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ORIGINAL RESEARCH

Investigation of Antiproliferative and Apoptotic Effects of Ethanol Extract of *Viscum album L.* on A549 Cell Line

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Abstract
Objective: This study aimed to investigate the effect of *Viscum album L.* extract on apoptosis and cell proliferation in the A549 and HEK-293 cell lines.
Material-Method: The cell proliferation test was conducted using the MTT method, and apoptosis analysis was performed using the Annexin V method with a Muse flow cytometer. *Viscum album* L. ethanol extract was applied to A549 and HEK-293 cells at concentrations of 1600, 1400, 1200, and 1000 µg/mL, and proliferation analysis was carried out using the MTT method.
Results: The IC_{50} value for A549 cells was 1090 µg/ml at the 72nd hour and 413.6 µg/ml for the HEK-293 cells at the 48th hour. Apoptosis analyses of A549 cells were performed with the AnnexinV method in a Muse/Flow cytometry device. A549 cells, treated with *Viscum album L.* extract at the IC_{50} concentration obtained, underwent late apoptosis at approximately 80%.
Conclusion: *Viscum album L.* methanol extract had antiproliferative and apoptotic effects on A549 cells. In addition, it had an antiproliferative effect on HEK-293 cells, a kidney epithelial cell line. Other studies have reported that *Viscum album L.* has an anticancer effect in different cell lines and can be used as a therapeutic agent. In most studies, its impact on cancer cells has only been investigated, and its effect on healthy cells has not been investigated. Our study shows that *Viscum album L.* extract also showed an antiproliferative effect on healthy cells. The mechanism of action of *Viscum album L.* should be elucidated with further molecular studies.
Keywords: *Viscum album L.*, A549, HEK-293, Proliferation, Apoptosis

INTRODUCTION

Lung cancer is the most common cancer worldwide and the leading cause of cancer death. Lung cancer has a survival rate of 5 years, but this rate is increasing due to not smoking and reducing smoking, early diagnosis, and targeted treatments[1]. Chemotherapy is the standard first-line treatment for lung cancer. Resistance to chemotherapeutic agents reduces the therapeutic effectiveness of these drugs. For this reason, targeted and combined treatments have been used as an alternative to chemotherapy. In traditional medicine, various plants have been used to treat many diseases[2]. Integrative treatments, which also use phytotherapeutic agents, aim to reduce physical and emotional symptoms and improve the quality of life in cancer patients[3].

Viscum album L.(VA) is a semi-parasitic plant that grows on various host tree species[4]. VA has been used for medicinal purposes since ancient Greek and Roman times[5]. In 1917, patients were given mistletoe preparations for cancer treatment[6]. The chemical content of VA varies depending on the species of the host tree, the harvest period, and the treatment of the plant before use[7]. Studies have shown that VA extracts have many biological activities, such as stimulating various cytokines, increasing the activity of natural killer (NK) cells, immunoadjuvant, anticancer, antimycotic, antibacterial, antiviral, and immunomodulatory activities[8,9]. Nowadays, VA preparations are used in addition to existing treatments to reduce the side effects of cancer treatment or to help patients recover[10]. Many chemical components of the VA

plant have been isolated in many studies. Among these chemical components, viscotoxins, lectins, alkaloids, amines, flavonoids, organic acids, terpenoids, phenols, lecithins, triterpenes, polypeptides, saponins, caffeic acid, acetylcholine derivatives, vitamin C, histamine, resins, thionins and cardenolides have been found[5,11] VA lectins play a growth inhibitory role on tumor cells[12-15]. Cell culture studies conducted with VA have shown that it has an anticancer effect. However, most studies have not investigated its impact on healthy and cancer cells. While the therapeutic effects of plants containing many active substances are being investigated, it is important also to investigate their impact on healthy cells. This study investigated the effect of VA extract on the apoptosis and proliferation of A549 cells, a lung cancer cell line. In addition to A549 cells, HEK-293 cells, a renal epithelial cell line, were used as a healthy control.

MATERIALS AND METHODS

Plant extraction

It was collected from *Salix alba L.* (willow tree), spreading on the 50th kilometer of the Konya-Seydişehir highway outside the city center in April-June. After the VA plant was dried in a closed and ventilated environment without exposure to direct sunlight for approximately one month, the trunk, branch, and leaf parts were ground into powder in a stainless steel chamber plant grinder (9000 rpm).

30 g of VA collected from the *Salix alba L.* plant and 300 mL of ethanol (solvent) were extracted in a volumetric flask at 50-55 oC in a Soxhlet extraction device for 8 hours. After extraction, the solvents in the extracts were removed with a Rotary Evaporator device at 45 ^{o}C. The extracts remaining in the flasks were sonicated with a minimal amount of solvent (4-5 mL ethanol) for 5 minutes at 37 °C using an ultrasonicator and transferred to vials. The vials were kept in a closed water bath at 55 °C for 1 week to allow the solvents to evaporate and the extracts to dry.

Preparation of VA concentrations

4 g of VA extract was dissolved in 25 mL of ethyl alcohol suitable for cell culture. Doses were prepared at concentrations of 1600 µg/mL, 1400 µg/mL, 1200 µg/mL, and 1000 µg/mL, not exceeding 1% ethyl alcohol.

Cell culture

The medium of A549 cells incubated at 37 ^{o}C was removed and washed with PBS. Trypsin was added to the petri dish containing the cells and incubated at 37 ^{o}C for 5 minutes. Cells separated from the surface with trypsin were transferred to the vial and centrifuged. A homogeneous cell suspension was obtained by discarding the supernatant and adding medium to the pellet. Cell suspension and Trypan Blue mixture was obtained and transferred to the Thoma slide. This dye exclusion assay determines the number of viable and/or dead cells in a cell suspension. Trypan blue is a large, negatively charged molecule. Trypan blue dye exclusion assay is based on the principle that live cells possess intact cell membranes that exclude this dye, whereas dead cells do not. Then, cell counts were performed under the microscope. Cells were planted in 96-well plates at $5x10^3$ in each well and incubated for 24 hours in a CO_2 incubator at 37 ^{o}C. At the end of 24 hours, the cells' medium was removed. 100 µL of the prepared different VA doses were added to each well. A new medium was added to the control wells. A new medium was added to the blank wells containing no cells. Six replicates were studied for each dose of VA at different concentrations. Plates were incubated separately for 48, 72, and 96 hours. 50% of the dose-medium mixture in each well was removed and discarded into each well where VA doses were applied every 24 hours. Fresh extract-medium mixture was added. All procedures performed for A549 cells were also applied to HEK-293 cells, which is the kidney epithelial cell line we used as control.

Proliferation analysis with MTT method

To prepare 1 mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution, 5 mg MTT powder was dissolved in 1 mL PBS. Cells incubated for 48, 72, and 96 hours were subjected to MTT testing on three days. 20 µL of the prepared MTT solution was added to each well after the medium was Removed and incubated at 37 °C for 4 hours. 150 µL DMSO was added to dissolve the formazan crystals formed after incubation. It was wrapped in aluminum foil and kept in the mixer for 20 minutes to ensure homogeneity. Absorbance was measured at 540 nm by spectrophotometric method.

Apoptosis analysis with annexin V method

A549 cells taken from the incubation were washed with PBS, removed with Trypsin, and then centrifuged, and the supernatant was discarded. The pellet part was homogenized with the medium, stained with Trypan blue, and counted under a microscope using a Thoma slide. Planting was done in a 6-well petri dish with $5x10^4$ cells in each well. It was studied in 3 repetitions. It was incubated for 24 hours in a

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37°C incubator with CO_2. VA extract was applied to the cells at the IC_{50} concentration determined by the MTT method. The same amount of medium was added to control cells. As in the MTT test, 50% of the dose-medium mixture in the cells was removed every 24 hours. The same amount of fresh extract-medium mixture was added. Apoptosis analysis of cells at 72 hours. Annexin V & Dead Cell Reagent were added, and Annexin V was measured in the MUSE Cell Analyzer. The results were evaluated.

RESULTS

IC_{50} values of VA extract applied A549 and HEK-293 cells determined by MTT method

VA extract was applied to A549 cells at doses of 1000 µg/mL, 1200 µg/mL, 1400 µg/mL, and 1600 µg/mL. Figure 1 shows the dose- and time-dependent variation of proliferation analysis results with the MTT method. In the same dose range, the same proliferation analysis was also applied to HEK-293 cells, a healthy kidney epithelial cell line. The results are shown in Figure 2.

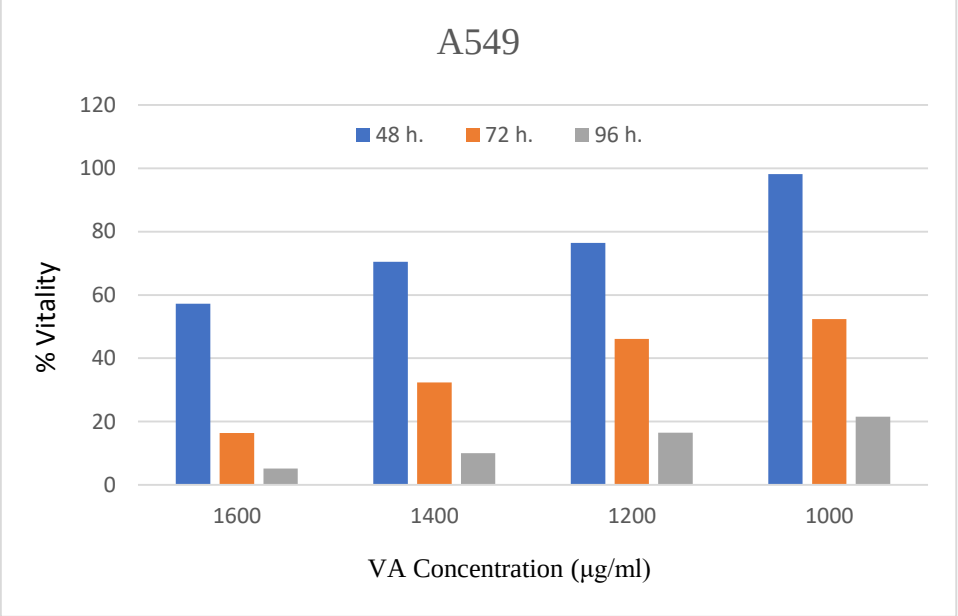

Figure 1. % viability of A549 cells applied with different concentrations of VA extract at 48, 72, and 96 hours

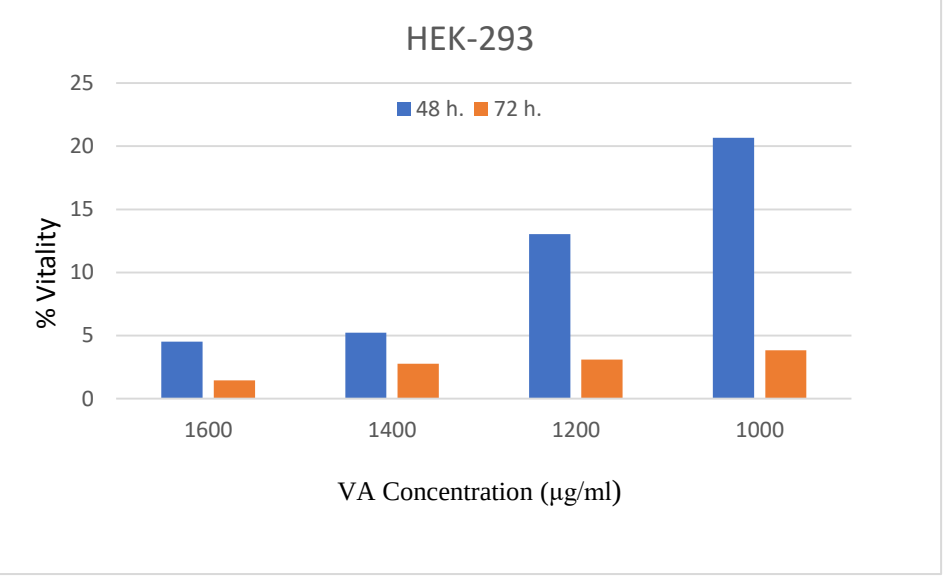

Figure 2. % viability of HEK-293 cells applied with different concentrations of VA extract at 48 and 72 hours

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The closest value to the IC_{50} value in A549 cells was obtained at a concentration of 1000 µg/mL at the 72nd hour, and the IC_{50} value was calculated using the equation in Figure 3.

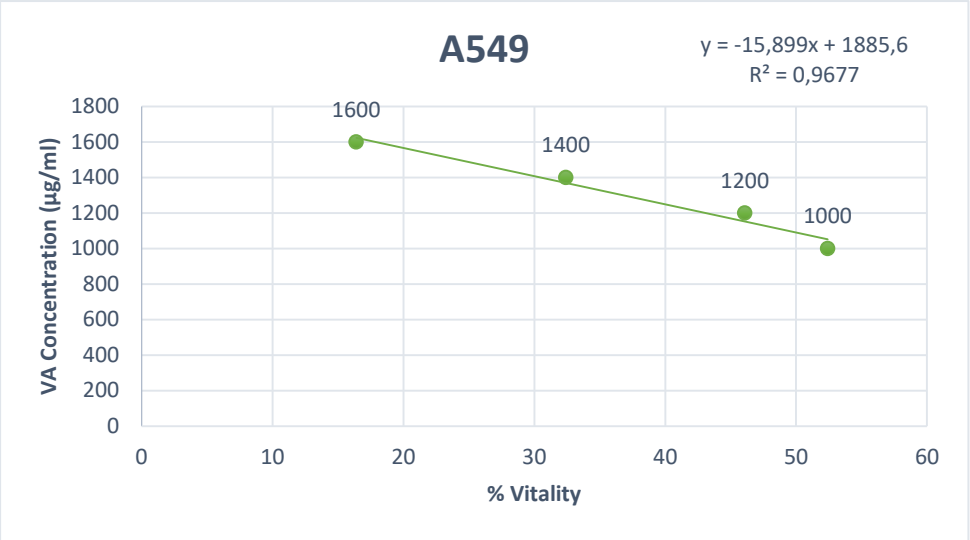

Figure 3. % viability, trend line, and line equation of A549 cells applied with different concentrations of VA extract at the 72nd hour (IC50: 1090 µg/mL).

Apoptosis analysis results with annexin V method on muse/flow cytometry device

A549 cells were seeded in 6-well plates at $5x10^4$ per well. Apoptosis analysis was performed using the Annexin V method on the Muse/Flow cytometry device. When the cell undergoes apoptosis, the phosphatidyl serine on the inner surface of the cell is translocated to the membrane's outer surface. Results were obtained according to the binding of Annexin V to phosphatidylserine translocated to the outer surface and the binding of 7AAD to the DNA in the cell nucleus. According to the results obtained from the Muse/Flow cytometry device (Figure 4, Figure 5, and Table 1), it was observed that A549 cells underwent late apoptosis at a rate of 80.60%.

Table 1. % viability and apoptosis table of VA extract-treated A549 cells and control cells at the 72nd hour

	% Viability	Early Apoptosis	Late Apoptosis	Debris	Total Apoptosis
Control	%93.9	%2.35	%2.25	%1.5	%4.6
A549	%2,25	%0.9	%80.6	%16.25	%81.5

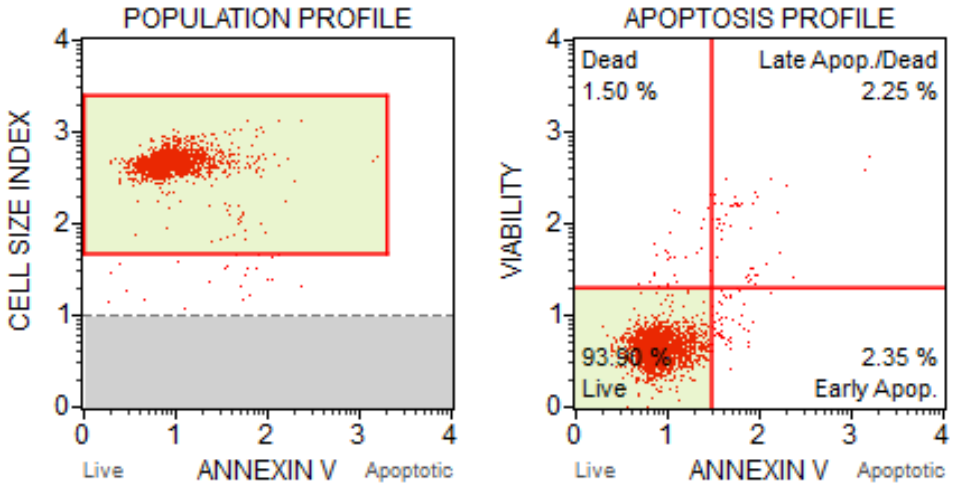

Figure 4. Apoptosis graphs of A549 control cells

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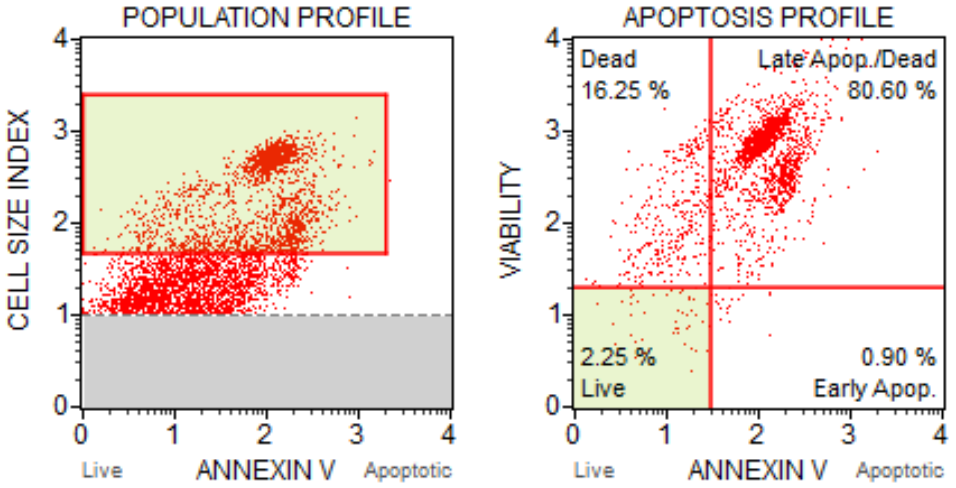

Figure 5. Apoptosis graphs of A549 cells were applied with an IC_{50} dose of *Viscum album* L. extract at the 72nd hour

DISCUSSION

Lung cancer is one of the most important and prevalent diseases worldwide. The prognosis for patients with advanced lung cancer is poor, with an average survival of one year. Lung cancer can be treated with surgery or chemotherapeutic drugs, depending on the type and stage of the disease. Due to the side effects of modern treatment methods such as chemotherapy, radiotherapy, and immunotherapy, studies have been conducted on the anticancer properties of medicinal plants traditionally used to treat many diseases, and it has been shown that they can be used in cancer treatment. VA has been used traditionally to treat many diseases for many years. Studies have proved the anticancer properties of the lectins and viscotoxins they contain.

In the phytochemical analysis of VA obtained from ethanol extract, flavonoid, and viscotoxin contents were the highest. Phytochemical compounds and viscotoxin contents are probably related to the antitumor effects of VA [16]. Cell death mechanisms include necrotic effects, depending on the impact of the host tree, time, and dose. Lectins in the *Viscum album*, especially viscotoxin and mistletoe lectins (ML-I, ML-II, ML-III), exhibit strong anticancer effects. These lectins can bind to the cell membrane, affect intracellular signaling pathways, and induce apoptosis. Lectins stop the growth of cancer cells by triggering apoptosis mechanisms leading to cell death.

Previous studies investigated the antiproliferative effect of VA extract in various cell lines (Table 2). Huyen et al. obtained an aqueous extract of VA cultivated on oak, apple, and pine trees, examined its cytotoxic effects on lymphoblastoid and monocytic cell lines, and reported its anticancer properties in these cell lines. Urech et al. demonstrated that viscin, betulinic acid, oleanolic acid, and ursolic acid inhibited growth and induced apoptotic cell death in Molt4, K562, and U937 leukemia cells. In the study by Pieme et al., the methanol extract of VA exhibited an antiproliferative effect in MB-MDA435 cells. Vlad et al. also indicated that ethanol and aqueous extracts of VA showed antiproliferative effects in HepG2 and MCF7 cells.Sarpataki et al. investigated the antiproliferative effect of ethanol extract of VA on normal human fibroblasts (Hfl) and a tumor cell line (Hela). Their results showed that ethanol extract of VA had minimal effect on the proliferation of normal fibroblasts (Hfl) while significantly reducing the proliferation of Hela cells. In the study by Urech et al. in 2006, the antiproliferative and apoptosis effects of VA extract were measured in four human bladder carcinoma cell lines (T24, TCCSUP, J82, and UM-UC3). According to the results, VA showed anticancer effects in all four different cell lines.

Different cell lines are genetically distinct, and these differences affect the response of the cells to the *Viscum album*. For example, some cell lines may have more active pathways that activate apoptosis mechanisms, while others may suppress these pathways. The rate of cell proliferation can also affect the IC_{50} value. Fast-proliferating cell lines are generally more sensitive to anticancer agents.

Table 2. Previous cell culture studies with VA extract

Researcher	Cell Line	IC_{50} Value
Huyen et al. (2003)[17]	Lenfoblastoid ve monositik hücre hattı	6,25-12,5 µg/mL
	K562	252 µg/mL
	Molt4	118 µg/mL
Urech et al. (2005)[18]	U937	138 µg/mL
Pieme et al. (2012)[19]	MB-MDA435	172 µg/mL
Vlad et al. (2016)[20]	MCF-7, HepG2	75 mg/mL
Sarpataki et al. (2015)[21]	Hela	5 µg/ml
	UM-UC3	380 µg/mL
	T24	1640 µg/mL
Urech et al. (2006)[22]	J82	1880 µg/mL
	TCCSUP	1920 µg/mL
Our study	A549	1090 µg/ml
	HEK-293	413,6 µg/ml

In our study, the IC50 value for A549 is compatible with the literature. The antiproliferative and apoptotic effect of VA extract on A549 cells was demonstrated in this study. However, the point that should be noted is this: VA extract did not only show an antiproliferative effect on A549 cells, which is a lung cancer cell line but also showed an antiproliferative effect on HEK-293 cells, a healthy epithelial cell line. In fact, as a result of applying the same VA concentration, HEK-293 cells showed more antiproliferative effects than A549 cells. In this study, which we conducted with the hypothesis that VA could be used alone or as a supportive treatment with existing drugs in lung cancer, the fact that healthy epithelial cell lines were also affected showed that VA could not be used for therapeutic purposes in lung cancer. It is seen that in the majority of other studies, healthy cell lines were not used and were not compared with cancer cells. The content of herbal extracts varies depending on the region where they grow, the collection period, and the extraction method. In addition to its effect on cancer cells, its effect on healthy cells should also be investigated. Having an antiproliferative or apoptotic effect on cancer cells without knowing its effect on healthy cells may not indicate that it can be used as a treatment tool. In this regard, while investigating herbal extracts' antiproliferative and apoptotic effects, their effects on healthy cells should also be evaluated.

CONCLUSION

Our study demonstrated that *Viscum album* L. extract exhibits anti-proliferative and pro-apoptotic effects on the A549 lung cancer cell line while inducing significant cytotoxicity and apoptosis in HEK-293 healthy cells. These findings suggest that *Viscum album* L. has potential as a therapeutic or adjuvant agent in cancer treatment. However, considering its adverse effects on healthy cells, further studies are required to enhance the selectivity of the extract. Future research should focus on testing different concentrations and formulations, investigating cell-type-specific effects, and conducting comprehensive toxicity analyses supported by in vivo models. Such investigations would provide a solid foundation for the safe and effective therapeutic use of *Viscum album* L.

Research Limitations: A limitation of this in vitro study is that there was no comparison with VA using commonly used chemotherapeutic agents for lung cancer, such as cisplatin or etoposide, in the same cell line. The synergetic effect of VA with chemotherapeutic agents can be investigated.

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Ethical Approval: Approved by the Local Ethics Committee of the Selcuk University Faculty of Medicine with decision number 2019/12

Disclosure statement: The authors have no conflicts of interest to declare.

Author contributions: Fadime Ovali; conceptualization, data curation, formal analysis, ınvestigation, methodology, project administration, resources, validation, software, visualization, writing-original draft, writing-review&editing. Husamettin Vatansev; supervision, conceptualization, project administration, resources,

validation, writing-review&editing. Hakan Vatansev; data curation, formal analysis, ınvestigation, methodology, writing-review&editing.

Conflict of interest: There is no potential conflict of interest relevant to this article.

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