sterile yellow pipette tip. the wells were washed three times with PBS, and 1.5 ml of culture medium with IC_{50} concentration of the extract was

added. After 24 hr the wells were assessed and photographed with a light microscope[11]. The images were analyzed by Tathcratch software (MathWorks Inc).

Invasion assay

Invasion potential was examined using the CytoSelect™24-Well Cell Invasion Assay kit (San Diego, CA, USA). First, the inserts were incubated with 300 µl of the serum-free medium at 37°C for one hour. Then 500 µl of medium containing 10% FBS was transferred to the lower chamber of the plate. The cells (7×10^5) pretreated with IC_{50} concentration of extract were cultured in the upper chamber. After 24 hr, the cells adhering to the surface inside the Matrigel were removed with a swab. The inserts were transferred to a well containing 400 µl of day solution. After 10 min the inserts were gently rinsed with distilled water and dried. Finally, inserts were transferred to an empty well and 200 ml of extraction solution were added to each well and placed on a rotary shaker for 10 min. The adsorption of samples was measured at 560 nm. By comparing the absorption intensity of the treated samples with the control samples, the rate of change in the ability of the cells to invade under the influence of treatments was evaluated.

Adhesion assay

A 96-well plate well was coated with a Matrigel solution (5 mg / ml) and allowed to dry at room temperature. After 2 hr cells treated with IC_{50} concentration of extract for 24 hr were trypsinized and suspended in a medium. 500 cells were cultured in each well and incubated for 24 hr. Then the wells were washed with PBS and the remaining cells were fixed with 4% paraformaldehyde solution for 20 min and stained with 5% crystal violet dye solution for 10 min. Then the stain was dissolved in 70% ethanol and the absorbance of each sample was measured at 570 nm[12].

Real-time PCR

The effect of AJ extract on matrix metalloproteinase (MMP)-2 and 9 expression levels was evaluated by Real-time PCR. After treatment for 24 hr, total RNA was extracted by TRIzol reagent Thermo Fisher Scientific TRIzol reagent (Waltham, MA, USA). The quantity and quality of the RNAs were verified by a nanodrop spectrophotometer and electrophoresis methods, respectively. Complementary DNA was synthesized by Vivantis Technologies kit (Selangor DE, Malaysia) according to the manufacturer's protocol. β-Actine was served as an internal control. Real-time PCR was conducted using TaKaRa Bio Inc. SYBR Premix Ex Taq Technology (Otsu, Shiga, Japan) on the Applied Biosystems StepOne Real-time PCR System (Foster City, CA, USA). The fold change in the relative expression of each target mRNA was calculated on the based on the comparative Ct ($2^{-\Delta\Delta ct}$) method. All the primers sequences were designed using GeneRunner software and listed in Table 1.

TABLE 1. Sequence of primers used for RT-PCR studies.

Genes	Sequence
MMP-2	(F)5'TTGGCAGTGCAATACCTGAA3', (R)5'GAGTCCGTCCTTACCGTCAA3'
MMP-9	(F)5'CATCGTCATCCAGTTTGGTG3', (R)5'CAGAAGCCCCACTTCTTGTC3'
β-Actin	(F)5'GTGGGCGCCCAGGCACCA3', (R)5'CTCCTTAATGTCACGCACGATTT3'

Statistical analysis

The tests were repeated at least three times independently. The data were presented as mean ± standard deviation. Statistical evaluation was performed using one-way analysis of variance and Tukey's test with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software, and differences were considered significant when $p < 0.05$.

RESULTS

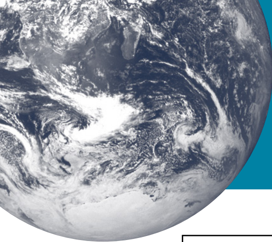
Effect of AJ extract on U87MG cell viability

The effect of 0, 62.5, 125, 250, 500, and 1000 µg / ml concentrations of AJ extract after 24, 48, and 72 hr of treatment on cell survival and proliferation was tested using trypan blue staining and MTT assay (Figures 1 A and B). The effect was along with changes in cell morphology, included volume reduction, rounding, granulation, and separation of cells from the substrate in a concentration- and time-dependent manner. The results showed that AJ extract reduced the viability of

TABLE 2. IC50 values after 24, 48 and 72 hr of treatment with AJ extract.

	24 hr	48 hr	72 hr
Trypan blue	201.37	169.39	41.25
MTT	181.65	154.45	119.65

The Values are presented as mean ± standard deviation.



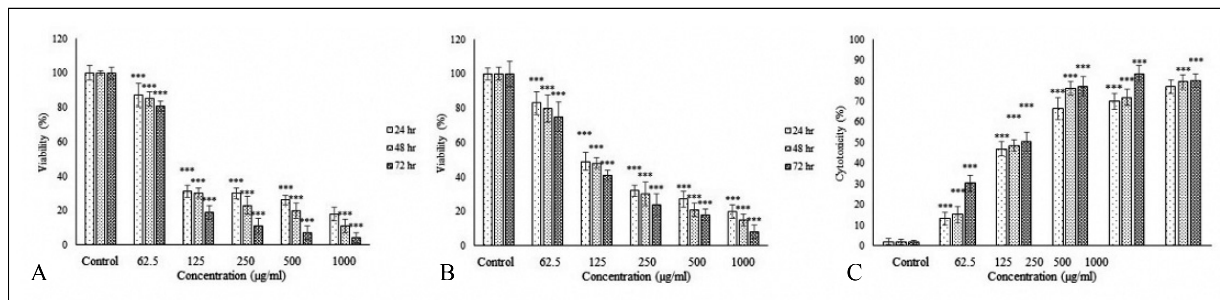


Fig. 1. The effect of AJ extract on the viability of U87MG cells. viability was evaluated after 24, 48, and 72 hr treatment with by *(A)* trypan blue, *(B)* MTT and *(C)* LDH tests. The control group received the same volume of serum-free medium (*indicates p<0.05, **indicates p<0.01, and ***indicates p<0.001 compared to control).

GBM cells in a concentration- and time-dependent manner. The IC_{50} values for each period were calculated and listed in Table 2.

The cytotoxic effect of the extract was further assessed by the LDH assay. The LDH activity in the cell culture medium was increased with the increasing concentration of the extract (Figure 1 C).

Effect of AJ extract on U87MG cells migration, invasion, and adhesion

Analysis of the images obtained from the scratch test by Tscratch software revealed that IC50 concentration of AJ extract decreased the U87MG cell migration by 34% (Figure 2). As shown in Figure 3, IC_{50} concentration of plant extract also decreased the cell invasion potential by 44%. The results of the adhesion assay revealed that AJ extract at the IC_{50} concentration lessened the cell adhesion by 34% (Figure 4).

Effect of AJ extract on MMP-2 and MMP-9 expression in U87MG cells

The results of gene expression analysis showed that treatment with AJ extract decreased the

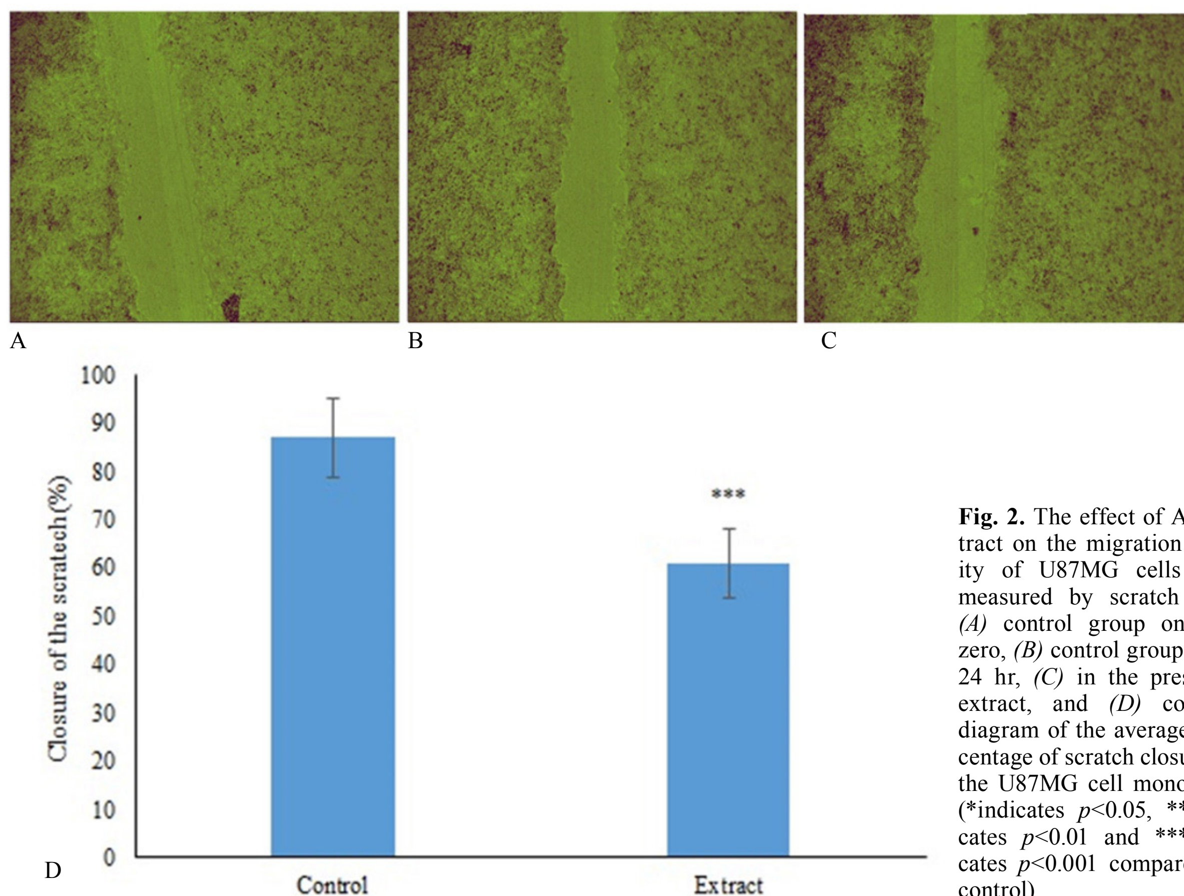


Fig. 2. The effect of AJ extract on the migration ability of U87MG cells was measured by scratch test. *(A)* control group on day zero, *(B)* control group after 24 hr, *(C)* in the presence extract, and *(D)* column diagram of the average percentage of scratch closure in the U87MG cell monolayer (*indicates p<0.05, **indicates p<0.01 and ***indicates p<0.001 compared to control).

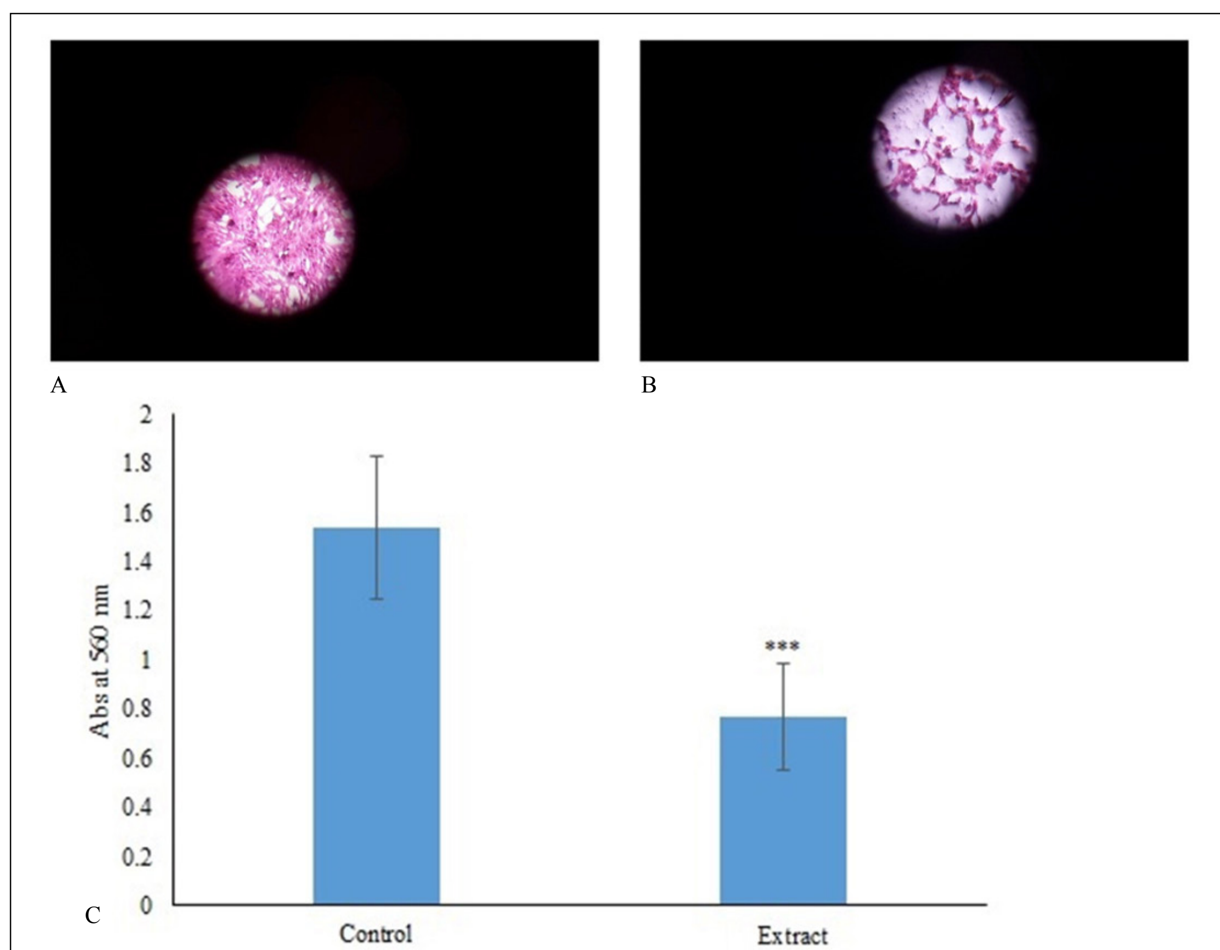


Fig. 3. The effect of AJ extract on the invasion ability of U87MG cells after 24 hr was measured by invasion test. *(A)* control group, *(B)* in the presence of extract, and *(C)* column diagram of the average absorption at 560 nm (*indicates $p<0.05$, **indicates $p<0.01$ and ***indicates $p<0.001$ compared to control).

MMP-2 and MMP-9 expression levels were by 29 and 41%, respectively (Figure 5).

DISCUSSION

In this *in vitro* study, the anti-cancer effects of AJ extract, which contains phenolic compounds, were investigated. First, the effect of the extract on cell viability was tested. The results showed that after each period of treatment, the viability of the cells gradually decreased with increasing concentration. After 24, 48, and 72 hr of treatment with the extract, the viability was significantly reduced in trypan blue staining and MTT test at concentrations of 62.5, 125, 250 500, and 100 µg / ml compared to the control group. In the next step, IC_{50} was calculated for all three treatment periods. The results of calculating the IC_{50} using both techniques were relatively close and in agreement.

Despite recent advances in cancer treatment, there is still no improvement in the life expectancy and quality of life of patients with GMB, one of the major causes of which is drug resistance. Therefore, the development of new strategies is necessary to overcome this problem.

Cell death is associated with loss of membrane integrity and release of extracellular cytoplasm contents. LDH is a relatively stable enzyme that is measured to assess for damage or toxicity to cells in cell culture media.

AJ extract showed concentration and time-dependent cytotoxicity for U87MG cells. Reduction of cell viability in treatment with AJ extract depends on the loss of cell membrane integrity and release of cell contents.

AJ is considered a rich source of anti-oxidants with several phenolic compounds, and these compounds may play a role in the anti-cancer activity of this plant. The total phenolic content in ethanolic extracts of leaves and red onion is between 27.83 to 98.23 mg GAE / g of the extract. DPPH method showed that ethanolic extracts from the leaves of this plant were the most effective free

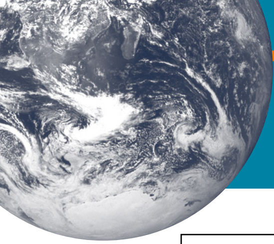


Fig. 4. The effect of AJ extract on the adhesion ability of U87MG cells after 24 hr was measured by adhesion test. *(A)* control group, *(B)* after treatment extract, and *(C)* column diagram of the average absorption of violet crystals at a wavelength of 590 nm (*indicates $p<0.05$, **indicates $p<0.01$ and ***indicates $p<0.001$ compared to control).

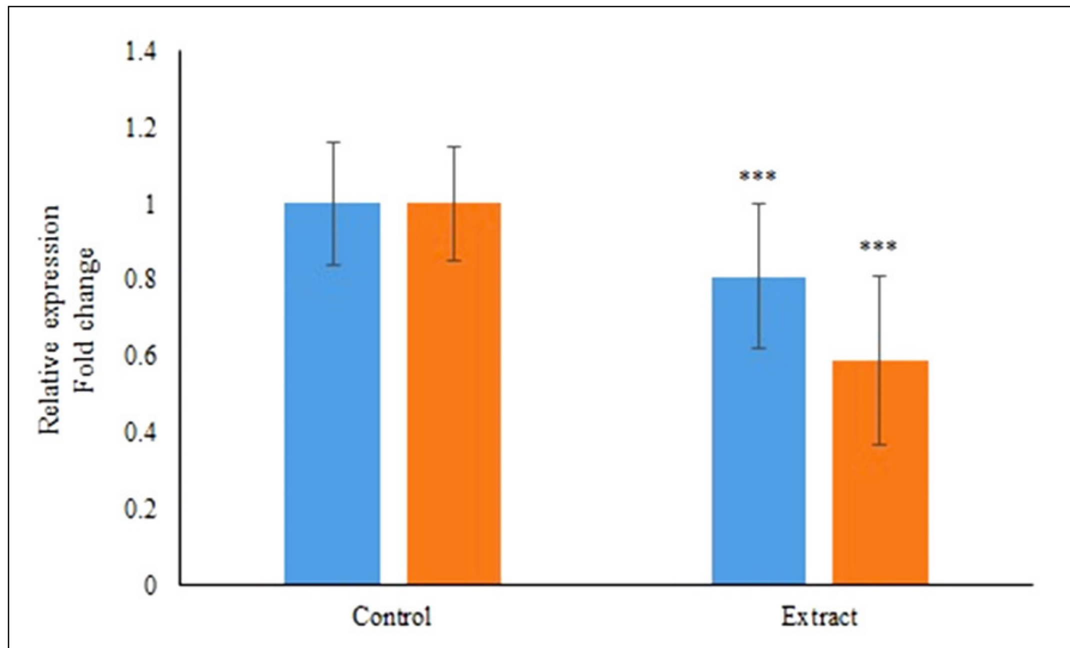


Fig. 5. The effect of AJ extract on the expression of MMP-2 and MMP-9 enzyme genes in U87MG cells after 24 hr was measured by Real-time PCR. (*indicates $p<0.05$, **indicates $p<0.01$ and ***indicates $p<0.001$ compared to control).

radical scavenging agent. Therefore, onions and leaves are considered as a natural source of anti-oxidants[13].

Phenolic compounds are secondary metabolites in plants with an aromatic ring that have one or more hydroxyl groups. More than 8000 natural phenolic compounds have been identified to date. They include simple phenols, flavonoids, lignins and lignans, tannins, xanthones, and coumarins. These compounds are known to have potent anti-cancer activity as well as to combat various diseases associated with oxidative stress. Previous studies have shown that the beneficial effects of dietary phenols on health are due to their ability to exhibit anti-oxidant, anti-inflammatory, and anti-clastogenic activities. The anti-cancer effects of phenolic compounds are primarily due to the ability of the following:

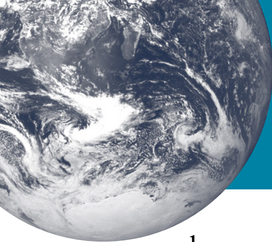
A) Arrest of the cell cycle;
B) Inhibition of cancer signaling cascades controlling cell proliferation, angiogenesis, and apoptosis;
C) Reactive oxygen species level adjustment;
D) Amplification of tumor suppressor proteins such as p53;
E) Increasing the ability to differentiate and transform into normal cells, etc.[14].

The results also showed that AJ extract reduced the migration, invasion, and adhesion potential of U87MG cells.

Tumor cell migration and invasion are considered as main features of malignant cancers, and this is especially important in GBM. GBM cells cannot metastasize to other organs, but their invasion and proliferation in brain tissue are the leading causes of death in patients. A disseminated growth pattern is one of the characteristics of GBM[15]. Hence, the ability to invade, along with the inherent resistance to temozolomide, has been a major barrier to successful therapy, and its control can be a useful strategy for efficient treatment.

MMP family has been shown over the years to play a role in tumor progression. MMPs, particularly MMP-2 and MMP-9 play a major role in the regulation of cancer cell migration, ECM invasion, and metastasis. They catalyze the degradation of extracellular matrix components. MMP-2 and MMP-9 are also important in glioma because their expression is directly correlated to the degree of malignancy and the rate of progression of cancer. Expression of both MMP-2 and MMP-9 genes is increased in human glioma tissues compared to normal brain tissue, and this increase is particularly evident in GBM tumors. Since the ability of tumor cells to migrate in the intercellular matrix is primarily attributed to the secretion of their MMP enzymes, inhibition of these enzymes can be a good treatment option[16].

To define the inhibitory effects of AJ extract on the invasive properties of GBM, we focused on MMP expression, because extracellular matrix degradation is required for tumor metastasis. We evaluated the expression of MMP-2 / -9 because several reports have shown a correlation between



decreased expression of MMP-2 / -9 and the potential for invasion in different cancer cells. Our results showed that AJ extract reduced the ability of U87MG cells to migrate, adhere and invade. This decrease is also associated with a decrease in the expression of MMP-2 and MMP-9.

CONCLUSIONS

According to the results of the present study, AJ extract had an inhibitory effect on cell proliferation and reduced the ability of cells to migrate, invade and adhere. So, this herb is potentially beneficial for the further development of new anti-cancer agents. The present data open a new possible approach in the cure of GBM. Future *in vivo* and clinical studies are suggested.

Conflict of interest:

There is no conflict of interest.

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