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Research Article

Cytotoxic, apoptotic, and necrotic effects of silver nanoparticles biosynthesized using *Origanum majorana* extract

Ramazan ERENLER[1,2*], Esmanur GEÇER[2], Büşra MORAN BOZER[3]

[1]*Research Laboratory Practice and Research Center, Igdir University, 76000 Igdir, Türkiye*

[2]*Department of Chemistry, Faculty of Arts and Sciences, Tokat Gaziosmanpasa University, 60240 Tokat, Türkiye*

[3]*Scientific Technical Research and Application Center, Hitit University, Corum, Türkiye,*

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*Corresponding author e-mail: rerenler@gmail.com

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ABSTRACT

Nanotechnology has gained great interest due to its widespread application. Anticancer activity of *Origanum majorana* extract and silver nanoparticles was investigated by MTT assay using human pancreatic adenocarcinoma cell lines (Capan-1), mouse normal fibroblast cell lines (L929), and human colon adenocarcinoma cell line (Caco-2). AgNPs@Om exhibited the excellent cytotoxic effect on Capan-1 cell lines with the cell viability of 29.17% and 25.70%, 22.49%, 21.16% at 1.0, 0.5, 0.25, 0.125 µg/mL respectively. However, the extract was determined to show moderate activity. A considerable fall in the viable cell number in Caco-2 cell lines was observed after the treatment of AgNPs@Om. The viable cells of Caco-2 cell lines were detected as 32.0% at 1.0 µg/mL with the treatment of nanoparticles. Yet, the viable Caco-2 cells were determined as 48.2% with the treatment of *O. majorana* extract. Both extract and nanoparticles had no impact on the viability of non-tumor cells (L929). Moreover, nanoparticles induced apoptosis. Consequently, AgNPs@Om may be a promising anticancer drug candidate.

Keywords: Silver nanoparticles, natural products, *Origanum majorana*, anticancer activity.

Origanum majorana özütü kullanarak gümüş nanoparçacıkların sitotoksik, apoptotik ve nekrotik etkileri

ÖZ

Nanoteknoloji, yaygın uygulaması nedeniyle son zamanlarda büyük ilgi görmektedir. *Origanum majorana* özütü ve gümüş nanopartiküllerin antikanser aktivitesi, insan pankreas adenokarsinoma hücre hatları (Capan-1), fare normal fibroblast hücre hatları (L929) ve insan kolon adenokarsinom hücre hatları (Caco-2) kullanılarak MTT metodu ile araştırıldı. AgNPs@Om, sırasıyla 1.0, 0.5, 0.25, 0.125 µg/mL'de %29.17 ve %25.70, %22.49, %21.16 hücre canlılığı ile Capan-1 hücre hatları üzerinde mükemmel sitotoksik etki sergiledi. Ancak ekstraktın orta düzeyde aktivite gösterdiği belirlendi. AgNPs@Om etkileştirilmesinden sonra Caco-2 hücre hatlarındaki canlı hücrelerin sayısında önemli bir azalma gözlendi. Caco-2 hücre hatlarının canlı hücreleri, nanopartiküllerin etkisi ile 1.0 µg/mL'de %32.0 olarak tespit edildi. Ancak *O. majorana* özütü ile muamele edildiğinde canlı Caco-2 hücreleri %48.2 olarak belirlendi. Hem ekstrakt hem de nanopartiküller, tümör olmayan hücrelerin (L929) canlılığı üzerinde önemli bir etkiye sahip değildir. Ayrıca, nanopartiküller apoptozu indükledi. Sonuç olarak, AgNPs@Om umut verici bir antikanser ilaç adayı olabilir.

Anahtar Kelimeler: Gümüş nanopartikül, doğal ürünler, *Origanum majorana*, antikanser aktivite.

1. INTRODUCTION

Medicinal plants have been employed for therapeutic purposes since ancient times.[1-5] These plants reveal a large spectrum of biological activities owing to their bioactive compound contents.[6-8] The discovery of spectroscopy in the 19th century led to natural products becoming the focus of science.[9-12] Secondary metabolites are substances synthesized by plants that have many functions such as protecting them against enemies. These compounds show a wide variety of interactions on the plant itself and other living organisms.[13-14] Because of

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acting as reducing and stabilizing agents, plant secondary metabolites have been used in the green approach for the synthesis of nanoparticles.[15]

Nanoparticles can be synthesized from nano to micron sizes and have many applications, including bioseparation and biocatalysts with optical, magnetic, electrical, and magnetic field strengths, thanks to their superior properties.[16] The most important advantages of metal nanoparticles are their relatively large surface area compared to their size and their unique properties.[17] Moreover, low cost, ease of use, and high stability are other advantages of metal nanoparticles.[18] In addition, the fact that metal nanoparticles do not have any toxic effects on the environment creates the ideal environment for the use of these nanoparticles.[19] It is accepted by the scientific world that metal oxide nanoparticles have antimicrobial effects, and it is reported that the greatest effect on agents causing the infection is obtained by silver nanoparticles.[20]
While nanoparticles are smaller in size than human cells, they are the same size as biological macromolecules such as receptors and enzymes. Therefore, nanoparticles can be used as probes by conjugating them with peptides, antibodies, or nucleic acids of the same size.[21] Thus, molecular changes and cellular movements related to pathological regions can be observed. These probes provide high sensitivity, stability, and absorption coefficient over a wide spectral range.[22]

Cancer is an illness described by the unrestrained increase and proliferation of abnormal cells in the body and the killing of normal cells and frequently causing death and it is one of the most serious and terrible diseases in the world.[23] Chemotherapy, radiation, and surgical treatments are among the traditional therapeutic strategies used in cancer treatment. These therapies have several limitations, such as high dose requirements, non-specific toxicity, and multidrug resistance. Besides, drugs used in chemotherapy have poor water solubility and have a high incidence of side effects. In addition, chemotherapy drugs cause significant damage to healthy tissues, causing adverse reactions. Therefore, current studies are now focused on safer and more effective treatment of cancer. In this sense, nanotechnology may be the solution to overcome these limitations of conventional chemotherapy. For this reason, the development of anticancer drugs produced from nanoparticles obtained from plants is of great importance.[24]

Origanum genus includes aromatic and medicinal plant species that are used effectively in food, traditional medicine, and the pharmaceutical industry.[25-26] This genus is also well known for its essential oils that are used in the food, cosmetic and pharmaceutical sectors.[27-28] *Origanum majorana* L., a species belonging to the *Origanum* genus of the Lamiaceae family, is a perennial medicinal plant called sweet marjoram.[29]

In this study, silver nanoparticles were synthesized from *Origanum majorana* L. and cytotoxic, apoptotic, and necrotic effects were investigated using Capan-1, Caco-2, and L929 cell lines.

2. MATERIALS AND METHODS

2.1 Chemicals

L-glutamine, trypsin-EDTA, and FBS were purchased from Merck (Darmstadt, Germany). Human pancreatic adenocarcinoma cell lines (Capan-1), mouse normal fibroblast cell lines (L929), and human colon adenocarcinoma cell line (Caco-2) were supplied from Kırıkkale University.

2.2 Synthesis of nanoparticles

Origanum majorana was utilized for silver nanoparticle synthesis. The spectroscopic studies such as X-ray diffraction, Ultraviolet-visible, Fourier-transform infrared as well as Scanning electron microscope revealed the formation of silver nanoparticles. The experimental procedure and spectral data as well as the antioxidant activity of green synthesized AgNPs@Om were reported previously.[30]

2.3 Cell culture

Human pancreatic adenocarcinoma cell lines (Capan-1), mouse normal fibroblast cell lines (L929), and human colon adenocarcinoma cell lines (Caco-2) were used for anticancer activity. Cells were incubated in DMEM-F12 or RPMI culture medium containing 1% antibiotic and 10% FCS in flasks in a carbon dioxide oven at 37 °C.

The medium was changed at 2-day intervals until the cells reach enough. After the cultures reach a certain number, they were removed with trypsin and inoculated into well plates (5.000-10.000 cells/well). When cells reach a certain number, media containing serum and antibiotics were replaced with media containing serum and antibiotics. It was then used to determine necrotic, apoptotic, and cytotoxic activities.[31]

2.4 Cytotoxicity assay

Capan-1, Caco-2, and L929 were inoculated into 96 well plates, with 10.000 cells per well. Substances of varying amounts were added to the cells and incubated for 24 hours. Afterward, *Origanum majorana* and AgNPs@Om (1.0, 0.5, 0.25, 0.125 and 0.0625 mg/mL) were added to the well and incubated for 24 h. Latex and media rubber was used for respectively positive and negative control. The media was incubated at 37°C for 2.5 hours after being replaced with 50 µL MTT solution (1.0 mg/mL). A new solution of MTT in isopropanol (100 µL) was then added in place of the MTT solution.

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The control cell viability was accepted as 100%. Cell viability was assessed by ELISA at 570 nm and calculated with the formula (1)

$$\text{Cell viability } \% = [Ax/Ay] \times 100 \quad (1)$$

Ax is the sample of optical density, Ay is the control.[32-34]

2.5 Apoptotic and necrotic analysis

Fluorescent staining was performed after the cells prepared as mentioned above interact with Capan-1 cells, Caco-2 cells, and L929 cells at different rates. In this way, apoptosis and necrosis were determined. During staining, cytoplasmic RNA is destroyed by treating cells with ribonuclease A (Sigma R-500) for staining only DNA. Hoechst (33342) staining stains the nuclei of cells blue. Despite staining whole cells, apoptotic cells appear brighter and nuclear homogeneity is lost. In this way, true apoptotic cells are differentiated from normal cells. The other dye used in dual staining is Propidium Iodide (PI). It shows necrosis by staining the DNA red. PI does not pass through the membranes of normal cells but only passes through the membranes of cells with damaged or dead cells, indicating necrotic cells. Cells that were only treated with the drug and affected with drug and inhibitor co-administered complexes will be removed after 24 hours and staining will be performed.

The painting process was carried as briefly, Capan-1 cells, Caco-2 cells, and L929 cells (15×10^3 per well) were cultivated in DMEM with L-glutamine supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin for 24 h, 5% CO_2 atmosphere, and at 37 °C, in a 48-well plate.[35] Different concentrations of AgNPs@Om (50-500 µg/mL) were treated for 24 hours. Then, detached and attached cells were collected and stained with Hoechst dye (33342), PI, and DNAse free-RNAse for 20 minutes at room temperature after washing with phosphate-buffered saline (PBS). The control group included Capan-1 cells, Caco-2 cells, and L929 cells treated with cell medium alone.

2.6 Statistical analysis

GraphPad Prism (8.0.1), Tukey's multiple comparison test, and the one-way ANOVA test were used for statistical analysis, and results were expressed as mean values ± SDs of three independent trials ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Cytotoxic effect

In this study, the cytotoxic effect of the extract and silver nanoparticles using Capan-1, Caco-2, and L929 were investigated by the MTT test. Table 1 shows the cytotoxic effect of *Origanum majorana*-mediated synthesized silver nanoparticles on Capan-1, Caco-2, and L929 cell lines. Cell viability of the Capan-1 cell lines treated with the nanoparticles and extract was determined as 29.17% and 69.35% at 1.0 µg/mL respectively. However, the cell viability decreased after the treatment of nanoparticles at 0.5 µg/mL, treatment of extract caused the increase of the cell viability at the same concentration (0.5 µg/mL). The Capan-1 cell lines' viability decreased with the decrease in concentration. Nevertheless, treatment of extract with Capan-1 cell lines increased the cell viability. In Caco-2 cell lines, nanoparticles were observed to have a significant effect on these cell lines. The cell viability was observed at 0.5 mg/mL as 27.91% after the treatment of AgNPs@Om. At the same concentration, the treatment with extract resulted in cell viability of 53.19%.

The concentration decrease caused an increase in cell viability. Concerning the L929 cell lines, cell viability was determined as 93.7% after the treatment with AgNPs@Om at 1.0 mg/mL, extract treatment resulted in the cell viability of 78.2%. Both extract and nanoparticles caused the increase in cell viability. According to this result, it was concluded that nanoparticles were more effective than the extract at a concentration of 1.0 µg/mL.

Table 1. Cytotoxic effect of AgNPs@Om and extract on cell lines. Data are shown as % viable cells, different letters indicate the significant difference in the same column ($P < 0.05$).

Concentration (mg/mL)	AgNPs@Om			Extract		
	Capan-1	Caco-2	L929	Capan-1	Caco-2	L929
1	29.17±3.6^{c}	31.95±2.7^{c}	93.70±4.4^{a}	69.35±3.4^{a}	48.16±2.1^{a}	78.15±5.3^{a}
0.5	25.70±5.9^{b}	27.92±2.6^{a}	101.75±3.1^{c}	76.11±5.4^{b}	53.19±24.1^{b}	120.69±7.2^{b}
0.25	22.49±1.9^{a}	28.69±2.9^{b}	96.91±2.0^{b}	89.39±4.5^{c}	69.85±2.8^{c}	122.72±7.8^{bc}
0.125	21.16±1.4^{a}	34.07±3.9^{d}	101.27±1.8^{c}	131.31±1.1^{d}	73.60±2.0^{d}	121.97±8.4^{b}

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3.2 Apoptotic and necrotic cells analysis

In many physiological situations, apoptosis, or programmed cell death, plays an important role in controlling cell number. Many human tumours have impaired apoptosis, implying that apoptotic function disruption contributes significantly to the transformation of a normal cell into a tumour cell. Apoptosis is also a major contributor to chemotherapy-induced tumour cell death.[36] The apoptotic and necrotic index of silver nanoparticles on Capan-1, Caco-2, and L929 cell lines is shown in Table 2. The cell death pathway was confirmed using the double staining method. The apoptotic index was found to be higher than the necrotic index in all cell lines, indicating that cell death occurred via apoptosis. The fluorescent dye Hoechst (33342) in the double staining solution binds to DNA, giving the cell nucleus its blue colour.

Table 2. Apoptotic index and necrotic index of AgNPs@Om on Capan-1, Caco-2, and L929 cell lines at different concentrations (mg/mL).

Concentration (1 mg/mL)	**AgNPs@Om**			**Extract**		
	Capan-1	**Caco-2**	**L929**	**Capan-1**	**Caco-2**	**L929**
% Apoptosis	15,30±1,40[b]	21,50±2,30[b]	8,40±0,70[b]	35,20±2,40[b]	35,20± 1,80[b]	0
% Necrosis	4,40±0,50[a]	4,20±1,30[a]	0,50±0,01[a]	8,20±0,40[a]	12,60± 2,30[a]	0

Apoptotic cell nuclei were distinguished from other nuclei by their blue color, which was caused by the deformation, brightening, and distortion of their borders. PI dyed the necrotic cell nuclei, which appeared red under fluorescent light (Figure 1). The morphological change was not observed in the control groups' cell nuclei. Nonetheless, apoptotic cell nuclei were stained with a blue fluorescence when compared to non-apoptotic cells.

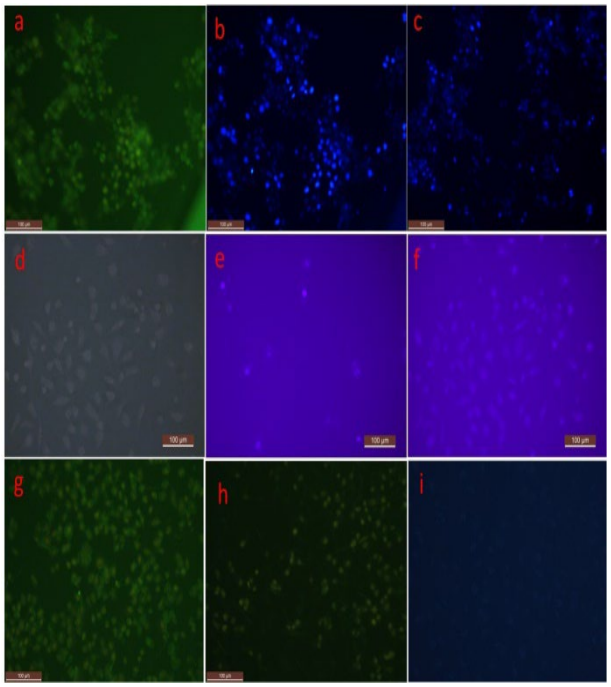

Figure 1. Fluorescence inverted microscopy image of Caco-2, Capan-1, L929 cells treated with extract and silver nanoparticles, a: control, Caco-2, b: Caco-2, extract, DAPI, c: Caco-2, AgNPs, DAPI, d: Capan-1, control, e: Capan-1, extract, DAPI, f: Capan-1, AgNPs, DAPI, g: L929, extract, FITCH, h: L929, AgNPs, FITCH, i: L929 control.

4. CONCLUSION

The synthesized silver nanoparticles from *Origanum majorana* revealed an excellent antiproliferative effect on the corresponding cell lines (Capan-1, Caco-2).

Furthermore, AgNPs@Om induced the cells to apoptosis. Cytotoxic effects of extract and AgNPs@Om were investigated. The percent viability of cell lines was calculated by comparison of absorbance values of control cells. AgNPs@Om did not reveal significant inhibition on L929 cell lines (normal cells). Moreover, observation of the apoptosis pathway in both cell lines after treatment of nanoparticles revealed that the nanoparticles could be a drug candidate. A key mechanism by which cytotoxic drugs kill tumor cells is the activation of apoptosis pathways.

Conflict of Interest

The authors declare that there is no conflict of interest with any person, institution, company, etc.

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