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Cytotoxic effects of silver nanoparticles synthesized using *Asphodelus aestivus* Brot. aqueous extract

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ABSTRACT: The aim of the study is to examine the cytotoxic, apoptotic effects and gene expressions of *Asphodelus aestivus* water extract (ASP) and silver nanoparticles (AgNPS) on decided cancer lines. Breast cancer cell lines MCF-7 and MDA- 231; melanoma cancer lines MEWO and CHL-1; fibroblast cancer lines WI-38 and HEL 299 were selected for biological activities. xCELLigence system was used for cytotoxicity. Annexin V-EG FP Apoptosis detection kit was used for apoptosis and gene expressions were assessed by real time online RT-PCR by using cancer cell lines Qiagen kits. AgNPS showed significant cytotoxicity in all cell lines. The most prominent apoptosis was determined in MCF-7 cell line for AgNPS. It has been observed that the most important progress in gene expression, the suppression capacity of MUC1-C, a breast cancer- associated oncoprotein, is greatly increased by nanoparticle formation. In addition, cells that were not affected at all in MDA-MB-231 breast cancer oncoprotein started to suppress with nanoparticles. In conclusion, it was determined that silver nanoparticles increased the effect especially in breast cancer cell lines and could be considered for use.

KEYWORDS: Green synthesis; apoptosis; cancer; melanoma; cancer cell lines; silver nanoparticles.

1. INTRODUCTION

In recent years silver nanoparticles (AgNPS) have attracted the attention of researchers in the field of nanotechnology. Silver nanoparticles are mostly biologically active and there are various physical and chemical methods for synthesis. These are sol gel process, chemical reduction, reverse micelle method, hydrothermal method, laser-mediated synthesis and biological methods [1-3]. They need high energy, temperature and toxic chemicals for production. Consequently, the green synthesis from plants is an eco-friendly, cost effective and non- toxic method. Therefore, green synthesis has gained importance around the world. Additionally, using plant extracts and the ratio of metal ions are found to be much faster and its stability is much better than microorganisms. There are several research in the literature about green synthesis of AgNPS using different plant extracts. *Azadirachta indica, Cassia tora, Elephantopus scaber, Oryza sativa* and *Arbutus unedo* can be given as examples [4-6]. The genus *Asphodelus* (belongs to Liliaceae family) has 5 sections, includes 16 species. The genus has a wide distrubition among Mediterrenean. *Asphodelus aestivus* Brot. is one of the *Asphodelus* L. species in Turkey. The genus *Asphodelus* is locally known as "çiriş otu" and "yalancı çiriş". It is used in traditional medicine and as a food material for years in Turkey. In traditional medicine, it is used for its wound healing, diuretic, antihemoroidal effects and used for alopecia, eczema [7-9]. There are much research about biological activities of *A. aestivus'* various extracts such as antifungal, antioxidant, cytotoxic [10, 11]. The chemical composition of *A. aestivus* showed that, it contains valuable compounds like flavonoids, triterpenes, glycosides, anthraquinones [12-14]. These secondary metabolites are biologically active and also known to assist bioreduction of Ag ions.

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The aim of the recent study is to investigate cytotoxic, apoptotic effects and gene expressions of ASP and AgNPS. Our purpose is to evaluate the effects in silver nanoparticles where we expect the activity increase based on our previous research.

2. RESULTS

2.1. Characterization of Silver Nanoparticles

According to the characterization results, a peak between 320-520 nm specific to monodisperse silver nanoparticles was observed in UV-Vis spectroscopy. The high shift in the major bands 3397 to 3398.84, 1612 to 1618.74 and 1366 to 1384.47 in FT-IR analysis indicated the formation of nanoparticles. The XRD results crystalline patterns were 2θ= 38.1, 44.3, 64.4, 81.5 which respond to (111), (200), (220) and (222) (JCPDS card no. 040783) for cubic and monoclinic nanoparticles. According to EDX C, O and Ag were seen. TGA proved the thermal stability and zeta potential (-22.2 mV) indicate the strong stability of the silver nanoparticles.

2.2. Cytotoxicity of AgNPS and ASP

Breast cancer lines MCF-7 and MDA-MB-231 and melanoma cancer lines MeWo and CHL-1 and fibroblast cell lines WI-38 and HEL 299 (control cell lines) were treated with AgNPS and ASP for 72h to analyze the cytotoxic effects. The IC_{50} values of AgNPS on MCF-7, MDA-MB-231, MeWo, CHL- 1, WI-38, and HEL 299 cells were determined as 25,73 µg/ml, 13,21 µg/ml, 30,00 µg/ml, 3,26 µg/ml, 30,66 µg/ml, 35,44 µg/ml respectively. AgNPS showed significant cytotoxicity in all cell lines. For ASP, IC_{50} values in MCF-7, MDA-MB- 231, MeWo, CHL-1, WI-38, and HEL 299 cell lines were also determined as 4.39 mg/ml, 25.56 µg/ml, 74.18 µg/ml, 29.44 µg/ml, 98.98 µg/ml and 146.10 µg/ml, respectively. ASP had less cytotoxicity in other cell lines except for the MCF-7 cell line compare to AgNPS (Figure 1).

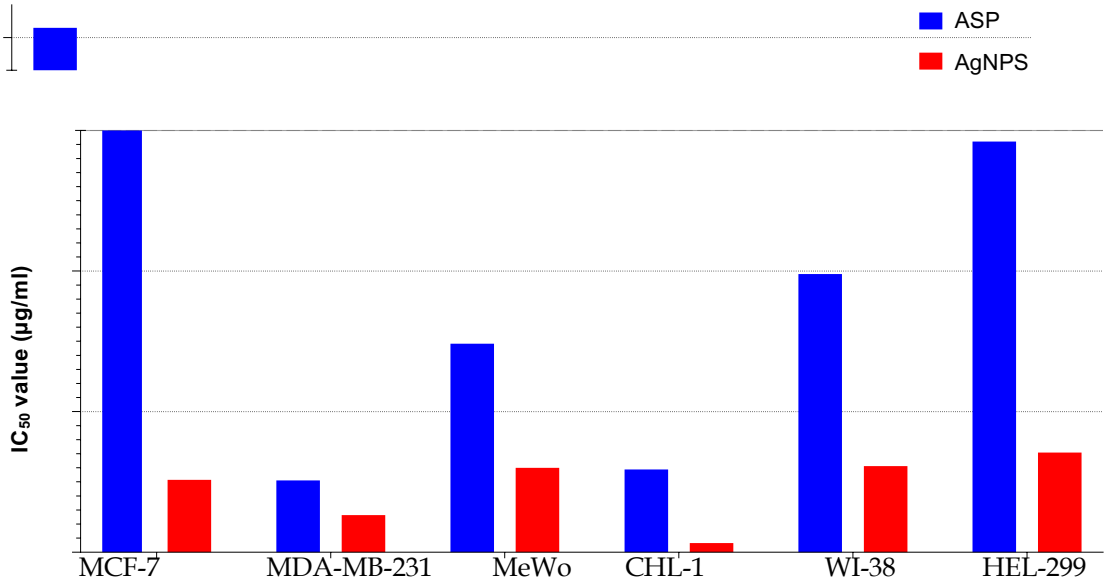

Figure 1. IC_{50} values of ASP and AgNPS in cell lines

IC_{50} values of AgNPS decreased 17-fold in MCF7 cell line and 9-fold in CHL cell line compared to ASP. AgNPS showed significant cytotoxicity at lower doses in Breast cancer and melanoma.
ASP: *A. aestivus* aqueous extract, AgNPS: *A. aestivus* silver nanoparticles

2.3. Apoptotic effect of AgNPS and ASP

The effects of synthesized nanoparticles and ASP on IC50 values in cancer lines on mitochondrial membrane potential were evaluated by Aneksin V. The effect of AgNPS to IC50 values on mitochondrial membrane potential (MMP) was determined in the mammary cell line (MCF-7), and nanoparticle treatment caused a more than twofold decrease in MMP compared to the control group and it was concluded that it induced apoptosis.

The apoptotic effect of AgNPS IC50 values were determined and apoptosis was increased 1,42 fold in MCF-7 cells, 1,42 fold in MDA-MB-231, 1,23 fold in CHL-1 cells, 0,74 fold in MeWo cells, 6,17 foldin WI-38 cells and 1,58 fold in HEL 299 cells compared to the control group and ASP induced 4.93 fold, 1.21 fold,-1.03 fold, -2 fold, 1.55 fold, and 31.85 fold, respectively. In breast cancer cell lines, the apoptotic effect of IC50 values of AgNPS and ASP were evaluated. The most prominent apoptosis, 4.93-fold increase, was determined in MCF-7 treated with ASP (Figure 2).

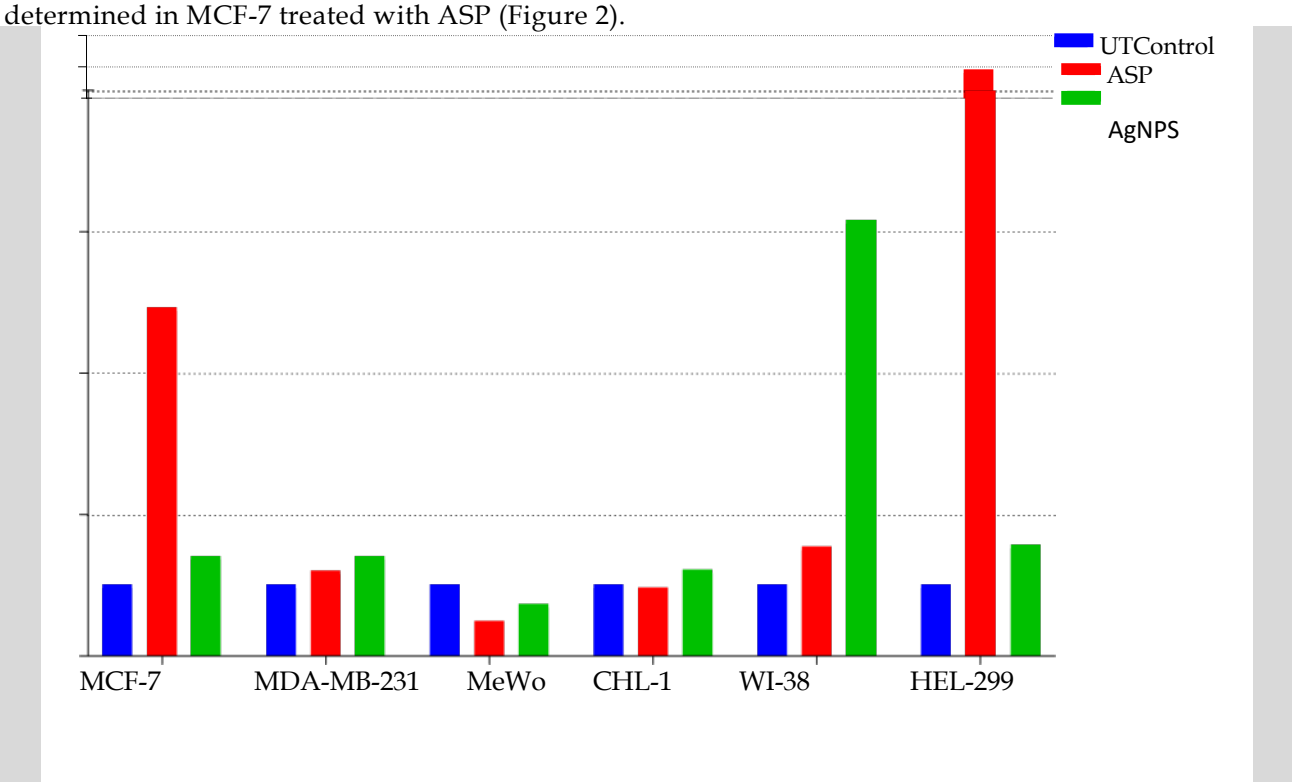

Figure 2. Apoptosis of ASP and AgNPS in cell lines.
ASP increased apoptosis in the MCF-7 cell line approximately 5-fold compared to the untreated cell line. While apoptosis was not observed in other cancer cell lines, it was detected in control cell lines. ASP: *A. aestivus* aqueous extract, AgNPS: *A. aestivus* silver nanoparticles.

2.4. Effect of AgNPS on gene expression

When the effects of water extract and AgNPS IC50 values on breast cancer gene expressions in breast cell line (MCF-7) were examined, water extract decreased the expression of 11 genes 2-7 times compared to the control group and increased the expression of 4 genes 2-11 times. AgNPS decreased the expression of 16 genes 2 - 8 times compared to the control group, and increased the expression of 4 genes 2 - 10 times. There was no significant change in other gene expressions compared to the control. When the effects of water extract and AgNPS IC50 values on gene expressions effective in breast cancer were examined in the breast cell line (MDA-MB-231), water extract increased the expression of 4 genes 2-9 times. AgNPS reduced the expression of 9 genes 2-9 times compared to the control group. There was no significant change in other gene expressions compared to the control group (Table 4). 8 genes showing co-sense gene expression changes were identified in both breast cancer cell lines (MCF-7 and MDA-MB-231). Progesterone Receptor (PGR) gene expression

increased 9-10 fold by water extract and AgNPS treatment in hormone-dependent MCF-7 cell line. AgNPS treatment showed more than 2-fold reduction in RASSF1, SRC, TP73 and TWIST1 gene expressions in both cell lines. TWIST1 was the gene with decreased expression. Retinoic acid receptor, beta (RARB) gene expression also increased more than 3 times in water extract application. Other genes showed similar expression profile. When the effects of waterextract and AgNPS IC_{50} values on gene expressions in breast cancer in melanoma cell line (CHL-1) were examined, water extract increased the expression of 21 genes by 2-14 fold compared to the control group and decreased the expression of 12 genes by 2-13 fold. AgNPS increased the expression of 15 genes 2 – 8 fold compared to the control group, and decreased the expression of 9 genes 2 – 11 fold. There was no significant change in other gene expressions compared to the control (Table 5). When the effects of water extract and AgNPS IC_{50} values on gene expressions in breast cancer in melanoma cell line (MeWo) were examined, water extract decreased the expression of 8 genes 2-11 fold and increased the expression of 28 genes 3-15 fold compared to the control group. AgNPS reduced the expression of the CDH8 gene 3 times compared to the control group. There was no significant change in other gene expressions compared to the control (Table 6). Eleven genes were identified that showed co-significant gene expression changes inboth melanoma cancer cell lines (CHL-1 and MeWo). While BCL6, CXCL12, DNAJC15, RB1 and TPM1 gene expressions increased in water extract in both cell lines, they showed different expression profiles in both cell lines and decreased gene expressions in AgNPS application. Other genes showed similar expression profile.

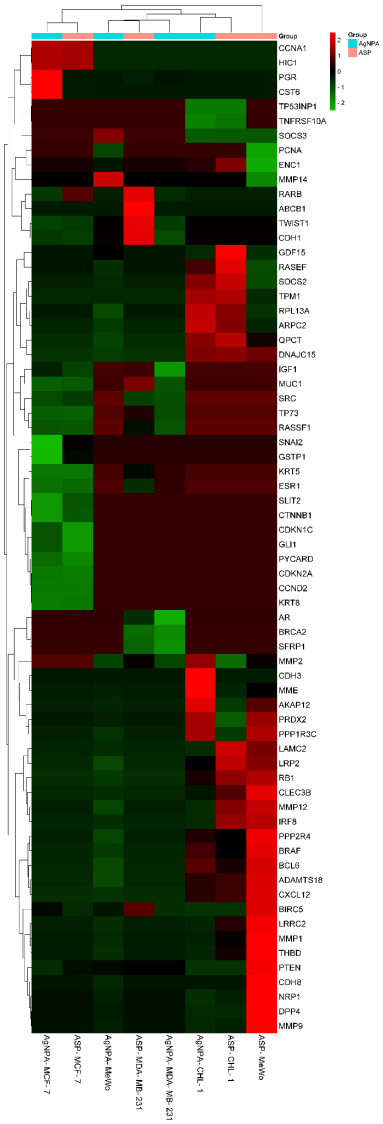

Figure 3. Gene expression analysis results of cancer cell lines

3. DISCUSSION

In the previous study green synthesized AgNPS was characterized with some methods as ultraviolet-visible spectroscopy (UV-vis), Fourier transform infrared spectroscopy (FT-IR), transmission electron microscopy analysis (TEM), scanning electron microscopy / energy-dispersive X-ray spectroscopy (SEM/EDX), thermal gravimetric analysis (TGA), X-ray diffraction analysis (XRD) and zeta potential [22]. Cytotoxicity studies of the characterized nanoparticles were carried out by various methods. ASP has low cytotoxicity in the MCF-7 cell line, and the IC_{50} value in the MCF-7 cell line at 72^{nd} hours was found to be 4.39 mg/ml. Aslanturk and Çelik (2013) found 185.92 µg/ml at the 24^{th} hour and 89.68 µg/ml at the 72^{nd} hour in the MCF-7 cell line [11]. This difference in cytotoxicity may due to the use of different parts of the *A. aestivus* plant. The tubers were used in Aslanturk and Çelik (2013)'s studies while aerial parts of *A. aestivus* were used in recent study. AgNPS had significant cytotoxicity in all cell lines. The significant cytotoxicity of AgNPS (25.73 µg/ml in breast cancer and 30.00 µg/ml in melanoma) was evaluated positively in terms of the effectiveness of the nanoparticle [11]. The cytotoxicity of *A. aestivus* extract has not been investigated in melanoma. In breast cancer cell lines, the apoptotic effect of AgNPS and IC_{50} values of the water extract

in MCF-7 was determined most clearly in the water extract, and a 4.93-fold increase in apoptosis was determined when compared to the control group. No apoptotic effect was observed in melanoma celllines. In the studies of Aslanturk and Çelik, a similar rate of apoptosis was observed for the water extract. They suggested that the prooxidant flavonoids in the water extract may cause oxidative DNA damage in cancer cells [11]. In present study, it is considered that the apoptotic effect of ASP in MCF-7 is due to oxidative DNA damage. However, we think that the reason why water extract does not show apoptotic effect in other cancer cell lines is that it cannot cause oxidative DNA damage because the antioxidant systems work efficiently in these cell lines. Steroid hormones regulate the transcriptional regulation of target genes, cell growth and function through nuclear receptors. These receptors bind to ligands and stimulate transcription with transcriptional cofactors. SRC, one of the nuclear receptor coactivators, is known to interact with nuclear receptors and enhance transactivation function in a ligand-dependent manner. SRC overexpression induces 17β-estradiol-stimulated cell proliferation and increases transcriptional activation of exogenous and endogenous 17β-estradiol-responsive genes [22]. In breast cancer cell lines, SRC expression was 2-fold down-regulated by AgNPS. It had been considered that the induction of SRC-mediated cell proliferation is also effective in the significant cytotoxicity of AgNPS, in breast cancer cell lines. TWIST, the essential helix-loop-helix transcription factor, is an important regulatorof mesenchymal phenotypes. It is expressed in human mesodermal tissues. It has been shown to be required for neural tube closure and is a repressor of myotome differentiation outside the somites [23]. Mironchik et al. (2005) performed persistent overexpression of TWIST in the MCF-7 cell line, and they showed that the cell morphology changed to a fibroblastic-like phenotype with mesenchymal transformation and protein markers. They also showed increased vascular endothelial growth factor (VEGF)expression in these cells. They suggested that TWIST overexpression in breast cancer cells induces angiogenesis, correlates with chromosomal instability, and promotes an epithelial-mesenchymal-like transformation that is crucial for its transformation into an aggressive breast cancer phenotype [24]. In recent study, TWIST1 expression was decreased two-fold in both cell lines with both nanoparticle treatments. It has been suggested that AgNPS treatment can reduce angiogenesis and metastasis in breastcancer with the support from the literature. Bai et al. showed that they induce apoptosis by overexpressionof BCL6 and downregulation of BCL2 and BCL-XL in diffuse large B-cell lymphoma [25]. It was determined that water extract and AgNPS treatment increased BCL6 gene expression in melanoma cell line (CHL-1) andwater extract treatment increased BCL6 gene expression in MeWo cell line. It has been evaluated that water extract and AgNPS can provide suppression of BCL2 and BCL-XL in apoptosis induction. The MUC1-C oncoprotein is associated with breast cancer. When the gene expressions in mcf-7 were examined, the suppression capacity increased from 4.65 to 7.69. On MDA-MB-231, on the other hand, gene expression was not affected at all by ASP, while 3.96 suppression was observed with nanoparticle formation. The effectof *A. microcarpus* on human malignant melanoma cells was investigated. It was determined that the obtained fractions had an effect IC_{50} value between 83-65 µg/ml and were suitable for further *in vivo* studies [25]. In another study, the migration and invasive ability of breast cancer cells was examined and it was observed that it inhibited its metastatic feature [26]. *A tenuifolius* has been shown to inhibit cell proliferation in melanoma cancer cells [27]. In another study with *A. aestivus*, inhibition was determined in MCF7 and different cancer cell lines [28]. As a result, AgNPS synthesized in this study showed significant cytotoxicity inbreast cancer and melanoma cell lines, induced apoptosis and carried out gene expression regulation that can be considered important especially for breast cancer treatment.

4. CONCLUSION

The anti-cancer activity of AgNPS was determined *in vitro*. In breast cancer and melanoma cell lines, AgNPS showed lower cytotoxicity than ASP. It has been suggested that AgNPS induces caspase- independent apoptosis. It is recommended that this finding is going to be supported by relevantmethods. In addition, it has been shown that AgNPS regulates gene expressions that can be considered important in terms of

prognosis in breast cancer and melanoma cell lines, and it is recommended that the gene expressions regulated by AgNPS be evaluated in vivo animal models.

5. MATERIALS AND METHODS

5.1. Plant material

A. aestivus L. was collected from Kuşadası (Aydın, Turkey) in January 2014. The plant was identified by Prof. Dr. Bijen KIVÇAK from Ege University Faculty of Pharmacy, Department of Pharmacognosy, Izmir, Türkiye and voucher specimen (No. 1520) is deposited in Herbarium of Ege University, Faculty of Pharmacy, Department of Pharmacognosy.

5.2. Preparation of plant extract

Plant extract was prepared by 5% infusion method by using the aerial parts of the plant. Infusion was then filtered and solvent was evaporated using vacuum evaporator [26].

5.3. Synthesis of AgNPS

Silver nitrate (Sigma-Aldrich, St Louis, USA) solution was used for the synthesis of AgNPS. 10 ml aqueous extract was mixed with 100 ml of 1 mM $AgNO_3$ (silver nitrate). Mixture was incubated by stirring at 60°C for 30 min. AgNPS were obtained after the color change from yellow to brown. Thesolution of silver nanoparticles was centrifugated at 5000 rpm for 30 min. (minute) for purification. After the procedure, it was lyophilized and stored [29].

5.4. Characterization of silver nanoparticles

Characterization of AgNPS were evaluated by Ultraviolet-Visible (UV-Vis) spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, Transmission electron microscopy analysis (TEM), Scanning electron microscopy / energy-dispersive x-ray spectroscopy (SEM/EDX), Thermal gravimetric analysis (TGA), X-ray diffraction analysis (XRD) and zeta potential characterization methods [29].

5.4.1. Ultraviolet-Vis spectroscopy (UV-Vis) analysis

Proof of synthesis and stability of nanoparticles will be performed with a device named Thermo Evolution Array UV-Visible spectrophotometer. During synthesis, values will be monitored before and after the incubation step and the presence of bathochromic shift will be evaluated. Measurement will be continued at 30 minutes intervals (30, 60, 90, 120, 150 minutes) until the absorption bands in the visible region stabilized [30, 31].

5.4.2. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

Fourier transform infrared spectroscopy analysis (Perkin Elmer Spectrum 100) will be performed to determine the interaction state and functional groups between the components of silver nanoparticles. Comparisons will be made by taking the FT-IR spectra of water extract and nanoparticles separately by Fourier Transform Infrared Spectroscopy between 4000-400 cm^{-1} [30, 31].

5.4.3. Morphological imaging with Scanning Electron Microscopy (SEM)

Scanning electron microscopy (Quanta250 FEG, Scanning Electron Microscopy-SEM) will be used to examine the morphology (particle structure, surface shape) of silver and iron nanoparticles. [30, 31].

5.4.4. X-Ray Diffraction (XRD) analysis

Crystal structure determination of silver nanoparticles, XRD analysis, will be performed using diffractometry [30, 31].

5.4.5. Energy-Dispers X-Ray (EDX) analysis

Elemental composition will be determined with the EDX detector. EDX analysis will be done with the SEM250 FEG Quanta device using the EDX detector [32].

5.4.6. Thermal gravimetric analysis (TGA)

Thermogravimetric analyzes are really important to know about thermal stability. It was carried out with a heating rate of 20°C/min using a Perkin Elmer TGA-4000 thermal analyzer. Analyses were achieved between 20 and 500°C temperatures range [31].

5.4.7. Zeta potential measurement

It will be determined by dynamic light scattering method using Malvern Zetasizer 1000 HS (Malvern Instruments, England). Three trials will be made from each sample [31].

5.5. Cell lines

Breast cancer lines MCF-7 and MDA-MB-231; melanoma cancer lines MeWo and CHL-1; fibroblast cancer lines WI-38 and HEL 299 (control cell lines) were selected for the recent study. Cell lines were cultured and maintained in mediums (EMEM for MCF-7, MeWo, HEL-299, WI-38 cell lines, DMEM for CHL-1 cell line and L15 for MDA-MB-231 cell line) containing 1% L-glutamine supplemented with 10% inactivated FBS and 1% penicillin/streptomycin and were maintained in a humidified incubator at 37°C with 5% CO_2. Analysis of cell viability was carried with trypan blue test [33].

5.6. Cytotoxicity assay

Breast cancer (MCF-7, MDA-MB-231), melanoma (MeWo, CHL-1) and control fibroblast (WI-38 and HEL 299) cell lines were seeded at 1×10^4 cells/well in 3 replicates on gold-coated plate96 well E-plates in xCELLigence system (ACEA Biosciences). Cells were incubated overnight. Synthesized AgNPS and ASP at varying concentrations (3.13 – 100.00 µg/ml) were added. The cells were incubated at 37°C for 72 hours in an incubator containing 5% CO_2 and 95% humidity, and the impedance of the E-plate was recorded every 15 minutes during the period. Cytotoxicity activity was evaluated with normalized cell index using xCELLigence software, and IC_{50} values and statistical analyzes were determined by sigmoidal dose-response analysis. The analysis was performed in 3 repetitions.

5.7. Analysis of apoptosis

AnnexinV-EGFP Apoptosis Detection Kit Biovision was used for determination of apoptotic effects. 5 x 105 cells were treated with their IC50 doses of each for 72 h. At the end of 72 h, cells were collected, washed and resuspended. The cells were incubated after the addition of AnnexinV- EGFP and propidium iodide (PI). AnnexinV-EGFP and propidium iodide (PI) [33].

5.8. Gene expression analyzes

In breast cancer and melanoma cell lines, effects of AgNPs and ASP on gene expressions were assessed by real time online RT-PCR (LightCycler 480 II) by using RT² Profiler PCR Array Human Breast Cancer and Human Melanoma PCR Array, Qiagen Kits. Total RNA extraction and cDNA were performed according to the kit manual, by using RNeasy Mini Kit (Qiagen) and RT2 First Strand Kit

cDNA Synthesis kit (Qiagen), respectively [33].

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