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Cytotoxic, Genotoxic and Anti-Metastatic Activity of *Alkanna Orientalis* (L.) Extract on Glioblastoma Multiforme Brain Cancer

Özge Çağlar Yildirim[1*], Sena Öner[1], Cihat Aksakal[1], Ceren Karagöz[1], Cemal Metehan Akkuş[1], Hasan Türkez[2]

[*1]*Department of Molecular Biology and Genetic, Faculty of Science, Erzurum Technical University, Erzurum, Turkey*

[2]*Department of Medical Biology, Faculty of Medicine, Atatürk University, Erzurum, Turkey*

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Abstract

This study aimed to evaluate, for the first time, the anti-proliferative and anti-metastatic effects of the aqueous extract of Alkanna orientalis (L.) on U87-MG glioblastoma cells. The cytotoxicity of this extract on normal human dermal fibroblasts and its anti-cancer activity on glioblastoma cells were assessed by using MTT assay. The potential genotoxic effects of the Alkanna orientalis (L.) extract on cell nuclei were further examined by Hoechst 33258 fluorescence staining and analyzed microscopicall. In addition, the inhibitory effect of the extract at a selected dose on the metastatic capacity of glioblastoma cells was investigated using in vitro scratch assay. Even the lowest tested dose exhibited notable anti-proliferative activity against U87-MG glioblastoma cells, while no significant levels of cellular toxicity were observed at low and moderate concentrations. However, the highest concentration resulted in a partial decrease in viability, particularly in HDF cells. Hoechst 33258 staining revealed no evidence of genotoxic nuclear alterations in HDF cells treated with 400 µg/mL extract compared to untreated controls. Moreover, the in vitro scratch assay revealed a pronounced inhibition of U87-MG cell migration 24 hours after treatment with this plant extract, indicating a potential anti-metastatic effect. These findings suggest that the aqueous extract of Alkanna orientalis (L.) may represent a promising alternative therapeutic candidate for the treatment of glioblastoma.

Keywords: glioblastoma, *Alkanna orientalis*, aqueous plant extract, anti-proliferative, anti-metastatic

[*]**Correspondence**: Özge Çağlar Yildirim
Affiliation, Ömer Nasuhi Bilmen Mahallesi .Farabi Bulvarı No:12
Yakutiye/Erzurum
E-mail: ozge.caglar@erzurum.edu.tr

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Introduction

Cancer remains the second leading cause of death worldwide, with projections indicating that the global cancer-related mortality rate will rise to approximately 11.5 million by 2030(1). Glioblastoma (GB) is one of the most common and aggressive cerebral neoplasms, associated with a dismal patient prognosis. The incidence of glioblastoma multiforme (GBM), the most prevalent form of primary brain tumor, is estimated at 1 in 20,000 individuals, with a maximum reported survival of only 14.6 months (2). The poor clinical outcomes of GBM are largely attributed to its pronounced genetic heterogeneity, extensive vascularization, and highly invasive growth pattern (3). Although current treatment modalities, including surgical resection, radiotherapy, and chemotherapy, have improved disease management to some extent, their severe side effects and inherent limitations underscore the urgent need for novel therapeutic strategies. Consequently, scientists remain actively committed to identifying more effective alternatives for cancer treatment (4).

Medicinal plants have constituted an integral component of healthcare systems throughout human history, providing a wide array of therapeutic applications across different cultures and time periods. Indeed, it has been reported that there are currently more than 800 medicinal products of herbal origin that have been recognized and employed in the treatment of various diseases (5). In recent decades, there has been a proliferation of research focusing on the potential of certain plant species to serve as novel therapeutic agents, particularly in the management of cancer. It is noteworthy that the World Health Organization reports that traditional medicines, which include a significant proportion of plant-based remedies, account for approximately 80% of healthcare practices worldwide (6). This highlights their enduring significance and widespread use. Preliminary evidence from certain studies indicates a potential correlation between the regular and long-term consumption of specific medicinal plants and a reduction in cancer incidence. However, the existing data are limited and insufficient to draw definitive conclusions. Consequently, it is imperative to conduct further rigorous investigations to identify and characterize novel plant-derived candidates that may contribute to the development of safe, effective, and targeted anticancer therapies (7).

Species of the Alkanna are among the medicinal plants that have been recognized since ancient times for their diverse medicinal, pharmaceutical, and other beneficial properties. These perennial herbaceous plants, comprising more than 50 identified species, represent an important member of the Boraginaceae family. Alkanna species are widely distributed across regions such as Syria, Lebanon, northern Iran, and Turkey (8). Notably, these plants are typically found at elevations around 2.400 meters above sea level, particularly on rocky or volcanic slopes (9). Among the various parts of this plant, the roots have been the most extensively studied due to their distinctive colored pigments, which have been shown through detailed analyses to contain bioactive compounds such as alkannins and shikonins. Traditionally, Alkanna roots have been employed in the treatment of various conditions, including skin injuries, stomach ulcers, and in the formulation of cosmetic products. In contrast, research investigating the potential medicinal applications of Alkanna leaf extracts remains remarkably limited. Nevertheless, a few studies have reported that the leaves exhibit inhibitory effects against certain infectious human pathogens, suggesting that this part of the plant may also harbor biologically active constituents worthy of further exploration(10).

Alkanna orientalis (L.) Boiss. var. orientalis is a perennial herbaceous plant characterized by its yellow flowers and known for producing commercially valuable alkannin and shikonins derivatives in its roots. Indeed, previous phytochemical studies have confirmed the presence of these bioactive compounds in the roots of A. orientalis(11). However, to date, no studies have systematically evaluated the potential medicinal and pharmacological activities of extracts derived from the leaves of this species. In light of this gap in the literature, the present study aimed, for the first time, to investigate the in vitro anti-proliferative and anti-metastatic effects of A. orientalis (L.) leaf extracts against brain cancer cells.

Materials and Methods

Preparation of Alkanna orientalis (L.) plant extracts: The plant samples were collected during the spring season from the Erzurum-Aşkale region. Following collection, the plants underwent multiple washing cycles with distilled water (dH_2O) to remove impurities, after which they were thoroughly dried using ambient air. Subsequently, 100 grams of the dried plant material were ground into a fine powder. This powdered sample was then subjected to hot extraction with distilled water at 60°C for 3 hours. The resulting extract was filtered through Whatman filter paper to obtain a clear solution for further analysis (12).

Preparation of Human Dermal Fibroblast (HDF) and Glioblastoma (U87-MG) Cell cultures: The HDF (ATCC® PCS-201-040) and U87-MG (ATCC® HTB-14) cell lines were obtained from the American Type Culture Collection (ATCC) for use in previous studies. Cells were cultured in T25 cm^2 flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. Cultivation was carried out in a humidified incubator at 37°C with 5% CO_2 until reaching 70–80% confluency. The culture medium was replaced every three days by removing the old medium and adding fresh medium.

Toxicity of Alkanna orientalis (L.) extract on HDF cell culture: The cytotoxicity of the plant extracts was assessed using MTT assay on healthy HDFa cultures. Initially, the old culture medium was removed, and the cells were washed twice with fresh phosphate-buffered saline (PBS). Subsequently, 3 mL of Trypsin-EDTA solution was added to well palate, and the cultures were incubated at 37°C with 5% CO_2 for 5 minutes to detach the cells. Following detachment, 6 mL of fresh DMEM medium was added to neutralize the trypsin. The cell suspension was transferred to a tube and a centrifugation process was carried out at 5000 rpm for 5 minutes. The sample was then resuspended in fresh medium for cell counting. Cells were diluted 1:1 with trypan blue and viable cells were counted using a Thoma slide(13). Subsequently, the cells were seeded into 48-well plates at a density of 5×10^4 cells per well and incubated for 24 hours prior to treatment.

The A. orientalis (L.) extract stock solution (g/mL) was prepared and subsequently diluted to obtain a range of concentrations (3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, and 400 µg/mL). Each concentration was applied to the cells in triplicate, and the plates were incubated for 24 hours. Two control groups were included in the experimental design: (i) a negative control consisting of untreated HDF cells, and (ii) a positive control consisting of HDF cells treated with Triton X-100. All experiments in this study were conducted in triplicate. At the end of the incubation period, the culture medium was aspirated from the wells, and the cells were washed several times with PBS. Subsequently, 200 µL of fresh medium was added to each well. Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly,

MTT reagent was added to the medium at a final concentration of 10%, and the plate was incubated at 37 °C for 3 hours to allow formazan crystal formation. Subsequent to the conclusion of the incubation period, dimethyl sulfoxide (DMSO) was added into each well to dissolve the formed formazan crystals. Absorbance was measured at 570 nm using a microplate reader.

Anti-proliferative effect of Alkanna orientalis (L.) extract: The anti-proliferative effect of Alkanna orientalis (L.) extract on glioblastoma cells was evaluated using the MTT assay. For this purpose, U87-MG cells were seeded at a density of 2×10^4 cells per well in 48-well plates, with three replicates per concentration. The extract was applied to the cultures at a range of concentrations (3.12–400 µg/mL). After 24 hours of incubation, the plates were removed from the incubator, and MTT reagent was added to each well. The plates were then incubated in the dark at 37 °C for an additional 3 hours to allow formazan crystal formation. Subsequently, dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader to quantify cell viability.

Evaluation of genotoxicity: In this study, the nuclear anomalies were evaluated using Hoechst 33258 fluorescence staining. Briefly, HDF and U87-MG cells were seeded at a density of 2×10^4 cells per well in 48-well culture plates. The cells were then treated with the predetermined dose of the plant extract and incubated for 24 hours. Untreated HDF and U87-MG cells served as the control group. At the end of the incubation period, the cells were gently washed several times with PBS and subsequently fixed on the plate surface by using 4% glutaraldehyde. Following fixation, the cells were washed again with PBS and stained with 1 µM Hoechst 33258 fluorescent dye for 5 minutes in the dark. The nuclear morphology was examined under a fluorescence microscope at 20× magnification, and representative images were recorded for further analysis (14).

Assessment of anti-metastatic activity: The anti-metastatic activity of the extract was assessed in U87-MG cells using the in vitro scratch assay(15). Additionally, the wound healing potential of the extract was evaluated in HDF cells using the same assay. For this purpose, 5×10^5 cells were seeded in triplicate into 24-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO_2 until reaching full confluency. A linear scratch was then introduced across the cell monolayer using the tip of a sterile 200 µL pipette. Detached cells were gently removed by washing with PBS, and the selected dose of the extract was applied to each well. The closure of the scratch area was monitored over a 24-hour period, and anti-metastatic activity was quantified by measuring the remaining wound width relative to the initial scratch.

Ethical Approval Statement: This study did not involve any human participants, animal experiments, or the use of personal data, and therefore did not require ethical approval.

Statistical analysis: All data are expressed as the mean ± standard deviation (SD) of at least three independent experiments performed in triplicate. Statistical significance among groups was determined using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $p \leq 0.05$.

Result

Toxicity of Alkanna orientalis (L.) extract: The cytotoxicity of A. orientalis (L.) extract was evaluated in HDF cells using the MTT assay. The extract did not induce significant toxicity in HDF cells across the range of tested concentrations. After 24 hours of treatment with the lowest concentration of the extract, HDF cell viability was maintained at 87.6%. In contrast,

treatment with the highest tested concentration (400 µg/mL) reduced cell viability to 45.1% compared to the untreated control group (Figure 1, $p<0.05$).

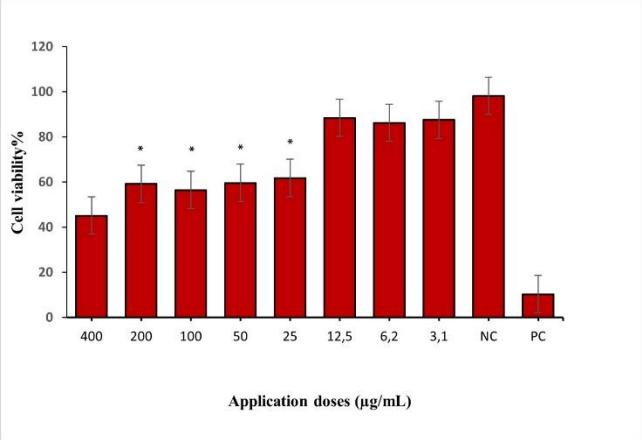


Figure 1. Cytotoxicity of A. orientalis (L.) extract on HDF cell culture by using MTT analysis for 24 h. * Indicates a statistically significant difference ($p<0.05$)

Antiproliferative effect of Alkanna orientalis (L.) extract: The antiproliferative effect of A. orientalis (L.) extract was assessed in U87-MG glioblastoma cell cultures. The extract significantly inhibited cancer cell proliferation in a dose-dependent manner compared to the control group. As the concentration of the extract decreased, the cell viability correspondingly increased. After 24 hours of treatment, the cell viability was 52% at the lowest tested concentration, whereas it decreased to 35% at the highest concentration (Figure 2, $p<0.05$).

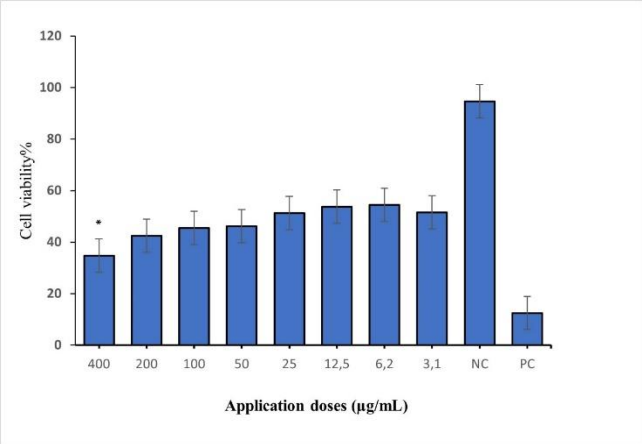


Figure 2. Anti-proliferative effect of A. orientalis (L.) extract on U87-MG cell culture by using MTT analysis for 24 h. * Indicates a statistically significant difference ($p<0.05$)

Evaluation of genotoxicity: This study was designed based on the findings of prior toxicity and anticancer assays, which identified 400 µg/mL as the highest dose for further evaluation. The potential of the plant extract at 400 µg/mL to induce nuclear abnormalities was assessed in healthy and glioblastoma cells using Hoechst 33258 fluorescence staining. Nuclear morphological alterations observed in stained cells following treatment were classified into three distinct categories: micronuclei (MN), lobulated nuclei (LB), and notched nuclei (NC). The mean nuclear abnormality index (MNi) was calculated for each group as the proportion of abnormal nuclei among a total of 1000 cells, and the results are summarized in Table 1. Compared to the untreated control groups, A. orientalis (L.) extract did not induce significant nuclear abnormalities in HDF cultures, even at the highest dose tested. In contrast, a marked increase in nuclear abnormalities was observed in U87-MG glioblastoma cells. The mean nuclear abnormality index was determined to be 0.004 ± 0.002 for HDF cultures and 0.011 ± 0.005 for U87-MG cells.

Evaluation of anti-metastatic activity: The migration of cancer cells represents the initial stage of the metastatic process and is therefore regarded as one of the leading causes of mortality among cancer patients. In this study, the effect of A. orientalis (L.) extract on the migratory capacity of cancer cells was evaluated using an in vitro scratch assay. Treatment with 400 µg/mL of the extract for 24 hours significantly reduced the migration of U87-MG glioblastoma cells compared to the untreated control group (Figure 3).

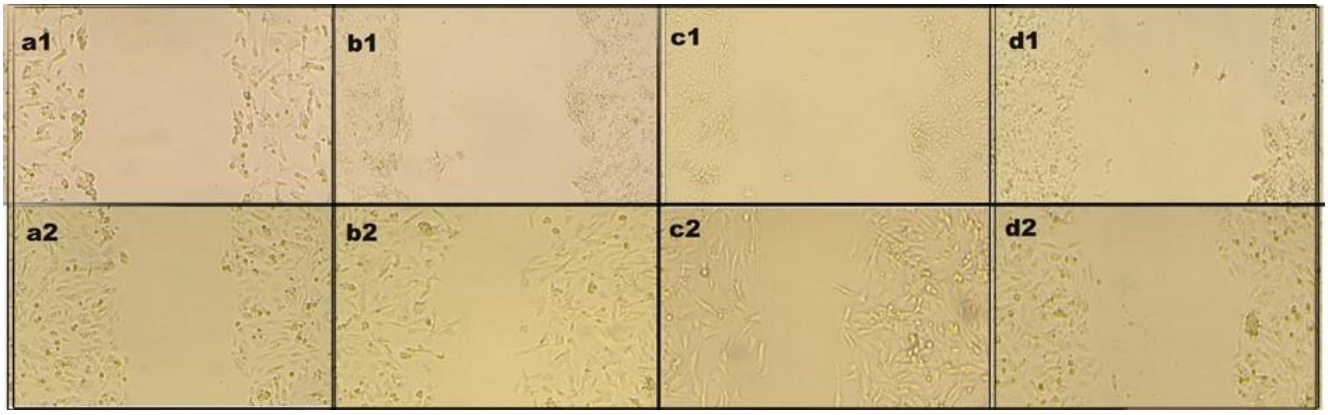


Figure 3. Effect of plant extract on HDF and U87-MG cell culture at 20x magnification in vitro scratch assay. a1. Untreatment HDF at 0 h, a2. Untreatment HDF at 24h, b1. Treatment HDF at 0 h, b2. Treatment HDF at 24 h, c1. Untratment U87-MG at 0 h, c2. Untreatment U87-MG at 24 h, d1. Treatment U87-MG at 0 h, d2. Treatment U87-MG at 24 h.

Treatment with 400 µg/mL of the extract for 24 hours significantly reduced the migration of U87-MG glioblastoma cells by 12.57%, compared to 25.55% in the untreated control group. However, the same treatment enhanced the migration of HDF cells by 58.87%, compared to an increase of 23.73% in the untreated HDF control group. This indicates that the extract has a dual effect on different cell types: anti-migratory activity in cancer cells and pro-migratory activity in normal fibroblasts.

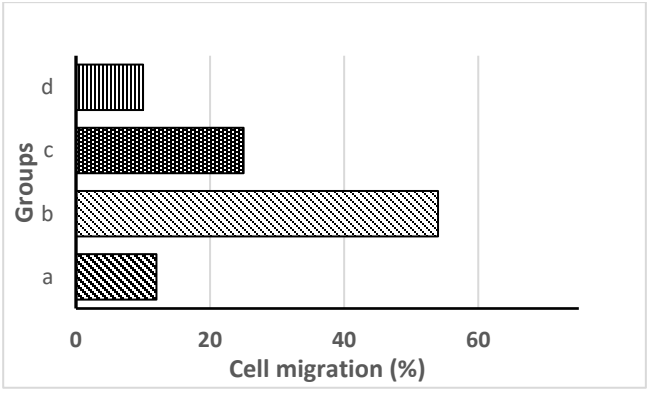


Figure 4. Cell migration rates at the end of a 24-hour period in all groups. a. Untreatment HDF, b. Treatment HDF, c. Untratment U87-MG, d. Treatment U87-MG.

Discussion

Cancer remains a major public health challenge worldwide. Various approaches, including chemotherapy, radiotherapy, and surgical intervention, are commonly employed to prevent and treat the disease. However, these conventional methods are often insufficient for achieving complete remission and are associated with severe side effects on healthy tissues. As a result, there is increasing interest in developing novel therapeutic strategies that selectively target cancer cells while sparing normal cells. In recent years, many medicinal plants with largely unexplored potential have attracted attention as promising candidates in cancer research (16)

In this study, Alkanna orientalis (L.) extract was prepared, and its cytotoxicity on healthy fibroblast (HDF) cells and anticancer activity on glioblastoma (U87-MG) cells were evaluated for the first time. The cytotoxicity assays revealed that A. orientalis extract was non-cytotoxic to HDF cells and even promoted proliferation at low concentrations. Conversely, even the lowest tested dose of A. orientalis extract exerted significant cytotoxicity against U87-MG glioblastoma cells. This selective cytotoxic effect represents a clear advantage over conventional chemotherapeutics, which typically damage both normal and cancerous cells. The cytotoxicity data presented here are encouraging and suggest that A. orientalis extract holds promise as a potential therapeutic agent for glioblastoma. A literature search revealed no prior

studies investigating the effects of A. orientalis extract on glioblastoma. However, a previous report demonstrated the anticancer activity of Alkanna tinctoria extract on U87-MG cells (17). Our findings are consistent with that study, indicating that these two members of the Alkanna genus may share similar anticancer properties, likely attributable to their richness in alkannin/shikonins derivatives (18). Although previous studies have focused on root-derived alkannin/shikonins compounds, the phytochemical profile of A. orientalis leaves has not yet been sufficiently characterized. This lack of detailed phytochemical characterization is a limitation of the present study and necessitates further phytochemical analysis in future research.

The metastatic potential of cancer cells is directly associated with their proliferative capacity(19), and the highly proliferative nature of glioblastomas is a key factor contributing to poor patient prognosis. Therefore, effective treatment strategies must also target metastasis. Recent in vitro studies have evaluated the anti-metastatic effects of natural compounds through various experimental models. In our study, the anti-metastatic effect of A. orientalis extract was assessed using an in vitro wound-healing (scratch) assay. The results demonstrated that treatment with A. orientalis extract significantly inhibited the migration of U87-MG glioblastoma cells.

Several plant species are well known for their anti-inflammatory, antibacterial, and wound-healing properties (20). Although the wound-healing and skin-regenerative potential of other Alkanna species has been previously reported (21), no data are available regarding the effects of A. orientalis extract in this context. In the present study, we performed a scratch assay on fibroblast cells, which play an active role in the early stages of the wound-healing process (22). Our findings showed that A. orientalis extract did not impair HDF cell proliferation; on the contrary, it exerted a positive effect, suggesting that the extract may also possess wound-healing potential in addition to its anti-metastatic activity.

Apoptosis, a programmed form of cell death, plays a critical role in the elimination of damaged or abnormal cells. In this study, Hoechst 33258 fluorescence staining was used to assess the genotoxic potential of A. orientalis extract on both HDF and U87-MG cell lines. The mean nuclear abnormality indices indicated that the extract exhibited stronger genotoxic effects on glioblastoma cells compared to healthy fibroblasts. Previous studies have shown that shikonins found in Alkanna species interfere with the proteasome-mediated degradation of unfolded or misfolded proteins (23), while the naphthoquinones in their structure have been reported to possess anti-inflammatory activity (24)and to inhibit the enzyme topoisomerase I (25). These anti-tumor and anti-inflammatory properties of naphthoquinone derivatives such as alkannin and shikonins may contribute to the induction of apoptotic pathways in cancer cells.

Conclusion

This study presents a comprehensive evaluation of the anticancer and anti-metastatic effects of the aqueous extract of A. orientalis (L.) on glioblastoma cells as well as its cytotoxic effects on healthy fibroblasts. The results show a clear anticancer effect that is dose-dependent with negligible toxicity for normal cells. This indicates a significant therapeutic potential. Future studies should focus on detailed mechanistic investigations and in vivo validations to advance clinical application.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships

that could have appeared to influence the work reported in this paper.

Author Contributions

Conceptualization: Özge Çağlar Yildirim, Hasan Türkez, And Sena Öner, Formal Analysis: Özge Çağlar Yildirim, Sena Öner, Hasan Türkez, And Ceren Karagöz, Investigation: Özge Çağlar Yildirim, Ceren Karagöz, Cihat Aksakal, And Cemal Metehan Akkuş, Methodology: Özge Çağlar Yildirim, Cemal Metehan Akkuş And Ceren Karagöz, Cihat Aksakal, Project Administration: Özge Çağlar Yildirim And Hasan Türkez, Writing – Original Draft: Özge Çağlar Yildirim, Cemal Metehan Akkuş, Sena Öner, And Ceren Karagöz, Writing – Review & Editing: Özge Çağlar Yildirim And Hasan Türkez

ORCİD:

First Author https://orcid.org/0000-0003-1412-8411
Second Author https://orcid.org/0000-0001-7886-8561
Third Author https://orcid.org/0009-0003-7519-5500
Fourth Author https://orcid.org/0009-0004-6156-3488
Fifth Author https://orcid.org/0009-0007-8777-2853
Sixth Author https://orcid.org/0000-0002-7046-8990

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