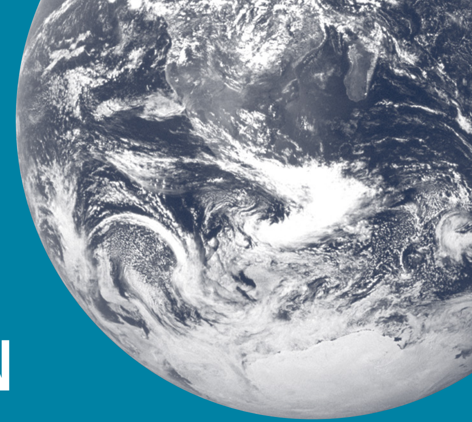

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ANTI-INVASION AND ANTI-METASTASIS EFFECTS OF DANDELION *(TARAXACUM OFFICINALE)* HYDROALCOHOLIC EXTRACT ON GLIOBLASTOMA MULTIFORME CELL LINE MODEL

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Abstract – *Objective:* *Glioblastoma multiforme is one of the malignant brain tumors and despite recent advancements in cancer treatment remains largely incurable. Cancer invasion has a cascade of interrelated and sequential steps, including cell adhesion, extracellular matrix degradation, and cell movement. Hence, inhibition of the invasion-associated steps could be a potential strategy for prolonging the life of patients. This study aimed to evaluate the anti-invasion and anti-metastasis effects of Dandelion* (Taraxacum officinale) *hydro-alcoholic extract on glioblastoma multiforme cell line (U87MG).*

Materials and Methods: *The hydro-alcoholic extract was prepared, and the cell line was treated with 1000, 500, 250, 125, and 62.5 µg / ml of extract for 24, 48, and 72 hr. Cell viability was evaluated. The effect of extract (IC50 concentration) on cancer cell invasion potential was tested. The expression levels of MMP-2, MMP-9, TIMP-1, TIMP-2, uPA, uPAR, p38MAPK, ERK1/2, and SAPK/JNK were analyzed. Comparisons between groups were performed by Tukey's test one-way analysis of variance and differences were considered significant when* $p<0.05$.

Results: *After treatment with extract, the cells viability was decrease in a concentration- and time-dependent. IC50 concentration of dandelion extract significantly decreased the cell migration by 32% (*$p<0.05$*), cell invasion potential by 77% (*$p<0.05$*) and cell adhesion by 51% (*$p<0.05$*). Also, the expression levels of proteolytic enzymes associated with matrix and base membrane degradation (MMP-2, MMP-9, and uPA) were decreased and the levels of their endogenous inhibitors (TIMP1 and TIMP2) were increased. Moreover, the p38MAPK and SAPK/JNK signaling pathway, which stimulates proteolytic enzymes and matrix degradation, was inhibited by extract treatment.*

Conclusions: *Dandelion extract reduced the viability and invasion potential of the glioblastoma cells by regulating proteolytic enzymes and matrix dynamics through the p38MAPK and SAPK/JNK pathway.*

KEYWORDS: *Glioblastoma multiforme, Dandelion, Migration, Invasion, Adhesion.*

INTRODUCTION

Glioma is the most common form of central nervous system neoplasm and originates from glial cells[1]. The high potential of this tumor to invade and the rapid cell proliferation are prominent features of this type of cancers[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]. It has an incidence of 3.21 per 100,000 population and this number is expected

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to increase in future[4]. The gold standard treatment for GBM patients (surgical resection combined with radiotherapy and adjuvant chemotherapy) leads 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].

Tumor invasion is the major cause of death in GBM patients[6]. It is a multifaceted process that results from coordinated events[7]. Malignancy is progressed by the degradation of the basal membrane (BM) and extracellular matrix (ECM) by proteolytic enzymes, followed by invasion[8]. Matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) are two proteolytic enzymes for degradation of the ECM and BM. Studies showed that the expression of MMP-2, MMP-9, uPA, and uPA receptor (uPAR) is correlate with cancer cell invasion potencial[9,10].

It is becoming increasingly clear that mitogen-activated protein kinase (MAPK) pathways are involved in the development of metastatic cells from hyperproliferating cells. MAPK signaling pathways help regulate tumor cell adhesion, motility, and degradation of ECM and BM in the early stages of metastasis[11-15].

Natural products, particularly of plant origin, have historically made a major contribution to pharmacotherapy, especially for cancer, despite the lack of scientific evidence of their effectiveness and safety[16,17]. Dandelion (*Taraxacum officinale*) from Asteraceae or Compositae family, is an edible plant spread worldwide. It has been believed that dandelion originated in Greece, or perhaps the Northern Himalayas, and spread across temperate areas to Europe and Asia Minor. It is traditionally proposed as a dietary supplement and herbal remedy for the prevention, management, and treatment of various human diseases. In a review the pharmacological and therapeutic features of dandelion in traditional medicine and scientific documents are estimated[18].

Due to the need to identify new therapies strategies for GBM, this study aimed to investigate anti-invasion and anti-metastasis effects of Dandelion (*Taraxacum officinale*) hydroalcoholic extract on glioblastoma multiforme cell line.

MATERIALS AND METHODS

Reagents and cell culture

Trypsin, trypan blue, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), crystal violet, ethanol, matrigel solution, 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), fetal bovine serum (FBS) and glutamine were procured from Gibco (Carlsbad, CA, USA). GBM cell line (U87MG) were obtained from the Pasteur Institute (Tehran, Iran) and maintained in a humidified incubator (37°C, 5% CO_2) in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin solution.

Plant material and extract preparation

Dandelion was collected from around the city of Kermanshah (located in the western part of Iran) in early spring 2020 and its impurities were removed. After approval by a botanist, plant material was washed, air-dried, and crushed. The extract was prepared using 95% ethanol (1 g in 10 ml ethanol) for 72 hr at 37°C with occasional shaking. Then, it was filtered via a Whatman no. 1 filter paper and evaporated using a rotary evaporator. Finally, the plant extract was dissolved in a serum-free culture medium and sterilized with 0.22 µm syringe filters[19].

Viability and cytotoxicity tests

The cells were seeded in a 96-well plate and incubated overnight. Following attachment, the cells were treated with 1000, 500, 250, 125, and 62.5 µg / ml of extract for 24, 48, and 72 hr. For trypan blue staining, after trypsinization, the cell suspension was mixed with trypan blue stain solution (0.4% in PBS) and incubated for 3 min at room temperature. A drop of the trypan blue/cell mixture was applied to a hemacytometer. The unstained (viable) and stained (nonviable) cells were counted by a microscope. The percentage of cell viability was calculated by dividing the number of viable cells by the number of total cells×100.

For MTT assay, 50 µl of MTT solution (5 mg / 1 ml in PBS) was added to each well and incubated in the dark. After 4 hr, 100 µl of DMSO was added to each well and the plate was placed on a shaker at room temperature for 20 min. Absorbance was read at a wavelength of 570 nm against a reference wavelength of 630 nm using a plate reader and the percentage of cell viability was calculated by dividing the absorbance of the treated group by the absorption of the control group×100.

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

Cytotoxicity assay was performed using the lactate dehydrogenase activity kit (Abcam Inc, Cambridge, MA, USA). After treatment, the medium on the cells was removed and after centrifugation, 50 µl of supernatant of each sample was added to the wells of a 96-well plate, and lactate dehydrogenase activity was measured per the kit instructions and using the standard NADH curve[20].

Migration assay

The cells were seeded at a density of 5×10^5 cells per well in 6-well culture plates for scratch assay. The monolayer of cells was then scraped with a 20–200 µL micropipette tip to create a wound. Cells were then washed with PBS and treated with the IC50 concentration of the extract. After 24 hr the wells were photographed with a light microscope[21]. The images were analyzed by Tathcratch software (MathWorks Inc).

Invasion assay

The cell invasion assays were performed using a Transwell chamber inserted with polyethylene terephthalate filter membrane containing 8 µm pores in 24-well plates (Corning, NY, USA). 1×10^5 control and pretreated cells with IC_{50} concentration of extract were suspended in 200 µL of serum-free medium and seeded onto the upper compartment of the Transwell chamber. The lower chamber was filled with medium containing 10% FBS. After 24 hr, the medium in the upper chamber was removed, and the filters were fixed with 70% ethanol for 10 min. The cells remaining on the upper surface of the filter membrane were then completely removed by wiping with a cotton swab, and the cells on the opposite surface of the filter membrane were stained with 0.5% Coomassie Brilliant Blue for 10 min. 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

The wells of a 96-well plate well were coated with a Matrigel solution (5 mg / ml) and allowed to dry at room temperature. The cells treated with IC50 concentration of extract for 24 hr were trypsinized and 500 cells were seeded in wells and incubated. After 24 hr, 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. The stain was dissolved in 70% ethanol and the absorbance of each sample was measured at 570 nm[22].

Real-time PCR

The expression levels of MMP-2, MMP-9, TIMP-1, TIMP-2, uPA, uPAR, p38MAPK, ERK1/2, and SAPK/JNK were analyzed by Real-time PCR. After treatment for 24 hr with IC50 concentration, total RNA was extracted by Thermo Fisher Scientific TRIzol reagent (Waltham, MA, USA) and verified by a nanodrop spectrophotometer and electrophoresis methods. Complementary DNA was synthesized by Vivantis Technologies kit (Selangor DE, Malaysia) according to the manufacturer's protocol. 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'
TIMP-2	(F)5'AAGCGGTCAGTGAGAAGGAGTGG3', (R)5'CCTTGGAGGCTTTTTTGCAGTTG3'
TIMP-1	(F)5'TTCGTGGGGACACCAGAAGTCAAC3', (R)5'TGGACACTGTGCAGGCTTCAGTTC3'
uPA	(F)5'GCCATCCCGGACTATACAGA3', (R)5'AGGCCATTCTCTTCCTTGGT3'
uPAR	(F)5'CTGGAGCTGGTGGAGAAAAG3', (R)5'TGTTGCAGCATTTCAGGAAG3'
P38 MAPK	(F)5'CGAAATGACCGGCTACGTGG3' (R)5'CACTTCATCGTAGGTCAGGC3'
SAPK/JNK	(F)5'TGCTGAAGGTCCTAGGAACT3' (R)5'GCATACAGCTTTCCAGCATC3'
ERK 1/2	(F)5'TCAAGCCTTCCAACCTC3' (R)5'GCAGCCCACAGACCAAA3'
GAPDH	(F)5'CAATGACCCCTTCATTGACC3', (R)5' TTCACACCCATGACGAACAT3'

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

	24 hr	48 hr	72 hr
Trypan blue	275.20±8.28	167.41±11.93	114.51±3.29
MTT	318.91±9.03	160.25±8.52	124.89±6.29

The values are presented as mean ± standard deviation.

Statistical analysis

The tests were repeated at least three times independently. All data are expressed as means ± standard deviation (SD). Comparisons between groups were performed by Tukey's test one-way analysis of variance and differences were considered significant when $p < 0.05$.

RESULTS

Dandelion extract effect on U87MG cell viability

The results of trypan blue staining and MTT assay indicated that extract decreased the viability of GBM cells in a concentration- and time-dependent manner (Figures 1 A and B). The IC_{50} values for each period were calculated and listed in Table 2. The LDH activity in the cell culture medium was increased in a concentration-dependent manner (Figure 1 C).

Dandelon extract effect on U87MG cells migration, invasion, and adhesion

Analysis of the images obtained from the scratch test showed that dandelion extract significantly decreased the U87MG cell migration by 32% (p<0.05) (Figure 2). As shown in Figure 3 plant extract also significantly decreased the cell invasion potential by 77% (p<0.05). The results of the adhesion assay revealed that extract treatment significantly lessened the cell adhesion by 51% (p<0.05) (Figure 4).

Dandelion extract effect on gene expression in U87MG cells

As shown in Figures 5, MMP-2, MMP-9, uPA, and uPAR expression levels were significantly decreased in treated cells compared with control cells (p<0.05). Alternatively, TIMP-1 and TIMP-2 levels were significantly increased after treatment (p<0.05). Also, the effect of dandelion extract on MAPK levels and signaling were tested (Figure 6). p38MAPK and SAPK/JNK expression were significantly reduced while ERK1/2 expressions level did not change significantly in treated cells compared with control cells (p<0.05).

DISCUSSION

In this study, we reported for the first time the synergistic effect of dandelion extract on the growth of GBM cells. Our results showed that extract inhibit cell proliferation in a time- and dose-dependent manner. In the next step, IC50 was calculated for all three treatment periods. The results of calculating the IC50 using both techniques (trypan blue staining and MTT assay) were relatively close and in agreement. Data also showed that dandelion extract reduce the potential of migration, invasion, and adhesion of U87MG cells. The invasion capability, along with intrinsic resistance to chemotherapy, is two main barriers to successful treatment of GBM.

The cause of death in most cancer patients is the spread and invasion of the tumor. Therefore, developing a strategy to inhibit this invasion can be very effective in reducing cancer mortality.

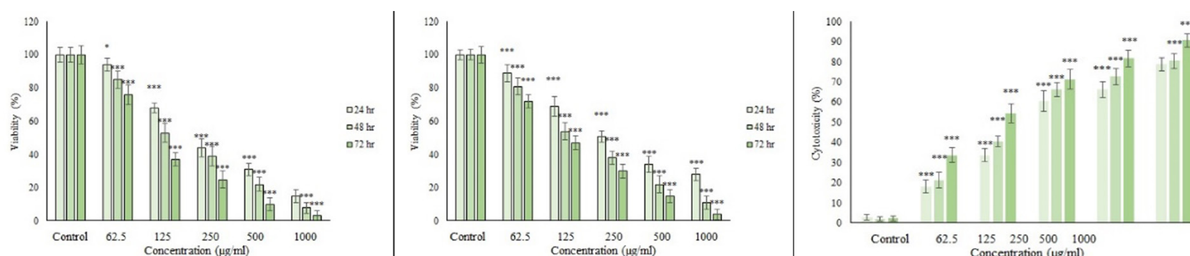
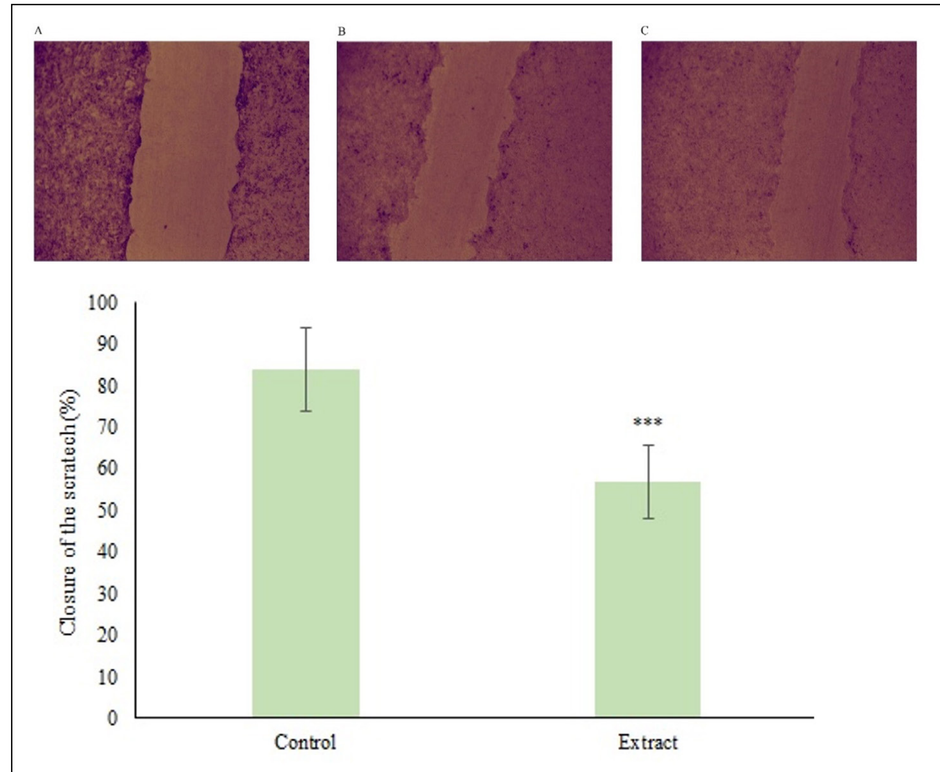


Fig. 1. The effect of dandelion extract on the viability of U87MG cells. Viability was evaluated after 24, 48, and 72 hr treatment with by (A) trypan blue, (B) MTT and (C) LDH tests. The control group received the same volume of serum-free medium (* indicates p<0.05, ** indicates p<0.01, and *** indicates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

Fig. 2. The effect of dandelion 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 $p<0.001$ compared to control).



Although there have been many recent studies on natural anti-cancer compounds[23,24], there has been little focus on their ability to inhibit cancer invasion[25].

Loss of plasma membrane integrity and release of cytoplasmic contents into the surroundings is a phenomenon that occurs in cell death. LDH is a relatively stable enzyme that is measured to assess for damage or toxicity to cells in cell culture media. Data from LDH activity assay shows that dandelion-induced cell death was associated with loss of cell membrane integrity.

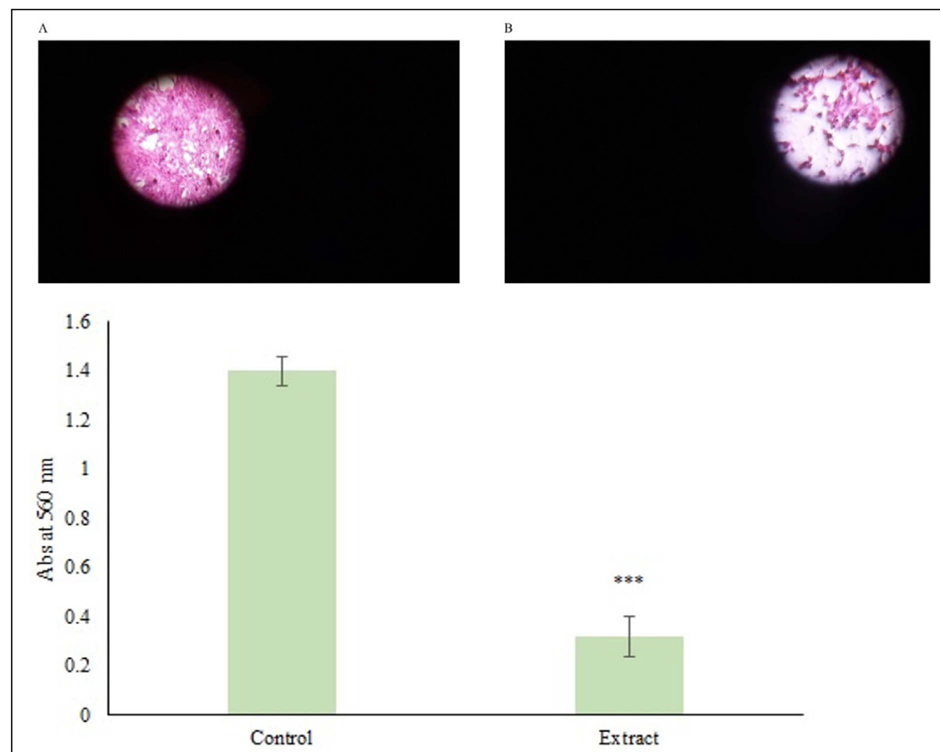
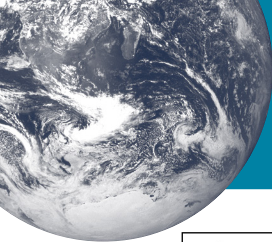


Fig. 3. The effect of dandelion extract on the invasion ability of U87MG cells after 24 hr was measured by invasion test. (A) control group, (B) in the presence of extract, and (C) column diagram of the average absorption at 560 nm (*** indicates $p<0.001$ compared to control).



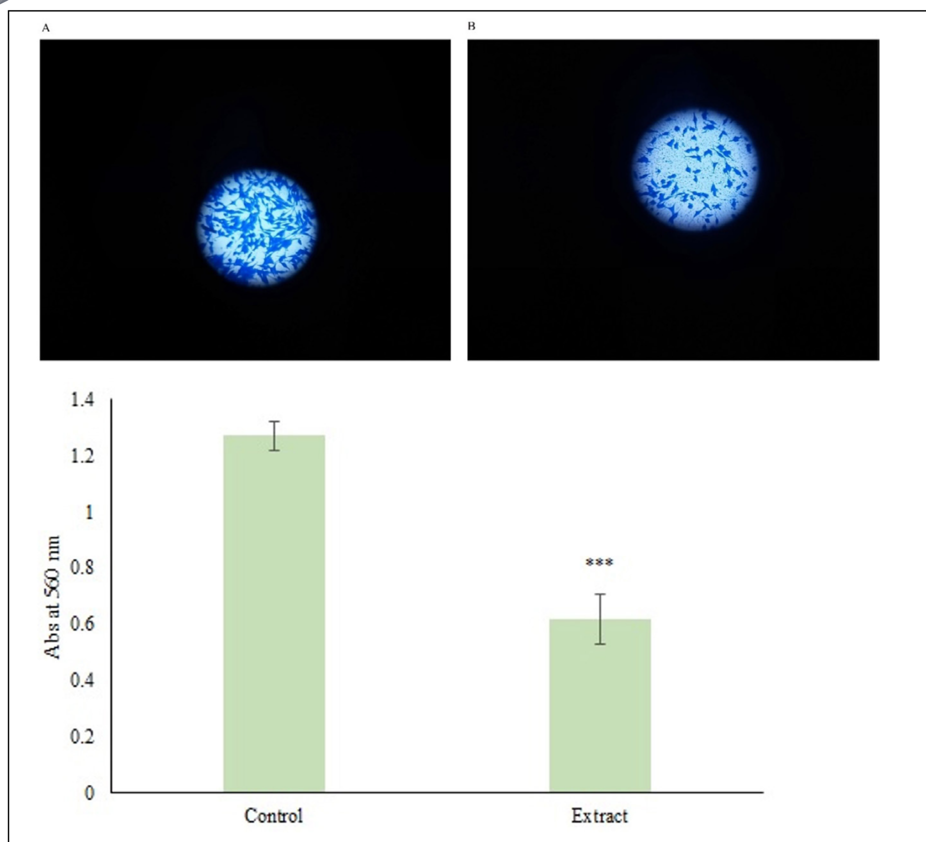


Fig. 4. The effect of dandelion extract on the adhesion ability of U87MG cells after 24 hr was measured by adhesion test. (A) control group, (B) after treatment extract, and (C) column diagram of the average absorption of violet crystals at a wavelength of 590 nm (*** indicates p<0.001 compared to control).

Considering the great history of the dandelion in herbal medicine, relatively little is known about the chemical constituents. The harvest time influenced the amounts of phytocompounds in dandelion. The most suitable period for plant collection from its natural environments is depended on its targeting medical applications. The dandelion root is a rich source of inulin: about 2% in the spring, increasing to about 40% in fall. As in the roots, the dandelion leaves contain sesquiterpenes as well as p-hydroxyphenylacetic acid and b-sitosterol. The most abundant phenolic compounds in the leaves and flowers are hydroxycinnamic acid derivatives, in particular caffeic acid esters such as chlorogenic, chicoric and monocaffeoyltartaric acids. Compared to the roots, the dandelion leaves are characterized by higher polyphenol contents. It also is a rich source of a variety of vitamins and minerals, including beta carotene, nonprovitamin A, carotenoids, xanthophylls, chlorophyll, vitamins C and D, many of the B-complex vitamins, choline, as well as minerals iron, silicon, magnesium, sodium, zinc, manganese, copper, and phosphorous. Sesquiterpene lactones confer a bitter taste to the plant, which is particularly notable in the Leaves but also in the roots especially when spring harvested. Saponins, flavonoids, alkaloids, phenols were very concentrated in the stem, root, and flower, with a higher concentration of flavonoids in the flower extracts. Phenols and steroids were also found in the investigated plant parts. The phytochemicals presented in dandelion, their structure and biological significance are listed in a review by Jalili et al[18].

In 1981, for the first time, it was shown that the hot water extract of dandelion possessed anti-tumor activity. After that several studies were conducted to approve the anti-cancer properties of dandelion and find the mechanism behind its effect on the adult and pediatric malignant cells[18].

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

The ECM and BM serves as a physical barrier to cancer cell invasion into the surrounding tissue. The degradation of ECM by different members of MMP family removes this physical barrier for cancer invasion. MMP-2 and MMP-9 are considered to be important for cancer invasion[26]. In cancer cells, MMP-2 and MMP-9 are controlled by their endogenous inhibitors (TIMP-1 and TIMP-2)[27]. Therefore, decreasing MMP activity or increasing TIMP activity could reduce cancer cell invasion[28]. In this study, the expression levels of MMP and TIMP was estimated using Real-time PCR. Our data showed that dandelion extract

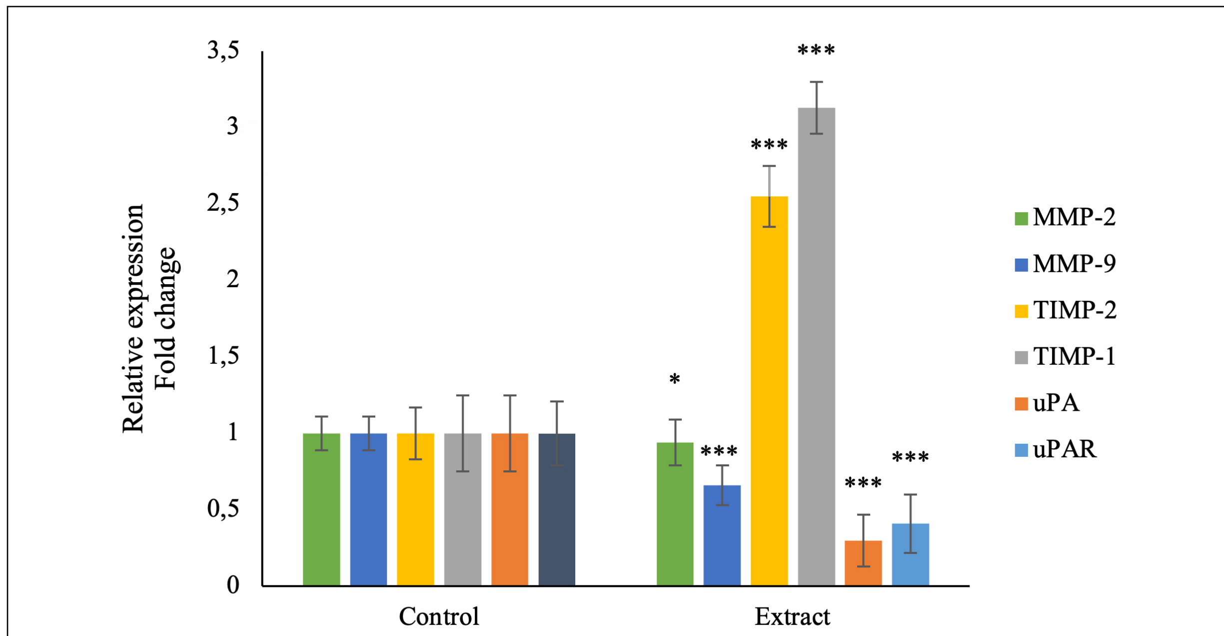


Fig. 5. The effect of dandelion extract on the expression levels of proteolytic enzymes in U87MG cells after 24 hr was measured by real-time PCR. (* indicates $p<0.05$ and *** indicates $p<0.001$ compared to control).

significantly reduced MMP-2 and MMP-9 and increased TIMP-1 and TIMP-2 expression levels.

uPA is another enzyme involved in ECM degradation. It converts plasminogen to plasmin, which directly mediates cancer cell invasion by degrading matrix proteins such as collagen IV, fibronectin, and laminin or indirectly by activating MMP-2, MMP-3, MMP-9, and uPA[29]. uPAR focuses uPA activity on the cell membrane, thus regulating cell surface-associated plasminogen proteolysis by uPA[30].

In this study the effects of dandelion extract on uPA and uPAR were tested. The results showed that extract decreased uPA and uPAR expression.

The MAPK signaling pathway is involved in MMPs regulation[31]. p38MAPK induces MMP expression and activates JNK and c-Jun pathways in human melanoma cells[32]. Also, p38MAPK

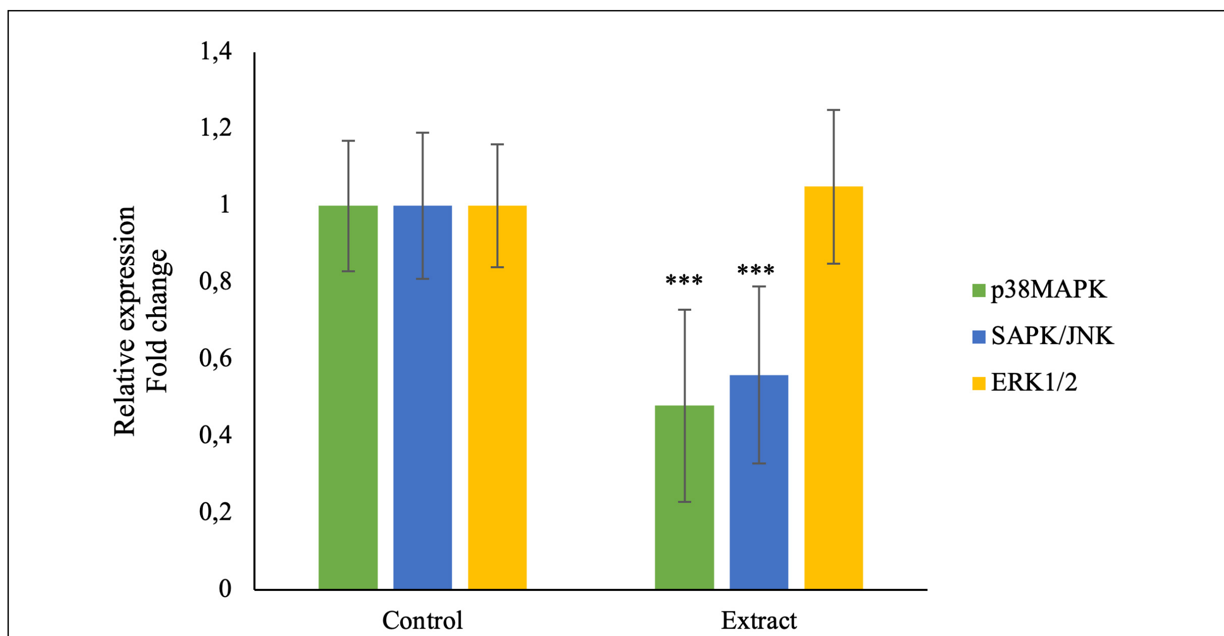


Fig. 6. The effect of dandelion extract on the expression levels of proteins involved in the p38mapk and sapk/jnk pathway in U87MG cells after 24 hr was measured by real-time PCR. (*** indicates $p<0.001$ compared to control).

could upregulate uPA expression[33]. Constitutive p38alpha MAPK activity is required for increased uPAR expression and matrix invasion by cancer cells[34]. Downstream from MAPK signaling, JNK activation helps regulate cancer cell invasion and expression of MMP-1, MMP-2, and MMP-9. So, inhibiting JNK decreases cancer cell invasion[35].

However, one of the main limitations of nutraceutical uses in clinical models is biodistribution in CNS and drug delivery systems could be useful to improve the intake in glioblastoma cells[36].

A disseminated growth pattern is one of the characteristics of GBM[37]. 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.

CONCLUSIONS

Dandelion extract had cytotoxic effects on U87MG cells which lead to a reduced cell viability, invasion, migration, and adhesion ability of the cells. These results could translate into beneficial effects of this plant.

Conflict of interest:

There is no conflict of interest.

Funding:

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