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ARAŞTIRMA MAKALESİ (Research Article)

THE EFFECTS OF PLANT ORIGIN EXOSOME EXTRACTS ON A549 AND HCT116 CANCER CELL LINES

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ABSTRACT

By applying the exosomes-like nanoparticles obtained from the leaves of the Gundelia munzuriensis plant on lung cancer (A549) and colon cancer (HCT116) cell lines, the effects of plant exosomes on cancer cells were investigated. WST1 (Water-soluble tetrazolium salt – 1 analysis) was performed and the cytotoxic effect of the exosome in these cell lines was examined. According to our findings, exosomes incubated with HCT 116 cell lines were found to affect the viability of cancerous cells by 40-50%. On the other hand, a rate of 50% decrease in A549 cell lines was observed in 24 hours with 250 µg/mL. As a result, our study demonstrates that plant-derived exosomes may be promising therapeutic agents for future cancer treatment, and the research provides an important source for future studies.

Keywords: *Nanoparticles, HCT116, A549, cancer, Gundelia munzuriensis.*

BITKI KÖKENLI EKSOZOM EKSTRAKTLARININ A549 VE HCT116 KANSER HÜCRE HATLARI ÜZERINE ETKILERI

ÖZ

Gundelia munzuriensis bitkisinin yapraklarından elde edilen eksozom benzeri nanopartiküller akciğer kanseri (A549) ve kolon kanseri (HCT116) hücre hatlarına uygulanarak bitki eksozomlarının kanser hücreleri üzerindeki etkileri araştırıldı. WST1 (Suda çözünür tetrazolyum tuzu – 1) analizi yapılıp bu hücre hatlarında eksozomun sitotoksik etkisine bakıldı. Bulgularımıza göre, HCT 116 hücre hatları ile inkübe edilen eksozomlar, kanserli hücrelerin canlılığını %40-50 oranında etkilediği tespit edildi. Öte yandan, 250 µg/mL ile 24 saatte A549 hücre hatlarında %50'lik bir azalma gözlendi. Sonuç olarak, çalışmamız bitki kaynaklı eksozomların gelecekteki kanser tedavisi için umut verici terapötik ajanlar olabileceğini göstermektedir ve araştırma gelecekteki çalışmalar için önemli bir kaynak sağlamaktadır.

Anahtar kelimeler: *Nanopartüküller, HCT116, A549, kanser, Gundelia munzuriensis.*

1. INTRODUCTION

The complex disease, which occurs when cells in an organism grow, divide and multiply uncontrollably, and develops under the influence of genetic and environmental conditions, is called cancer. Cancer is the second among the deadly and incurable health problems globally, following cardiovascular diseases. According to data by the World Health Organization (WHO), cancer is known to have caused the deaths of 10 million people in 2020. The most frequent types of cancer encountered in 2020 worldwide include breast cancer, lung cancer, and colon cancer. Furthermore, lung cancer is also the leading type of cancer resulting in death, and colorectal cancer ranks second [1].

Cancer treatment options were limited to traditional chemotherapy, radiotherapy, and surgery until recently. In recent years, different practices have been tried with immunotherapy applications, obtaining promising results [2]. However, while these methods are still inconclusive, they are costly and time-consuming. As the developing technology offers different solutions every day, scientists continue their search for the optimum alternatives.

The discovery of exosomes and understanding the importance of their biological function between cell-to-cell have paved the way for the use of these nanovesicles as therapeutic agents in many diseases. Cancer is one of the leading diseases among these, and exosomes are used in cancer research for early diagnosis, treatment, drug transport and many other purposes. Recent research confirms that engineered exosomes increase cancer-killing effectiveness and the ability of drugs to target cancer, thereby increasing the effectiveness of individual cancer treatments [3].

Exosomes are nano-sized particles that are secreted by various cells and absorbed by recipient cells. Exosomes are rich in ingredients such as proteins, lipids, mRNA and miRNA, and they can preserve these contents unimpaired [4]. Studies on exosomes in biomedical, biochemistry, oncology and cell biology fields are increasing, and there is promising research on the possibility that exosomes can play a role in the effective transport of bioactive compounds to target cells or tissues. The fact that they do not require any modifications and can be obtained from natural sources increases the interest in exosomes [5].

Newly discovered plant-derived exosomes compared to those obtained from mammalian cells have paved the way for different treatment pathways with their advantages and biochemical properties, and they have revived existing alternative medicine studies. It is known that many plants consumed as food today contain bioactive compounds with different functions. The data obtained so far demonstrate that exosomes can be investigated and applied in the fields of food and agriculture. Nevertheless, the number of studies in these areas regarding exosomes has been considered insufficient to date [5].

Plant-derived exosomes attract the attention of researchers because they can target the desired tissues, have high biocompatibility along with adequate biodistribution. They possess the nanocarrier potential to transmit chemical drugs, genes, and small molecules. Thus, they are able to introduce drugs targeting precise and specific tissues instead of generating systemic effects, which is thought to lead to improved therapeutic impacts and fewer side effects [6].

The primary purpose of this study is to isolate the exosomes from the leaves of the plant Gundelia munzuriensis, an endemic species, and to assess its potential therapeutic effects on Lung Cancer (A549) and Colon Cancer (HCT 116) cell lines in vitro. The fact that a study examining the effects of G. munzuriensis exosomes on cancer has not been done before makes this study significant. Hence, it is thought that by investigating their effects on cancer by isolating G. munzuriensis exosomes (GME) and giving them to mammalian cells, an alternative treatment for cancer, which is the most serious and increasingly common problem of our time, can be discovered.

2. MATERIALS and METHOD

Plant-derived exosomes attract the attention of researchers because they can target the desired tissues, have high biocompatibility along with adequate biodistribution. They possess the nanocarrier potential to transmit chemical drugs, genes, and small molecules. Thus, they are able to introduce drugs targeting precise and specific tissues instead of generating systemic effects, which is thought to lead to improved therapeutic impacts and fewer side effects [6].

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2.1. Experimental

In this study, effects of the exosomes isolated from the *G. munzuriensis* plant leaves on the in vitro models based on HCT 116 and A549 cell lines were investigated. The leaves of the *G. munzuriensis* plant used in the research were freshly collected in Ovacık – Tunceli (Koyungölü road, Ovacık, 3.2 km, 1285 m, 39°21'12" N / 39°10'48" E, 01,06.2021), they were transported in cool packs and stored in the deep freezer at -80^{o} C until the isolation process. Before starting isolation, the leaves were washed and shredded in distilled water with the help of a shredder. In the experiment setup, the isolation of *G. munzuriensis* exosomes (GMEs) was made by centrifuging according to the manufacturer's protocol and the ATPS (aqueous two-phase system) isolation method. Nanoparticle tracking analysis (NTA) was conducted to determine the dimensions and concentrations of the exosomes, while BCA analysis was made to assess their protein concentrations. Afterwards, to evaluate the cytotoxic effect of GMEs on cancer cells, WST-1 cell viability assay was performed on the cancer cells that were incubated for 24, 48 and 72 hours in five different exosome concentrations varying between 25 to 250 µg/mL.

2.2. Exosome Isolation

G. munzuriensis leaves were crushed in distilled water using a shredder, and the mixture was filtered using filter paper. The extract was centrifuged for 10 minutes at 1000 x g, 10 minutes at 20000 x g, and 30 minutes at 20000 x g. After each centrifugation, the supernatant was removed from the pellet and collected. The collected supernatant was filtered through a 0.22 µm filter. The PEG-DEX solution used for the isolation in the ATPS method was added at a 1:1 ratio (22.5 ml buffer for 22.5 mL extract). For the washing process, a 1:1 ratio washing solution (150 mL H_2O for 150 mL solution) was prepared. The buffered samples with washing solutions were centrifuged at 1000 xg for 10 minutes,

and 80% of the upper phase of the samples were discarded. Then, the washing solution was added at the same amount as the discarded part (36 mL), and the samples were centrifuged at 1000 xg for 10 minutes. The rinsing process was repeated. Finally, the part above the phase line was discarded. The isolated GMEs were stored at -20℃.

2.3. Nanoparticle Tracking Analysis (NTA)

The exosome quantification and their size distribution were estimated with a 488 nm laser using Nanoparticle Tracking Analysis (NTA). The isolated exosomes were analysed utilizing NanoSight NS300. The samples were placed into the vessel of the device, and the size dispersion (nm) was calculated using the NTA method. The video captured the nanoparticles at least five times with 60-second intervals, and the test was performed employing the NTA 3.3.301 software.

2.4. BCA Analysis

The GMEs were diluted at a 1:5 ratio (5 µm of exosomes, 20 µL of H_2O). The diluted exosomes (10 µL each) were placed into two wells, H_2O was placed into two other wells (10 µL), and standards were put into 14 wells (10 µL) of the 96-well plate. Next, B and A solutions were mixed at a 1:50 ratio (86.274 mL of solution B, 4313.726 mL of solution A), and each well was added 200 µL of this mixture. The plate was then incubated for 30 minutes. Measurements were made with the spectrophotometer (Thermo Scientific, USA) at 570 nm wavelength, and the results were recorded.

2.5. Cancer Cells and Passaging

Lung cancer and colon cancer cell lines were obtained from the American Type Culture Collection (ATCC). DMEM high glucose was used as the culture medium for the cell lines, and this medium was supplemented with 10% (v/v) FBS and 1% (v/v) PSA (10.000 units/mL penicillin, 10.000 µg/mL streptomycin, and 25 µg/mL amphotericin B). Cells were incubated in a humid 37 °C incubator with 5% CO^2 . In the passage of adherent cells, the surfaces of the flasks were first washed with 1x DPBS without Ca^{2+} and Mg^{+2}. Cell-surface interaction was dissociated with 0.025% (w/v) trypsin for 1-2 minutes at 37°C. The detached cells were collected in a cell culture medium and centrifuged at 1300 rpm for 5 minutes. Then, the pellet was dissolved in the cell culture medium. For the exosome treatment, high glucose DMEM was supplemented with 10% FBS (Exosome-Deficient). On the hemocytometer, 10 µL of cells with the medium were placed, and cell counting was performed with a microscope.

2.6. HCT 116 and A549 Cell Culture, Exosome Treatment and WST-1 Assay

PBS of 100 µL per well was added to the edge wells of two separate 96-well plates. To each well, 100 µL of cell suspension with medium (44 mL medium, 1230 mL cell suspension) was cultured, and the plate was placed into the incubator. After 24 hours of cell cultivation, the media in the wells was vacuumed, and clean media (150 µL per well) was given to the control groups. Experimental groups with concentrations ranging from 25 to 250 µg/mL were also given 150 µL from exosomes with media and left for incubation.

After 24 hours of the exosome treatment, the excess was discarded, leaving 90 µL in the wells. Each well was supplemented with 10 µL WST-1 dye. The culture was incubated for 30 minutes (CO_2 incubator). Measurements were made with the spectrophotometer (450 nm length). The same procedures were applied to cells that were kept under treatment for 48 and 72 hours.

2.7. Statistical analysis

Statistical analysis of the data was performed using two way ANOVA and IBM-SPSS 25 package program. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Nanoparticle Tracking Analysis (NTA) Results

NTA is a real-time visualization method that can rapidly determine the size, concentration, and biological protein biomarkers of exosomes in biological fluids [7]. The isolated GMEs were analysed using the NanoSight NS300 device. The nanoparticles were illuminated by a laser beam, and their movements were tracked under Brown. Then, the NTA was performed to assess the concentrations, sizes, and size densities of the nanoparticles. The size dispersion profiles obtained through the NTA are presented in Figures 1, 2 and 3. NTA analysis settings and results are given in Table 1. These dispersion profiles were then normalized to total nanoparticle concentrations and final cell counts. The particles were between 8 and 413 nm in diameter according to Figures 1 and 2. Since exosomes are generally considered to be 30-150 nm in diameter, the focus was on particles in this size range to measure their concentration. The NTA software identified and measured the particles expected in this range. According to the data we obtained, D10 was measured as 72.8±2.3, D50 was found to be 138.2±7.5, and D90 was 257.8±13.3 nm. In addition, the mean size of the exosomes was determined as 148.8±6.2 nm, and the mode was 97.3±10.3 nm. The concentration of nanoparticles was assessed to be 6.73e+11 particles/mL.

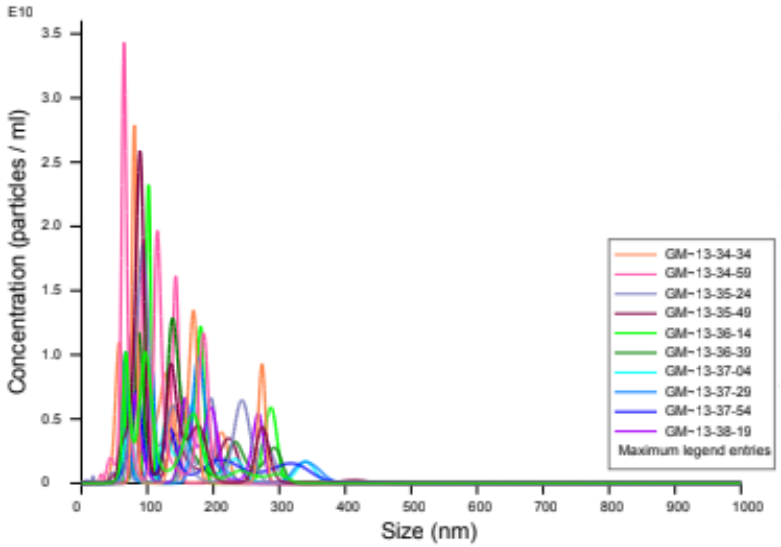

Figure 1. Average particle diameter and particle concentration of GMEs investigated with NanoSight.

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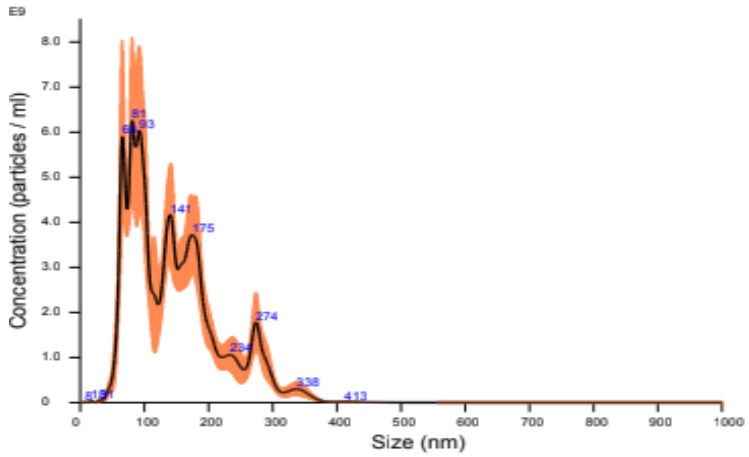

Figure 2. Average particle diameter and particle concentration of GMEs.

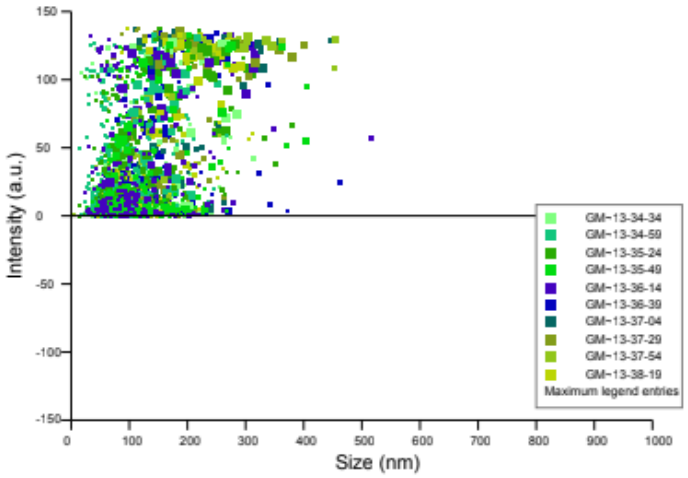

Figure 3. Density graph.

Table 1. NTA analysis settings and results.

Capture Settings		**Results (Mean±Standard Error)**	
Camera Type:	sCMOS	**Mean:**	148.8±6.2 nm
Laser Type:	Green	**Mode:**	97.3±10.3 nm
Camera Level:	15	**SD:**	66.5±3.6 nm
Slider Shutter:	1206	**D10:**	72.8±2.3 nm
Slider Gain:	366	**D50:**	138.2±7.5 nm
FPS	25.0	**D90:**	257.8±13.3 nm
Number of Frames:	499	**Concentration (Upgrade):**	6.73e+11±4.39e+10 particles/ml 11.4±0.6 particles/frame 14.2±0.9 centres/frame
Temperature:	21.8 - 22.0 °C		
Viscosity:	(Su) 0.953-0.956 cP		

Dilution factor:	1 x 10e4

3.2. Results of Protein Analysis

The protein concentration of the GMEs was determined with the BCA protein analysis method. The absorbances (A1, A2) of the plate at 570 nm were reported. The BCA test is a widely used colorimetric method to estimate the protein concentration in samples (Morton and Evans, 1992). The standards are presented in Figure 4. The protein concentration of the GMEs was estimated utilising the standard curve obtained from the serial dilution of the BCA, the graph of which is presented in Figure 4. An increasing trend was observed with the increasing number of fractions. The protein particle concentration (particle/ml-1) of G. munzuriensis was found to be 0.0246 µg/mL. When the total protein amounts obtained through BCA and the exosome concentration measured by means of the NTA (Figure 2) are compared, it was concluded that there were enough exosomes secreted to conduct the study.

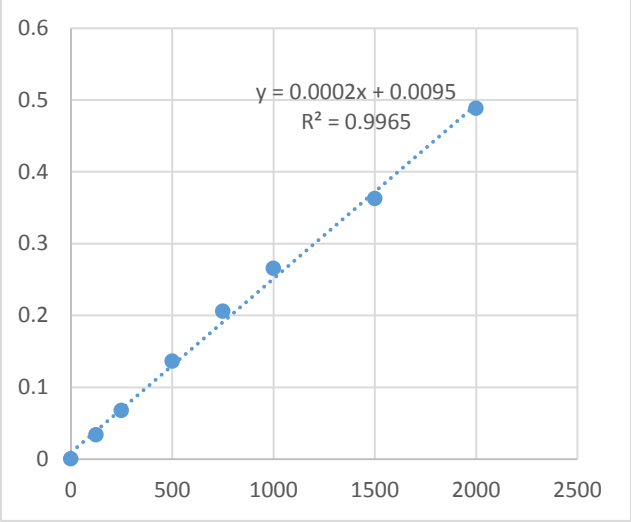

Figure 4. BCA Standard Curve Plot (Concentration/Absorbance).

3.3. Cell Viability (WST-1) Analysis Results

To determine the toxic dose of GME in cancer cells, a cell viability assay was performed using a WST-1 reagent-based assay. The operation principle of WST-1 depends on the division of tetrazolium salt by a cellular mitochondrial dehydrogenase to form a formazan (Yin et al., 2013). HCT 116 and A549 cells were added in 96-well plates. After the cells were added with 25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL and 250 µg/mL GME, the absorbance of the living cells was measured after 24, 48 and 72 hours of incubation.

3.4. HCT 116 WST-1 Analysis Results

Cell viability of the colon canser (HCT 116) cell line was presented in Figure 5 in response to increased doses of GMEs and treatment times. According to our findings, there was no linear decrease or increase in the 24-hour incubation compared to negative control due to dose, but each dose was found to affect the viability of the cells by an average of 40-50%. After the 48-hour incubation, GMEs are found to affect cell viability at rates of 30-40% in all doses compared to negative control. The

most significant decrease was observed in the 72-hour incubation, and the cytotoxic effects of GMEs against HCT 116 cells were clearly observed especially in 50 and 250 µg/mL groups. Cells waiting for 72 hours affected cell viability by 65-70%.

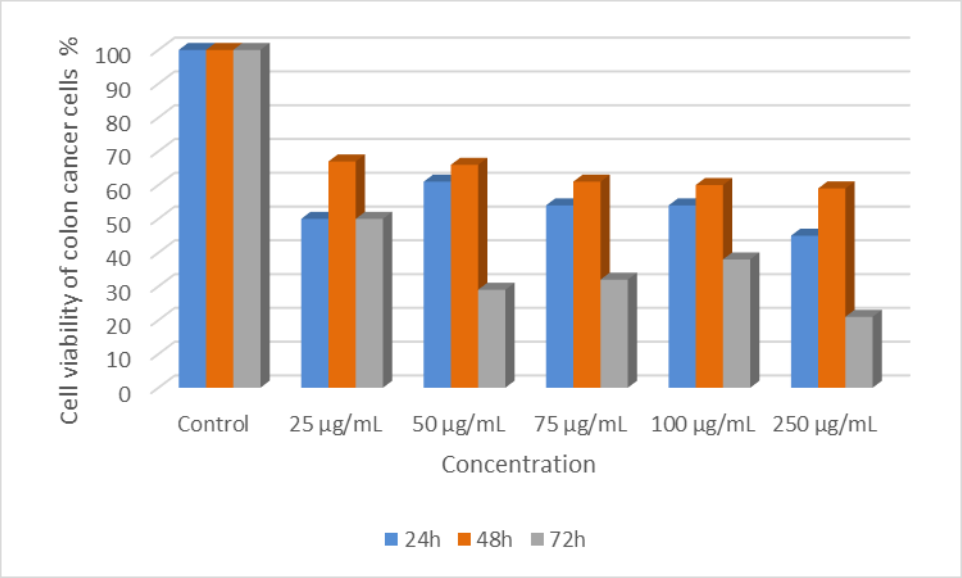

Figure 5. Cell viability of colon cancer cells.

3.5. A549 WST-1 Analysis Results

The cell viability response of the A549 cell line to different doses and administration times of GMEs is presented in Fig. 6. According to the findings, a significant decrease in cell viability was observed only at the 250 µg/mL concentration of the 24-hour incubation. However, 24 and 48 hours of incubation showed a steady decrease in cell viability due to increased concentrations compared to negative control. The effect of 24-hour incubation was 25-40% on cell viability. On the other hand, 48 hours of incubation with GMEs had an impact of 20-25% on cell viability. During the 72-hour incubation, increased concentrations of GMEs were found to increase the number of A549 cells compared to negative control.

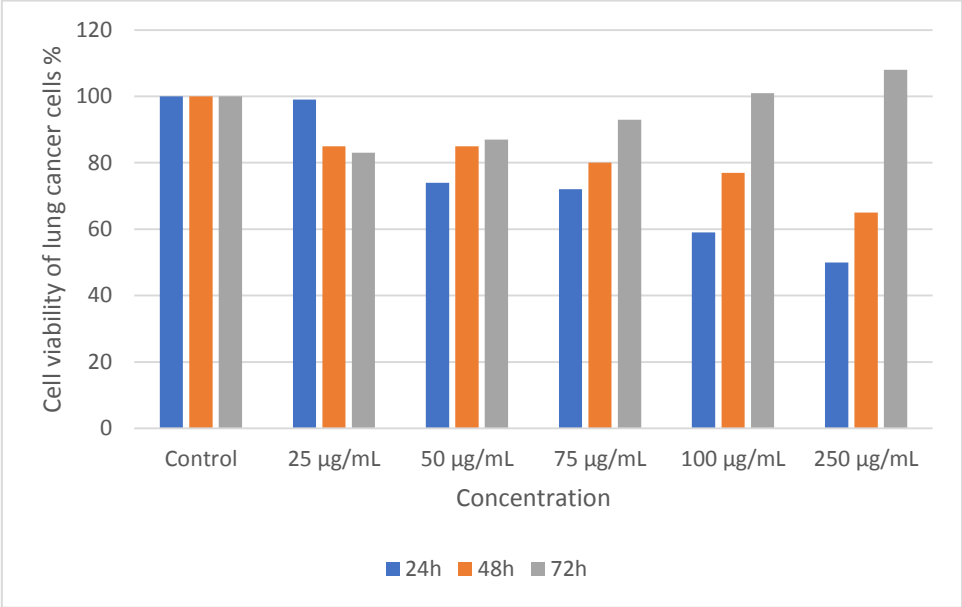

Figure 6. Cell viability of lung cancer cells (A549).

4. DISCUSSIONS

In recent years, the rapid development in the field of exosomes has attracted the attention of many scientists. As newly discovered agents of intercellular communication, exosomes have areas of application in the early diagnosis and treatment of tumours. Exosomes are 30-100 nm nanovesicles involved in maintaining cell-to-cell communication. New information about their content and functions has been revealed every day since their discovery in 1983. Various protein components of exosomes are surrounded by a double layer of lipids, and have a morphologically spherical structure [10].

It is known that plant exosomes are used in the diagnosis and treatment of various diseases besides mammalian exosomes. Studies demonstrating their therapeutic effects particularly on cancer are increasing [6, 3, 11]. Even though some therapeutic studies exist about several species of the genus Gundelia, the potential effects of the endemic species *G. munzuriensis* on cancer cells have not been investigated to date.

The aim of this study is to investigate effects of the exosomes isolated from the *G. munzuriensis* plant on A549 and HCT 116 cancer cell lines. The experimental procedure incorporates the isolation and characterisation of the exosomes of the *G. munzuriensis* plant which is dried through centrifuging and the two-phase gradient ATPS method with Polyethylene Glycol (PEG)-Dextran (DEX). According to the results, the isolation of relatively homogenous and stable exosome-like extracellular vesicles from the *G. munzuriensis* plant was successfully achieved. Evaluations of exosomes were carried out with nanoparticle tracking analysis (NTA) and BCA analysis for their size, morphology, and characterization of protein contents. Since their contents are variable, the choice was based on the distinctive size and morphology of the exosomes, which are known as some of the indicators of their carrier structures. Based on these findings obtained from the characterisation experiments, we arrived

at the conclusion that Gundelia exosomes were successfully isolated. According to the BCA results of GME, the protein particle concentration (particle/mL^{-1}) was determined as 0.0246 µg/mL. Besides, the diameter of the particles was between 8 and 413 nm according to the NTA results (Figures 1, 2 and 3) (Tablo 1). According to the data we obtained, the mean size of exosomes was found to be 148.8±6.2 nm and mod 97.3±10.3 nm. The concentrations of nanoparticles were measured as 6.73e+10. When the total protein amounts obtained with BCA (Figure 4) and the exosome concentration measured by NTA (Figure 2) were compared, it was determined that sufficient amounts of exosomes were obtained for our study.

After the necessary analyses, to evaluate the hypothetical cytotoxic effects of the *G. munzuriensis* exosomes on the A549 and HCT 116 cancer cell lines, WST-1 cell viability assay was performed on cells which were incubated with exosome samples of five different concentrations varying between 25 µg/mL and 250 µg/mL for 24, 48 and 72 hours. To determine the optimal treatment concentration, the cells were processed with exosomes at five different GME concentrations, including 50 µg/ml, 75 µg/mL, and 100 µg/mL, for 24, 48 and 72 hours. The results are presented in Figures 5 and 6. As the findings of the HCT 116 WST-1 studies demonstrate in Figure 5, the application of the GMEs on the cell lines analysed in this research resulted in a substantial decrease (40-50%) in cell viability depending on the dose. On the other hand, as seen in Figure 6, the application of GMEs on A549 cell lines did not lead to a significant decrease in cell viability according to the findings of the WST-1 assays on these lines, and it was observed that it increased cell proliferation in the 72-hour incubation depending on the dose.

In a similar study with *G. tournefortii*, Saraç et al. (2019) observed that the plant seeds applied in vitro on fibroblast (L929), HUVEC, MCF-7, prostate cancer (PC3) and stomach cancer (MKN-45) cell lines had a significant cytotoxic activity only on prostate cancer cell lines [12].
Similarly, the anticancer effects of the exosomes obtained from the edible *Vaccinium myrtillus* (European blueberry) plant on A549 cell line have also been investigated. The cytotoxic activities of the isolated exosomes were assessed through the MTT test. As a result of the investigation, it was determined that the 50 µm exosome solution demonstrated a cytotoxic effect on the A549 cell line in the 72-hour period [13].

In another study related to the effects of Gundelia on cancer, MCF-7 and HUVEC cell lines were treated for 24 and 48 hours with 50, 100, 250, 500 and 1000 µg/mL concentrations of *G. tournefortii* extracts, and XTT test was performed. All plant extracts are found to have cytotoxic activity on MCF-7 and HUVEC cells in both 24-hour and 48-hour periods as well as in all application doses in general. In addition, the root and leaf extracts were also determined to have generally similar levels of cytotoxic activity [14].

Another study found a decrease in VEGF and an increase in caspase 3 expression compared to the control group as a result of the treatment with exosomes obtained from the garlic plant in colon cancer (HCT 116) , breast cancer (MDA-MB 231) and small cell lung cancer (DMS 114) cell lines. However, the opposite was observed in the control cells with an increase in VEGF and a decrease in caspase 3 expression [3].
The effects of exosomes derived from lemon juice (Citrus lemon L.) on A549, human colorectal adenocarcinoma cell line (SW480) and chronic myeloid leukaemia (LAMA84) cell lines were examined in another study. These cell lines were treated in vitro with 5 or 20 µg/ml of nanovesicles

for periods of 24, 48 or 72 hours. The MTT viability test findings revealed that lemon nanovesicles inhibited tumour cell viability depending on dose and time compared to untreated cells [15].

5. CONCLUSIONS

Edible plants have been seen as an important alternative in the diagnosis and treatment of many diseases since prehistoric times. The plant type or species preferred in the use of these resources allow therapeutic applications in various diseases according to their content. Determining the phytochemical content of a plant enables the determination of the area of use for this plant. There are many phytochemical components derived from the leaves, roots, seeds, and flower buds of the Gundelia plant. Phytochemicals play essential roles in pathological pathways with their biological activities such as being anti-cancerogenic and antioxidants caused by antioxidant properties. Therefore, it is considered that Gundelia species will have significant roles in the diagnosis and treatment of cancer diseases. The fact that exosomes have a wide and advantageous place in cancer-related studies makes the exosomes obtained from these plants more important.

In our study, increasing concentrations of GMEs are found to demonstrate cytotoxic effects on HCT 116 cell lines in a three-day period. On the other hand, a 50% decrease was observed in the 24-hour 250 µg/ml application in the A549 cell lines. As a result, current studies with plant-derived exosomes suggest that these nanovesicles may be promising therapeutic agents for future cancer treatment as a single agent or in combination with other materials. When their toxic effects on cancer are considered, GMEs could be a new example of a series of plant-borne exosomes that can be used to treat cancer and other chronic diseases.

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