

EFFECTS OF WHOLE FRUIT EXTRACT OF *ELAEAGNUS ANGUSTIFOLIA* L. ON GLIOBLASTOMA CELL LINES

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ABSTRACT

Elaeagnus angustifolia L. (EA) is a natural plant food with therapeutic and medicinal properties for treating various diseases such as rheumatoid arthritis and osteoporosis. Since herbal agents are origins of bioactive compounds, they can be applied in complementary treatment protocols. In this study, EA whole fruit extract's cytotoxic effects were investigated on both animal and human glioblastoma cancer cell lines. The extract of whole fruit of EA was obtained by maceration in ethanol. The phytochemical compounds were identified using high-performance liquid chromatography (HPLC). The MTT and LDH assays were performed to evaluate the cytotoxicity effect of the extract and the determination of cell death. Wound-healing assay and colony formation analysis were employed for migration and proliferation evaluation. Based on HPLC analysis, the main flavonoid components of the extract were included rutin and apigenin. The results demonstrated that EA extract at the dose of 125 to 2000 µg/ml and 61.5 to 2000 µg/ml inhibited C6 and U87 cells' viability and induced significant cell cytotoxicity at both 48 and 72 h incubation times. Besides, EA extract significantly inhibited cell migration and colony formation at 250 to 1000 µg/ml concentration. Overall, the results showed that EA extract could inhibit several stages in glioblastoma carcinogenesis in vitro. Therefore, it can be suggested as an anticancer in the clinical treatment approaches of glioblastoma cancer.

Keywords: *Elaeagnus angustifolia* L., Glioblastoma, Cell line, Cytotoxicity, Wound-healing-assay, Colony-forming units' assay

INTRODUCTION

Elaeagnus angustifolia L. (EA), predominantly known as oleaster, Russian olive and, wild olive, belongs to the genus *Elaeagnus* of the *Elaeagnaceae* (*Araliaceae*) family. It commonly cultured in the western and central region of Asia (from Russia to Turkey and Iran) (Çakmakçı, Topdaş et al. 2015). Based on the phytochemical analysis, the EA fruit contains polysaccharides, amino acids, vitamins, carboxylic acids, flavonoids, sitosterols, terpenoids, coumarins, phenol, cardiac glycosides, carotenoids, saponins, and tannins (Faramarz, Dehghan et al. 2015, Torbati, Asnaashari et al. 2016). Various parts of this plant have been used traditionally for treating several forms of prevalent diseases such as rheumatoid arthritis, osteoporosis, wound, nausea, cough, asthma, fever, flatulence, jaundice, and diarrhea (Farzaei, Bahramsoltani et al. 2015).

Cancer has been recognized as the second leading cause of death worldwide after cardiac illness (Nagai and Kim 2017). The uncontrolled proliferation and extensive invasion of tumor cells are the hallmarks of cancer. These factors intensify the metastasis process and thus make treatment difficult (Fouad and Aanei 2017). Glioblastoma multiforme (GBM), as the fatal malignant type of primary brain tumor, has an invasive phenotype. These tumors usually diffusely infiltrate into the brain's multiple sections, making its surgical resection difficult. Hence, patients may suffer from prognosis and a 5-year survival rate, even against routine treatment protocols containing surgery, chemotherapy, and radiotherapy (Batash, Asna et al. 2017, Shergalis, Bankhead et al. 2018).

Traditional medicines are recognized as a valuable source of therapeutic approaches for modern drug development. Over recent decades, many types of alternative and complementary medicine have been employed to overcome cancer worldwide. Several studies established the mechanism of the antitumor effect of some important plants by regulating signaling pathways, induced apoptosis, inhibiting angiogenesis, modulating transcriptional activators, and increasing antioxidant enzymes (Desai, Qazi et al. 2008, Khan, Ali et al. 2020).

It has been shown that herbal drugs have limited toxic side effects, provide a better quality of life, prolong survival rate, and extend tumor response in cancer patients (Yin, Wei et al. 2013, Wu, Lu et al. 2015, Sheng, Zou et al. 2018). Also, the medicinal plant's use combined with routine treatment protocols could increase

efficacy and overcome the immunosuppressive tumor microenvironment (Lai, Hung et al. 2010, Lee, Bae et al. 2018).

The antitumor assessments of the *E. Angustifolia* leaf indicated inhibition features. *E. Angustifolia* fruit also showed a dose-dependent inhibition trend on hepatoma cells (Wang, Fan et al. 2013, Farzaei, Bahramsoltani et al. 2015).

Furthermore, the EA represented could induce suitable anti-neoplastic effects in some tumor models (Mehrabani Natanzi, Pasalar et al. 2012, Amiri Tehranizadeh, Baratian et al. 2016, Hamidpour, Hamidpour et al. 2017).

Some studies have considered the effect of EA or its derivate on carcinogenesis. A recent survey by Saleh et al. showed that flower parts of this plant prevent cell proliferation, migration, and colony formation, also cell cycle arrest (Saleh, Mohamed et al. 2018). A similar outcome was observed for chemopreventive effect against diethylnitrosamine (Katsamaklis, Slot et al.)-induced hepatocellular carcinoma (HCC) in rats (Amereh, Hatami et al. 2017).

Regarding glioblastoma cancer, the C6 cells are spindle-like cells that induce human-like GBM when injected into Wistar rats' brains (Giakoumettis, Kritis et al. 2018). U87 is a human primary glioblastoma cell line that is predominantly applied in brain cancer research. (Yu, Ping et al. 2008). There is no finding of the effect of whole fruit of EA (containing the fleshy part, endocarp, and kernel) in an aspect of glioblastoma cell line studies. The present study wants to assess the anti-tumoral effects of EA whole fruit extract on glioblastoma cell lines C6 and U87 for the first time. Studying the proliferation and survival ability of these tumor cell lines provide new glances into finding the complementary strategies for glioblastoma cancer therapy.

MATERIAL AND METHODS

Chemicals and Plant material

The methanol and ethanol used in this research were of HPLC grade and all chemicals and media were purchased from Sigma (Steinheim, Germany). The *Elaeagnus angustifolia* L. fruits (Fig. 1) were obtained from local market in Damghan, Semnan province of Iran. After manual cleaning, the fruits were milled and sieved to a defined particle size (0.5 mm).

The rat C6 and U87 human glioblastoma cell lines were provided by the Pasteur Institute of Iran (National Cell Bank of Iran, Tehran, Iran).



Figure 1 The photo of *Elaeagnus. angustifolia* L fruit

Extract preparation

The EA whole fruit powder (10 g) was macerated in ethanol (70% v/v) at 1:10 volume for 48 h in a shaker incubator. The solution was filtered using filter paper (Whatman no: 1), and the ethanol was evaporated in a rotary evaporator. The final residual extract was oven-dried at 40°C for 24 h, and then the dried extract was scrubbed from the surface of glass plates and used for the next analysis.

HPLC analysis

Phenolic compounds and flavonoids of the extract were determined using high-performance liquid chromatography (HPLC) apparatus (waters 2695, USA) equipped with a PDA detector (waters 996, USA). Millennium32 software was used for data acquisition and integration. The used column was a C18-Waters (15 cm×4.6 mm). Solvent A was methanol, and solvent B was distilled water in a gradient manner when the flow rate was 1 ml min⁻¹. The temperature was adjusted at 25°C and the wavelength at 195-400 nm. The quantification was performed using the linear calibration curves of standard compounds.

Cell Culture

The rat C6 and U87 human glioblastoma cell lines were cultured in DMEM-F12 culture media containing heat-treated fetal bovine serum, penicillin, and streptomycin by a monolayer manner (Gibco, Grand Island, USA). Then, they were placed in an incubator with a humidified atmosphere of 95% air and 5% CO₂ (37°C).

MTT Assay

Cell viability was measured via MTT assay kit (Sigma-Aldrich). Briefly, the glioblastoma cell lines were adjusted at 10⁴ cells/well and cultured (24 h) in 96-well plates. The cells were transfer to prepared mediums containing determined EA extract concentrations and then incubated for 24, 48, and 72 h. In the next step, the MTT solution was poured into wells, and incubated (37°C for 4 h). The formazan crystals formed were dissolved in 100 µl acid/alcohol (0.04NHCl in isopropanol) by mixing. A microplate reader was used to determine the optical density of samples (570 nm)(Sun, Liu et al. 2018). The evaluation of cell viability was performed in comparison to the non-treated cell.

Cytotoxicity assay

A cytotoxicity detection kit containing lactate dehydrogenase (LDH) was used to analyze cytotoxicity activity (Roche Applied Science, Germany). First, 10⁴ cells were cultured in wells (24 h) and then inoculated to a prepared medium (containing EA extract concentrations). For low and high control, the cells were cultured in a medium. After 48 and 72 h, 100 µL of Triton X-100 solution was poured into high control wells and mixed thoroughly to destroy the cell membranes. Afterward, the plates were centrifuged (10 min at 250 g), and the 100 µL of supernatants were relocated to another flat-bottom plate. In the next step, 100 µL of the reagent of LDH kit placed into each well with incubation at 21°C (Ghanghareh and Zare 2020). The absorbance was monitored in ELISA reader by 490 nm wavelength. The cytotoxicity was calculated as:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

Wound-healing assay

The wound-healing was evaluated to indicate cell migration (Bobadilla, Arévalo et al. 2019). The cells were cultured in 24-well plates in a culture medium (24 h). The wounds were made in monolayers of the cells and the detached cells were separated after washing with PBS. Cells were treated with a determined concentration of EA extract and incubated for 24 h (CO₂ incubator)(ben Sghaier, Pagano et al. 2016). After replacing the medium with PBS, the wound gap photos were taken using a digital camera on a microscope fitted state. The wound width

was determined in six random zones and processed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Wound closure distance was quantified according to:

$$\text{Wound Closure distance} = [A (0h) - A (24h)]$$

Colony formation assay

To assay the colony formation (Helderman, Löke et al. 2020, Jabeen, Sharma et al. 2020) the C6 and U87 cells were plated in 6-well plates (2×10³) and exposed to 250, 500, and 1000 µg/ml of EA extract as treatment groups and cell lines without treatment were considered as the control group for 72 h. The medium was refreshed by 3 days up to grow new colonies. At the cell numbers greater than 30, then washed by PBS and placed in paraformaldehyde. Before washing and drying, the cells were stained with crystal violet. The counting of cells was done on colonies containing more than 50 cells using a microscope. The colony formation ratio was determined according to the following equation:

$$\text{Plate colony formation inhibitory ratio} = \frac{\text{number of colonies treated with EA}}{\text{number of cells inoculated}} \times 100$$

Statistical Analysis

Statistical analyses were done by SPSS 16.0 and GraphPad Prism version 5.0 software. Comparisons between treatment groups were performed using variance test analysis (ANOVA) followed by post hoc Student's t-tests, and the results were stated as means ± SEM. Differences were considered significant when P ≤ 0.05.

RESULTS

Determination of phytochemical compounds of the extract

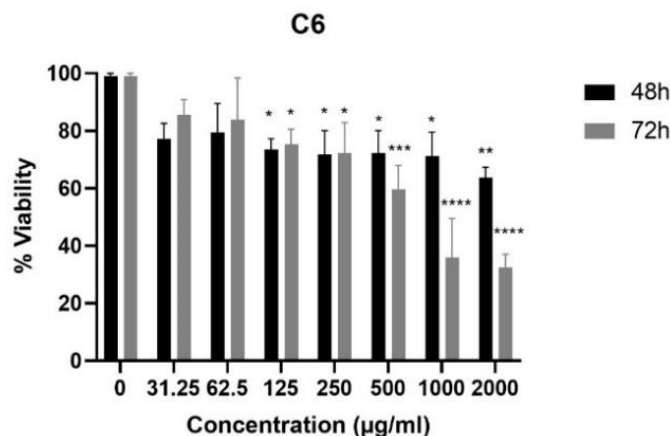
HPLC analyzed the phytochemical compounds of EA whole fruit extract are summarized in Table 1. The major compounds of EA extract were rutin and apigenin. These compounds are belonging to the flavonoid class act as core compounds in the pharmacological activities of EA extract. Also, the rutin and apigenin are such plant pigments that are found in certain fruits and vegetables.

Table 1 Major phytochemical compounds of EA extract analyzed by HPLC

Compounds	Retention Time (min)	Detection wavelength (nm)	Concentration (µg/5 mg extract)
Rutin	27	256.8	12.29
apigenin	38.6	276.4	5.12

Cell viability

The effect of EA extract on cell viability in glioblastoma cancer C6, and U87 cells was quantitatively evaluated using the MTT assay. Treatment with EA extract at the dose of 125 to 2000 µg/ml and 61.5 to 2000 µg/ml inhibited C6 and U87 cells' viability, respectively (Fig. 2). The IC₅₀ values of EA for C6 and U87 cells were 541.6 and 247 µg/ml, respectively.



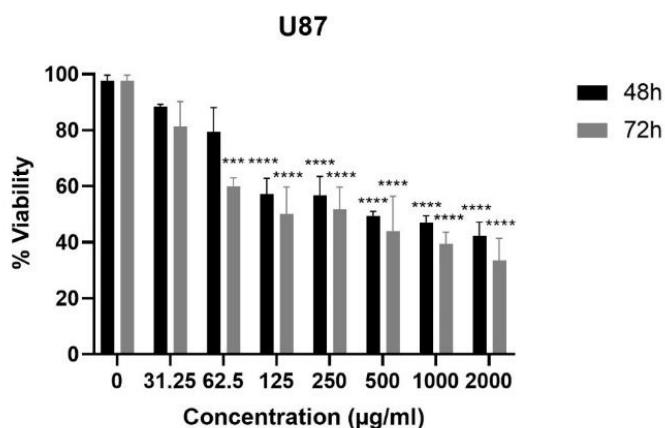


Figure 2 Effect of EA extract on C6 and U87 cells viability in 48 and 72 h expressed as mean ± SD of three replicates (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.00001 represent significant differences between treatment groups and control group (zero concentration of extract))

Cytotoxic Effect

The release of LDH is a marker for cell death. We, therefore, assessed whether treatment of cells with EA extract resulted in LDH release. The effects of a range of EA extract concentrations (0, 31.25, 62.5, 125, 250, 500, and 1000 µg/ml) on cell death in glioblastoma lines are shown in Fig. 3. The results revealed that a dose of 1000 at 48 h culture and 62.5, 250, 500, and 1000 µg/ml at 72 h culture resulted in a significant induction of death of the C6 cell line. In the U87 cell line, the concentration of 31.25 to 1000 µg/ml induced remarkable cell cytotoxicity at both 48 and 72 h incubation times, although this response is not dose-dependent.

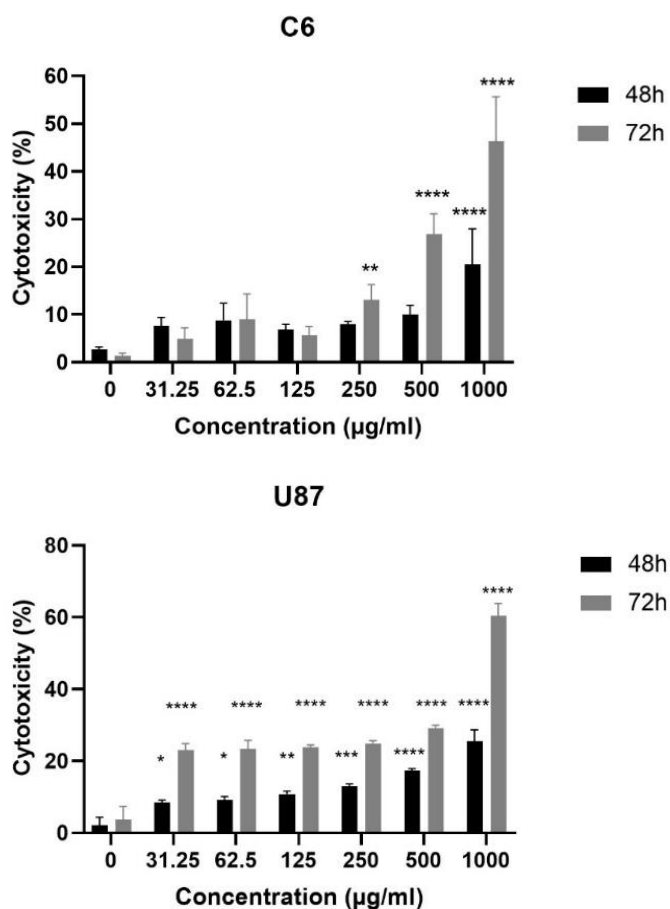


Figure 3 Cytotoxic effect of EA extract on C6 and U87 cells in 48 and 72 h expressed as mean ± SD of three replicates (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.00001 represent significant differences between treatment groups and control group (zero concentration of extract))

Cell migration

The effect of different doses (250, 500, and 1000 µg/ml) of EA extract on cell migration is quantified using the average distance of scratch width between its edges.

Fig. 4A showed after making a scratch and comparing the migrating cells after 24 h in the no-treatment group, and it was observed that the wounding space between C6 cell layers was almost complete. The wound closure distance is shown in the column bar in Fig. 4B. An observed dose-dependent effect in wound closure distance was observed in the presence of EA extract relative to untreated control cells by 24 hours.

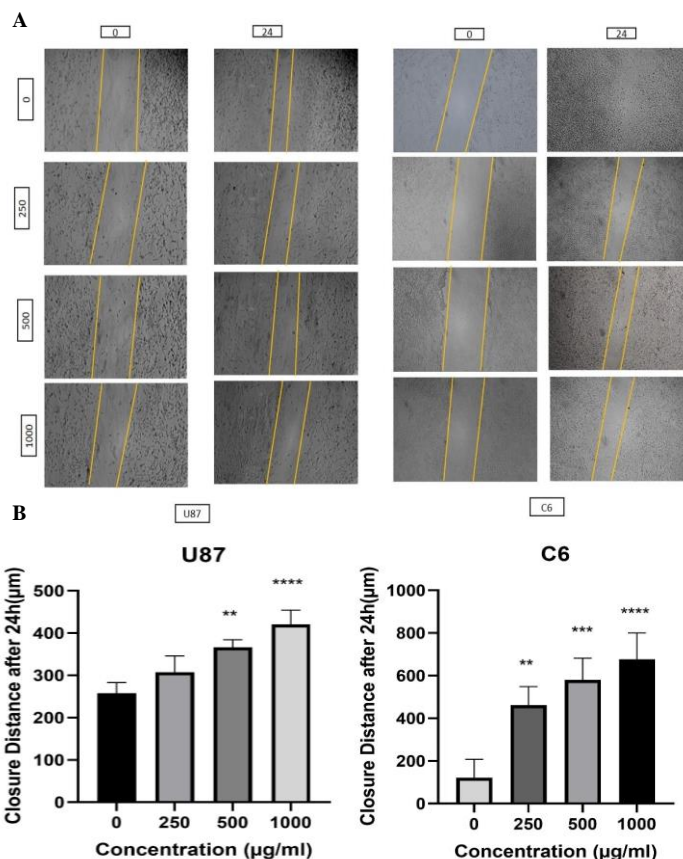


Figure 4 (A) The changes in cell migration in C6 and U87 cells treated with EA extract for 24 h; (B) The wound width of C6 and U87 cells treated with EA extract. The data are presented as mean ± SD of three replicates (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.00001 represent significant differences between treatment groups and control group (zero concentration of extract))

Colony formation

The effects of EA extract on tumor cell clonogenicity in C6, and U87 cells are illustrated in Fig. 5. As can be seen, the extract not only could notably inhibit the colony formation but also cause a significant decrease in colony formation number ratio in both cell lines. The culture of the C6 cell line with 1000, 500 and 250 µg/ml resulted in diminished colony size significantly. In the U87 cell line, the concentration of 1000 and 500 µg/ml induced a remarkable colony size decreasing. These results reflect the ability of EA extract to prevent the tumor cell from dividing.

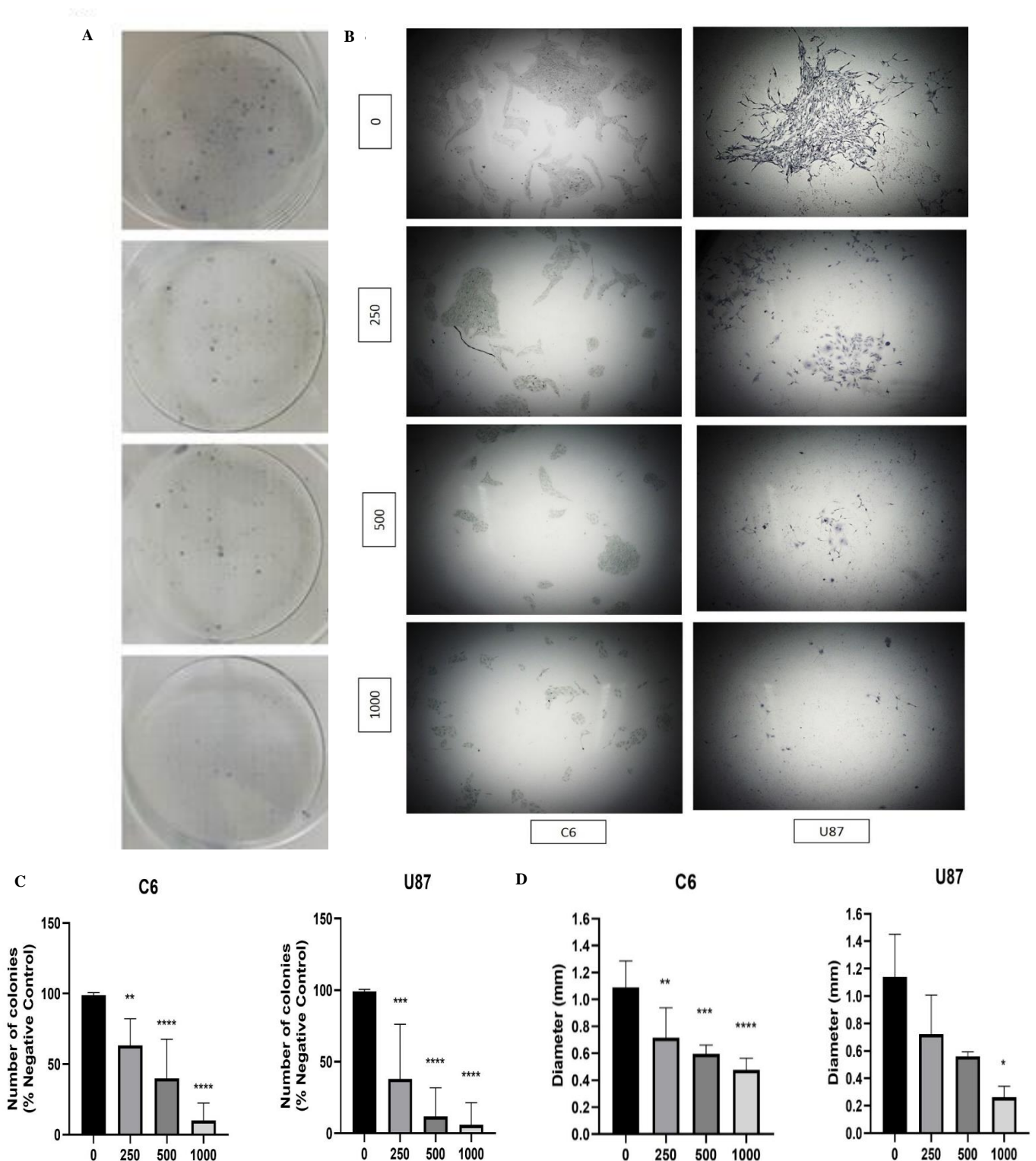


Figure 5 (A) The tumor cell-induced colony formation; microscopic view of C6 cells culture; (B) microscopic view of cells C6 and U87; (C) Colony formation number; and (D) colony size in C6 and U87 cell line treated with EA extracts expressed as mean ± SD of three replicates (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.00001 represent significant differences between treatment groups and control group (zero concentration of extract))

DISCUSSION

The phytochemical component of EA extract was identified and the impact of the extract was evaluated on viability, cytotoxicity, migration, and colony formation of determined glioblastoma cell lines. The main quantified flavonoid compounds in EA extract were including rutin and apigenin which is according to the previously reported flavonoid group for EA extract (Hamidpour, Hamidpour et al. 2017, Ozen, Yenigun et al. 2017). However, a broad different type of flavonoids and flavone glycosides are detected in other EA parts (Si, Qin et al. 2011).

It is well documented that flavonoids interfere with several signal transduction pathways in the cancer cells and thus restrict proliferation, angiogenesis, and metastasis and increase apoptosis (García-Lafuente, Guillaón et al. 2009, Ravishankar, Rajora et al. 2013). Furthermore, apigenin, as a phytoestrogen

aglycone that alone or in combination with chemotherapy led to apoptosis, inhibits invasion, and induces cell cycle arrest of cancer cells (Chan, Chou et al. 2012, Shi, Shiao et al. 2015, Meng, Zhu et al. 2017). Rutin, a Quercetin Glycoside, improves breast cancer cells' chemosensitivity, decreases the superoxide generation in colon cancer cells, and inhibits adhesion and migration of lung cancer (ben Sghaier, Pagano et al. 2016, Iriti, Kubina et al. 2017).

Considering the effect of EA fruit extract on two types of glioblastoma cell lines, the EA extract can decrease cell survival and increase tumor cell death in a dose-dependently manner. Our study also shows that the extract significantly inhibits the migration and proliferation of C6 and U87 glioblastoma cancer cells. Rat C6 glioblastoma cell line was initially induced in Wistar rats by N, N'-nitroso-methyl urea. This cell is very close to glioblastoma multiforme (GBM) morphologically when injected into rats' brains. Another cell line was U87, which is a cell line

derived from human malignant glioma (Benda, Lightbody et al. 1968, Jaworski, Sawosz et al. 2013).

Also, the present investigation demonstrated the proper wound healing effect of the extract of EA on mentioned glioblastoma cell lines. This extract had a good result in the migration rate of experimented cells. It seems this bioactive extract may influence epithelial and collagen around the wound. This finding is compatible with the reported wound healing impact of aqueous EA fruit extract in rats (Natanzi, Pasalar et al. 2012).

Our results are in agreement with other findings that have pointed to the anticancer properties of EA fruit. Amerreh et al. (2017) investigated EA fruit's chemopreventive effect in diethylnitrosamine-induced hepatocellular carcinoma in rats. They found that EA fruit possesses strong chemopreventive activity against primary liver cancer, which may be related to the antioxidant, anti-inflammation, and antimutagenic effects of the EA fruit (Amerreh, Hatami et al. 2017). In another study, Ya et al. (2014) concluded that different fractions of ethyl acetate of EA L. extract, especially in high concentration and polarity levels, increase antioxidant and scavenging activities and also enhances inhibitory effects on the proliferation of HeLa cells in vitro (Ya, Shang-Zhen et al. 2014). Li et al (2009) investigated the effect of the methanol extract of *Elaeagnus glabra* (Thunb), one of the species of family *Elaeagnaceae*, on tumor cell invasion. According to their results, *E. glabra* extract suppresses the expression of molecules responsible for the tumor cell invasion and thus reduces cancer cells' metastasis (Li, Baek et al. 2009). Saleh et al (2018) reported that EA extract prevents cell development, colony generation and reduces oral cancer advancement (Saleh, Mohamed et al. 2018).

Furthermore, Wang et al. showed that extracts of edible parts from *Elaeagnus Angustifolia* L. significantly inhibit the proliferation of the human hepatoma cell HepG2. Moreover, the extracts increased the spleen and thymus indices and promoted tumor-bearing mice's immune organ (Wang, Fan et al. 2013). Antitumor activities of EA might be due to bioactive components like flavonoids, polyphenols, triterpenoid, lignanoid, benzenoid, pro-anthocyanosides, and polysaccharides. These compounds can regulate mutant p53 genes, suppress Ras protein expression, block the cell surface tyrosine kinases, inhibit proliferative agents, and mediate inflammatory factors (Wang, Fan et al. 2013, Amiri Tehranizadeh, Baratian et al. 2016, Hamidpour, Hamidpour et al. 2017). However, our results are different from those of Abu-Dahab (2007), who investigated 67 plants for the antiproliferative activity against cancer cells. In their study, the cells' proliferation rate was not significantly different from that of the untreated cells (Abu-Dahab and Afifi 2007). So, it can be argued that the anticancer activity of EA substantially depends on the property of extracts, and the more potent inhibitory effects occur in, the higher concentrations of extracts. Thus, various concentrations can affect the outcome of studies.

Overall, our findings confirmed the antiproliferative and cytotoxic potency of EA fruit extract on C6 and U87 (rat and human) cancer cells. However, it is necessary to discover the main mechanism underlying these results, particularly the related molecular pathways.

Routine cancer treatment protocols, including surgery, chemotherapy, and radiation therapy, cannot reduce mortality and are accompanied by side effects as disadvantages. Therefore, several attempts have been focused on finding impressive therapeutic agents to reduce the unfavorable adverse effects. In this regard, natural compounds and food supplements with potential anticancer properties could apply as safe alternatives.

CONCLUSION

The efficiency assessment of the extract of *E. angustifolia* fruit revealed a significant effect on viability, cytotoxicity, migration, and colony formation of C6 and U87 glioblastoma cancer cell lines. Based on the phytochemical analysis, the major bioactive compounds of *E. angustifolia* extract was flavonoids including rutin and apigenin. The viability of both C6 and U87 were notably decreased by increasing the *E. angustifolia* extract concentration. Also, a realistic cytotoxicity effect was observed on cell lines after the determined incubation time. The *E. angustifolia* extract proved wound healing activity on evaluated glioblastoma cells. The colony proliferation rate and colony size were inhibited in treating the cancer cells by *E. angustifolia* extract. This study showed that *E. angustifolia* has a good potential for alleviating and inhibiting glioblastoma cancer. Considering the beneficial therapeutic properties of *E. angustifolia* it could be proposed as a safe medicinal plant to manage glioblastoma cancer. Further clinical trials are recommended to discover probable effects in various cancer.

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Ethics Approval: Iran National Committee approved this study's protocols for Ethics in Biomedical Research (ID: IR.SEMUMS.REC.1398.012).

Conflict of interest: The authors declare no potential conflicts of interest concerning the research of this article.

Abbreviations

EA	<i>Elaeagnus. Angustifolia</i> L.
GBM	Glioblastoma multiforme
HCC	hepatocellular carcinoma
HPLC	high-performance liquid chromatography
HUVEC	human umbilical endothelial cell
IL-10	Interleukin 10
LDH	lactate dehydrogenase
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
TNF- α	Tumour Necrosis Factor alpha
PBS	phosphate buffer saline

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