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Original Article

Differences in genotoxicity and cytotoxicity potentials of green and chemically synthesized silver nanoparticles

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ABSTRACT

Background and Aims: In recent years, metal nanoparticles have been extensively synthesized for a variety of applications and have been used in large-scale research in various fields, such as chemistry, physics, life science, material science, medical science, and engineering, depending on their size and shape adjustment properties. In this study, we aimed to compare the effects of silver nanoparticles synthesized using two different methods on DNA damage and cell viability in human lymphocyte cultures.

Methods: We introduced a green and simple method for the synthesis of AgNPs using endemic *Onosma papillosa* Riedl leaf extract as a reducing agent for the first time. Blood samples were collected in heparinized tubes from four healthy males, non-smokers, and healthy male. In this study, we used comet assay [Genetic Damage Index (GDI) and Damaged Cell Percentage (DCP)] and flow cytometry methods for genotoxicity and cytotoxicity.
For comparison, commercially obtained AgNPs synthesized by chemical methods were used, with consideration given to the size of AgNPs synthesized via the green method.

Results: Based on the results, it was determined that DNA damage caused by AgNPs synthesized through the green method in human lymphocyte cultures was not statistically significant compared with the negative control. AgNPs obtained by chemical synthesis caused, however, a statistically significant increase in the frequency of DNA damage compared with the negative control ($p<0.001$). The percentage of necrotic cells was 13.55±3.37 and 25.37±14.53 in cultures obtained by green and chemically synthesized AgNPs, respectively.

Conclusion: Essentially, green synthesis can be recommended for use because of its lower toxicity compared with chemical synthesis.

Keywords: *Onosma papillosa*, Green synthesis, Chemical synthesis, Comet assay, Flow cytometry, Genotoxicity, Cytotoxicity

INTRODUCTION

Nanotechnology has received great attention and significant progress in many fields of science such as medicine and in the production of commonly used materials (Nikalje, 2015). This innovative technology has a significant impact on society, influencing numerous sectors such as communication, textiles, medicine, engineering, agricultural products, and food technology (Francisco and Garcia-Estepa, 2018). Among the myriad of products that incorporate nanomaterials, silver nanoparticles (AgNPs) are among the most widely utilized. Because of their unique antibacterial properties, AgNPs can be found in medical bandages, surgical devices, and medical masks to minimise microbial functions (Das et al., 2020; Gkika, Vordos, Magafas, L., Mitropoulos, and Kyzas, 2021; Buzea, Pacheco, and Robbie, et al., 2007).

In recent years, metal nanoparticles have been extensively synthesized for a variety of applications and have been used in large-scale research in various fields, such as chemistry, physics, life science, material science, medical science, and engineering, depending on their size and shape adjustment properties. Silver is one of the most mercantile nanomaterials, with a half million tonnes of silver nanoparticle output per year (Larue et al., 2014). In addition, metal nanoparticles play a profound role in the fields of high-precision biomolecular identification, catalysis, biosensors, and medicine. They have been recognized for their robust inhibitory and bactericidal effects as

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well as their antifungal, anti-inflammatory, and antiangiogenesis activities (Palencia, Berrio, and Palencia, 2017; Veerasamy et al. 2011). Veerasamy et al., (2011) demonstrated that the use of a natural, low-cost biological reducing agent, *Garcinia mangostana* L. leaf extracts (aqueous), can produce metal nanostructures through efficient green nanochemistry methodology, avoiding the presence of hazardous and toxic solvents and waste. Biosynthesized silver nanoparticles using mangosteen leaf extract showed excellent antimicrobial activity. The AgNPs used in the study performed by Veerasamy (2011) et al. have a size of approximately 30 nm.

The effects of AgNPs on viability have been reported in many *in vitro* and *in vivo* studies. Moreover, the toxicity of AgNPs is critically determined by NP size. The AgNPs used in this study have a size of approximately 30 nm. As reported by Avalos et al., (2014), AgNPs with a smaller size exhibit a higher toxicity than larger ones, as they can more easily enter cells and promote reactive oxygen species (ROS) generation. Phagocytosis of AgNPs by macrophage cells results in increased ROS. Therefore, it generates an inflammatory signal and subsequently activates macrophage cells, which induce the secretion of TNF-α. Increment of TNF-α levels cause cell membrane damage and apoptosis (Mikhailova, 2020). It has been reported that silver nanoparticles did not cause any change in biochemical parameters in rats administered a dose of 5 mg/kg. However, significant increases were observed in biochemical parameters on the 29^{th} day in rats administered a dose of 10 mg/kg for 28 days (Nakkala, Mata, Sadras, 2017).

The chemical synthesis of metal nanoparticles requires the use of hazardous chemicals such as sodium borohydride (Alexandridis, 2011) and hydrazine (Iravani, Korbekandi, Mirmohammadi, and Zolfaghari, 2014) as reducing agents to convert metal ions into metal nanoparticles. Green synthesis, unlike chemical synthesis, is an environmentally friendly method in which environmentally friendly compounds are used as reducing agents instead of hazardous chemicals. In green synthesis, bacteria, fungi, plants, algae, and other microorganisms that are part of the biological system are used as reducing agents (Kahraman, Binzet, Turunc, Dogen, and Arslan, 2018). This new approach has emerged as non-toxic, eco-friendly, clean, and cost-effective. It also offers the possibility to be performed under mild conditions (Bharathi, Vasantharaj, Bhuvaneshwari, 2018; Tharani, Bharathi, Ranjithkumar, 2020; Nandana, Christeena, Bharathi, 2022; Hawar et al., 2022; Khane et al., 2022). Green synthesis can indeed be regarded as an alternative method to produce biocompatible nanomaterials, representing an optimal convergence of materials science and biotechnology (Rashidipour and Heydari, 2015; Heydari and Rashidipour, 2015; Heydari, 2017; Heydari, Koudehi, Pourmortazavi, 2019; Arya, Mishra, and Chundawat, 2019).

In this study, we aimed to compare the effects of AgNPs synthesised by chemical and green methods using the endemic *O. papillosa* Riedl plant on oxidative DNA damage using single-cell gel electrophoresis (comet) and cell viability using flow cytometry in human lymphocyte cells. The purpose of using the *O. papillosa* plant in this study is that it is an endemic species in Türkiye, and to the best of our knowledge, it has not yet been used in nanoparticle synthesis.

MATERIALS AND METHODS

Chemicals

Chemically synthesized AgNPs was purchased from SkySpring Nanomaterials, Inc 2935 Westhallow Dr., Houston, TX 77082 (15 nm, purity of Ag: 99.99. Product#0127SH; Lot#0127-031314). Roswell Park Memorial Institute (RPMI) medium, phosphate buffer solution (PBS), normal melting agarose (NMA), Trisma base, Triton X-100, ethylene diamine tetra acetic acid (EDTA), and ethidium bromide (EtBr) were purchased from Sigma. Sodium chloride (NaCl), sodium hydroxide (NaOH), and hydrogen peroxide (H_2O_2) were purchased from Merck. Low melting agarose (LMA), phytohemagglutinin (PHA), penicillin-streptomycin, Fetal Calf Serum were purchased from Bioshop and Biochrom AG, respectively.

Plant material

The *O. papillosa* samples were collected from Niğde province (Locality: Niğde, Ulukışla-Aksaray 8 km (37º 34' N 34º 25' E), slopes, open field and roadside, 1380 m. in June 2017 (Figure 1). The voucher specimen was identified by Dr. Rıza Binzet (2018) and has been deposited in the herbarium of Mersin University (MERA), Mersin Province.

Figure 1. ***Onosma papillosa***

Experimental design I: chemico-biotechnical analysis

Preparation of the leaf extract from O. papillosa

O. papillosa leaves were washed 2-3 times with water and left to dry in the shade at ambient temperature. The dried leaves were ground in a mill to obtain fine powder (Waring 8011, USA) for extraction. The fine powder (5 g) was placed into 250 mL round bottom flask and boiled under stirring for 30 min. in 150 mL of ultrapure water. At the end of the specified period, the mixture was cooled to room temperature, filtered through Whatman No. 1, and the filtrate was stored at 4 °C before use.

Green synthesis of silver nanoparticles

The green synthesis of AgNPs was performed by placing into flask 100 mL of 1.0 mM $AgNO_3$ and 100 mL of *O. papillosa* leaf extract. The reaction mixture was stirred vigorously at 60 ºC. The colour change from yellowish brown to dark brown ratifies the formation of AgNPs. The mixture was allowed to cool to room temperature. For characterization of AgNPs, the reaction mixture was centrifuged at 14000 rpm for 15 min. The supernatant was removed, and the resultant pellet was washed three times, centrifuged, dried, and stored for further characterization.

Structural characterization of silver nanoparticles

The formation of AgNPs was determined by monitoring the color change using a UV-Vis spectrometer (Shimadzu, Japan). The crystallinity nature of the nanoparticles was identified using an X-ray powder diffraction pattern (XRD) (Rikagu, Japan). FT-IR spectroscopy (PerkinElmer, USA), Scanning electron microscopy (SEM) (Zeiss, Germany), Energy-dispersive X-ray spectroscopy (EDX) (Zeiss, Germany), and dynamic light scattering (DLS) (Malvern, England) were applied to determine the morphology, composition, size, and stability of the AgNPs, respectively.

FT-IR analysis

It is stated that plant extracts play a dual role in nanoparticle synthesis. FT-IR analyses were conducted to determine the functional groups of phytochemicals that are thought to be involved in the synthesis of nanoparticles in the *O. papillosa* extract and in providing stable structures of the synthesized nanoparticles.

SEM/EDX analysis

SEM and EDX analyses were performed to determine the surface morphology and chemical composition of AgNPs synthesized using *O. papillosa* extract. For comparison, SEM analyses of chemically supplied AgNPs were also performed.

Preparation of the AgNP solutions

Five mg of both the AgNPs obtained via green synthesis and the chemically synthesized were weighed and dissolved in 10 mL sterile distilled water. The final concentrations were adjusted to 150 µg/mL (Battal et al., 2015).

Experimental design II: Toxicity and genotoxicity evaluation

Collection of blood samples

The blood samples (3 mL) used in this study were taken from four healthy male non-smoking donors (mean age, 24.75 ± 0.82 years). During blood collection, heparinized tubes were used. *The Mersin University Ethical Committee approved the experiments described in this study (06.04.2022-2022/241).*

Lymphocyte isolation, cell growth, and exposure to AgNPs in culture media

Human peripheral lymphocytes (HPL) are noncycling primary cells (G0 cells). They are easily collectable by venipuncture. In the presence of suitable culture media and stimulants *in vitro*, HPL enters the cell cycle and undergoes mitotic division (Johannes and Obe, 2019). Blood samples were collected from heparinized tubes from four healthy male non-smokers. Lymphocyte cultures were prepared according to previous studies (Moorhead, Nowell, Mellman, Battips, and Hungerford, 1960; Sınacı, Çelik, Yetkin, Çevik, S., and Güler, 2023). 5 mL of whole blood was added to 5 mL of Histopak-1077 and centrifuged for 30 min at 2000 rpm. Lymphocytes were separated from the medium using a micropipette. A mixture of 500 µL PBS and 500 µL lymphocytes was prepared. Subsequently, 300 µL of this mixture was transferred into labelled tubes containing 5 mL of RPMI 1640 medium. PHA (0.2 mL) was added to the lymphocyte culture. Subsequently, solutions of 150 µg/mL of each AgNP obtained from chemical and green synthesis were added. In the positive control group, 10 mM H2O2 was used. No chemicals were used in the negative control groups. The tubes were closed and incubated at 37 °C at an angle of 45° for 72 h. Subsequently, single-cell gel electrophoresis and flow cytometry were performed.

Application of the single cell gel electrophoresis (comet) method and slide scoring

Single-cell gel electrophoresis (comet) was performed with lymphocytes from four donors following the protocol outlined by Singh et al. (1988). Briefly, 100 µL of lymphocyte cell suspension and 100 µL of 2% low-melting-point agarose were mixed at 37 °C and then placed on a slide recoated with a thin layer of 0.5% normal-melting-point agarose. The cell suspension was immediately covered with a cover glass, and the slides

were held for 5 min at 4 °C to solidify the agarose. After removing the cover glass, the cells were placed in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4 ºC. After washing in distilled water, the slides were placed in a horizontal gel electrophoresis chamber filled with a cold electrophoretic buffer (1 mM EDTA, 300 mM NaOH, pH = 13), and the slides were kept at 4 ºC for 20 min to allow the DNA to unwind. Then, electrophoresis was conducted at 20 °C using 25 V and 300 mA for 20 min. After electrophoresis, the slides were treated three times with a neutralisation buffer (0.4 M Tris, pH = 7.5). All preparative steps were conducted in the dark to prevent undesired DNA damage. Finally, the slides were stained with ethidium bromide (0.1 mg/mL, 1:4) and analysed using a fluorescence microscope (Olympus BX 51) equipped with a CCD-4230 video camera.

To determine the DNA damage in each experimental sample, two slides and 50 cells per slide were evaluated. A researcher (G.G) blindly scored slides to prevent bias. Damages in cells were classified by eye into five categories on the basis of the extent of DNA migration as undamaged (*Type0*), very little damage (*TypeI*), moderate damage (*TypeII*), high damage (*TypeIII*), and ultra-high damage (*TypeIV*). In the comet assay, two different parameters were evaluated. i. Genetic damage index ii. Damaged cell percentage, in formula below;

Genetic damage index (GDI)=0xType0+1xTypeI+2xTypeII+ 3xTypeIII+4xTypeIV
Dameged cell percentage (DCP)=TypeII+TypeIII+TypeIV

Flow cytometry method/apoptosis assay

The Annexin V-FITC apoptosis detection kit with PI was utilized to specify cellular apoptosis or necrosis. This kit is based on the observation of phosphatidylserine translocation from the inner to outer surface of the plasma membrane, which is easily detected by staining with the fluorescent dye Annexin V. Annexin V, which has a high affinity for phosphatidylserine, was conjugated to FITC for visualization. In flow cytometry analyses, late and early apoptotic cells, live cells, and necrotic cells were counted. Early apoptotic cells are not stained with PI but with annexin V. Late apoptotic cells stained with Annexin V and PI. While necrotic cells are stained with PI, they are not stained with Annexin V. PI and Annexin V dyes do not penetrate living cells (Sliwinska et al., 2015). Approximately $4x10^5$ cells were analysed via flow cytometry to determine the apoptotic response of blood lymphocytes exposed to two different types of AgNPs.

Statistical analysis

Normality control for all parameters was performed using the Shapiro–Wilk test. The STATISTICA 13.0 analysis programme was used for statistical evaluation of the data obtained because of the experiment protocols. To examine if there was a statistically significant difference between the results, the concentrations applied were compared both among themselves and between the positive control values and the negative control values. The averages of the data obtained from the experimental protocols were used for all analyses. One-way ANOVA was used because the data showed normal distribution according to the Shapiro-Wilk test result. The One-Way ANOVA test statistic was employed to compare the averages of more than two independent groups. In case of a significant difference detected with ANOVA, Tukey's test was used as a Post Hoc test. The p value (confidence interval) was set at 0.05 for evaluation. When examining the relationships between Continuous Measurement parameters, the "Pearson Correlation" coefficient was used. "Spearman Correlation" coefficient was used for analyzing the relationships between continuous ordinary measurement parameters.

RESULTS

Structural characterisation of nanoparticles

UV-Vis spectroscopy

The formation of AgNPs synthesized by the green method was determined using UV-Vis spectroscopy. Figure 2a shows the UV-Vis spectrum of the precursor salt $AgNO_3$ and AgNPs. When examining the spectrum, no absorption peak of the precursor salt was observed in the wavelength range of 300–800 nm. It was determined that the absorption peak observed at 430 nm in the same wavelength range is related to the formation of AgNPs. In metal nanoparticles, the conduction and valance bands are so close that electrons move freely. These free electrons of AgNPs, in resonance with light, promote the colour of the AgNPs appearing dark brown. It is known that alkaloids, terpenoids, and polyphenolic compounds in the plant content play a role in the formation of nanoparticles. Flavonoids are polyphenolic compounds containing various groups that are involved in the formation of nanoparticles.

X-ray diffraction method (XRD)

The crystal structure of AgNPs synthesized using *O. papillosa* plant extract was determined by XRD analysis and is shown in Figure 2b. When examining Figure 2b, four characteristic 2θ values between 0° and 80° were determined. According to the results obtained from the XRD spectrum of AgNPs, diffraction peaks corresponding to (111), (200), (220), and (311) crystal structures were observed at 38.09°, 44.26°, 64.53°, and 77.35° angles. It has been determined that the results obtained are compatible with the card number 01-071-9155 obtained from library scanning.

Dynamic light scattering analysis

The particle size of AgNPs synthesised using *O. papillosa* plant extract was measured using DLS. The average particle size and zeta potential of the synthesised AgNPs were determined to be 14 nm and -20.5 mV, respectively (Fig. 2c and 2d). The negative values of the zeta potential indicate that the synthesised AgNPs have high stability. In addition, negatively charged groups on the surface of nanoparticles are believed to prevent the aggregation of nanoparticles.

FT-IR analysis

As depicted in Figure 2e, the band observed at 3272 cm^{-1} in *O. papillosa* extract corresponds to -OH stretching vibrations. In the synthesized AgNPs, a stretching vibration band was observed at 3230 cm^{-1} (Fig. 2e). The bands observed at 2919 and 2849 cm^{-1} in plant extract belong to aliphatic C-H stretches, and these bands shifted to 2916 and 2853 cm^{-1} values in AgNPs (Fig. 2f). The C=O stretching vibration band observed at 1734 cm^{-1} in plant extract was not observed in the spectrum of AgNPs. The bands observed at 1603, 1423 and 1012 cm^{-1} in the plant extract belong to aromatic C=C stretching vibration, asymmetrical C-H deformation and C-O-C bending, respectively. These values are 1600, 1414 and 978 cm^{-1} in AgNPs.

SEM/EDX analysis

As depicted in Figure 3a, AgNPs synthesised with *O. papillosa* extract were spherical and homogeneously dispersed. In addition, upon examination of the SEM images of commercially available AgNPs (Figure 3b), it is evident that they possess a spherical structure similar to the nanoparticles synthesized with plant extract. To determine the composition of the biosynthesize nanomaterial, EDX analysis was performed. As illustrated in Figure 3c, the synthesized nanomaterial consists of Ag, C, N, and O, indicating the natural product. The presence of Pt is attributed to the sample plating.

Evaluation of the genotoxicity and cytotoxicity of nanoparticles

In this study, the effects of AgNPs obtained using *O. papillosa* and chemically obtained AgNPs on oxidative DNA damage and cell viability in human lymphocyte cells were assessed. The study aimed to compare the effects of AgNPs obtained through two different methods. Oxidative damage to DNA was evaluated using single-cell gel electrophoresis and cell viability was assessed using flow cytometry.

Results obtained by single-cell gel electrophoresis

A total of 100 cells were counted from samples of each dose, and the counted cells were classified according to their tail length as *Type 0,Type I,Type II,Type III,Type IV*. Data obtained from single-cell gel electrophoresis are shown in Table 1.

According to data from single-cell gel electrophoresis studies, the genetic damage index and the percentage of damaged cells caused by AgNPs obtained by green synthesis in human lymphocyte cultures were not statistically significant compared with the negative control ($p = 0.124$ and $p = 0.613$). The genetic damage index and the percentage of damaged cells caused by the AgNPs obtained through chemical synthesis reached 165.25 ±27.72 and 50.50 ±12.66, respectively, and for the positive control, these values reached 322.50 ±16.36 and 90.75 ±3.94, respectively. The genetic damage index and the percentage of damaged cells caused by AgNPs obtained through chemical synthesis and by the positive control were statistically significant when compared with the negative control ($p < 0.01$). When comparing green synthesis and chemical synthesis, the genetic damage index and the percentage of damaged cells caused by the nanoparticles obtained by chemical synthesis reached 165.25 ±27.72 and 50.50 ±12.66, on the other side, the genetic damage index and the percentage of damaged cells caused by the nanoparticles obtained by green synthesis reached 80.50 ±9.46 and 17.50 ±5.80, respectively. The increase in the genetic damage index value in cultures exposed to AgNPs obtained by chemical synthesis increased 2-fold compared with that in cultures exposed to AgNPs obtained by green synthesis. The percentage of damaged cells in cultures exposed to AgNPs obtained by chemical synthesis increased 3-3.5-fold compared to this percentage in cultures exposed to AgNPs obtained by green synthesis. This increase is statistically significant ($p < 0.001$). Comet formation of lymphocyte cells observed under fluorescence images is shown in Figure 4 a-e.

Results of the flow cytometry analysis

Table 2 presents data related to flow cytometry for the four donors. According to the data from flow cytometry studies, the number of early apoptotic cells increased in human lymphocyte cultures treated with silver nanoparticles obtained through green and chemical synthesis compared with the negative control. It is found that this increase is not statistically significant. The number of late apoptotic cells in cultures treated with silver nanoparticles obtained through green and chemical synthesis reached to 13.57±4.78 and 17.95±6.85, respectively, whereas it reached to 0.025±0.05 for negative control. There was a significant difference between negative control cultures and cultures treated with silver nanoparticles obtained through green ($p < 0.01$) and chemical ($p = 0.01$) synthesis for the number of late apoptotic cells. When comparing the green synthesis and chemical synthesis groups, the number of late apoptotic cells in lymphocyte cultures treated with AgNPs obtained through chemical synthesis was higher (Figure S1). While number of surviving cells in negative control cultures is 99.8±0.08, this value decreased to 59.82±9.27 and 44.57±11.86, green synthesis and chemical synthesis cultures, respectively. A 1.67-fold decrease in the number of cells in cultures exposed to AgNPs obtained from green synthesis was observed compared with

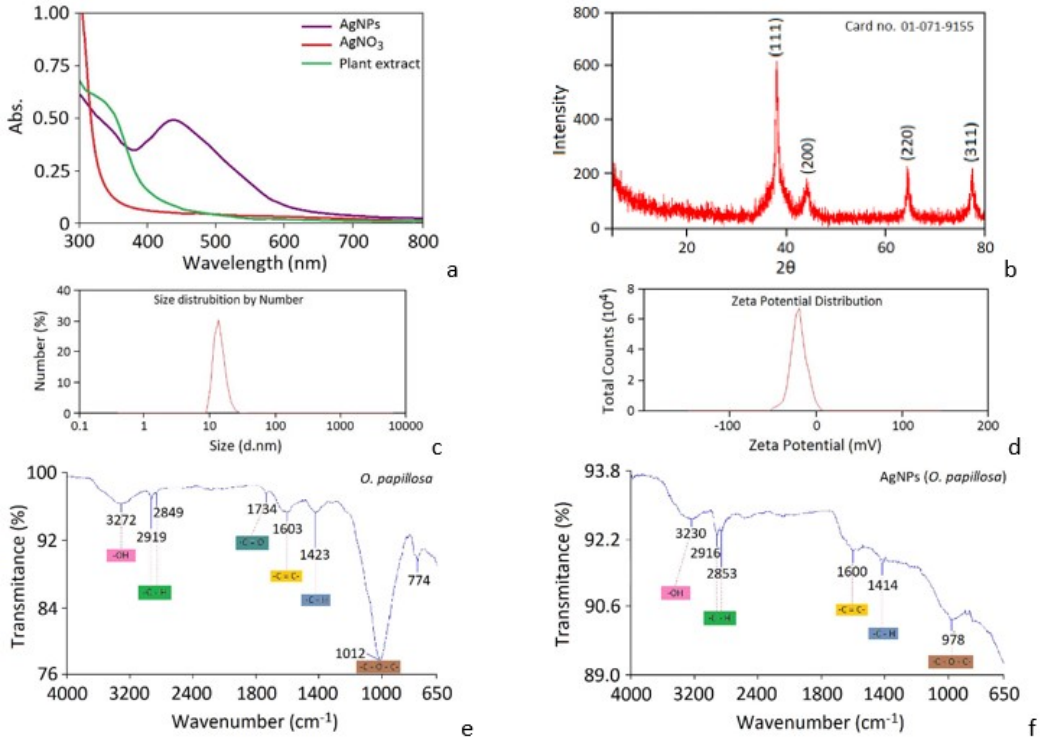

Figure 2. (a) UV-Vis spectrum of plant extract, precursor $AgNO_3$ salt and AgNPs, (b) X-ray diffraction pattern, (c) The particle size, (d) the zeta potential of green synthesized AgNPs, (e) FT-IR analysis of *O. papillosa* extract and (f) FT-IR analysis of green synthesized AgNPs.

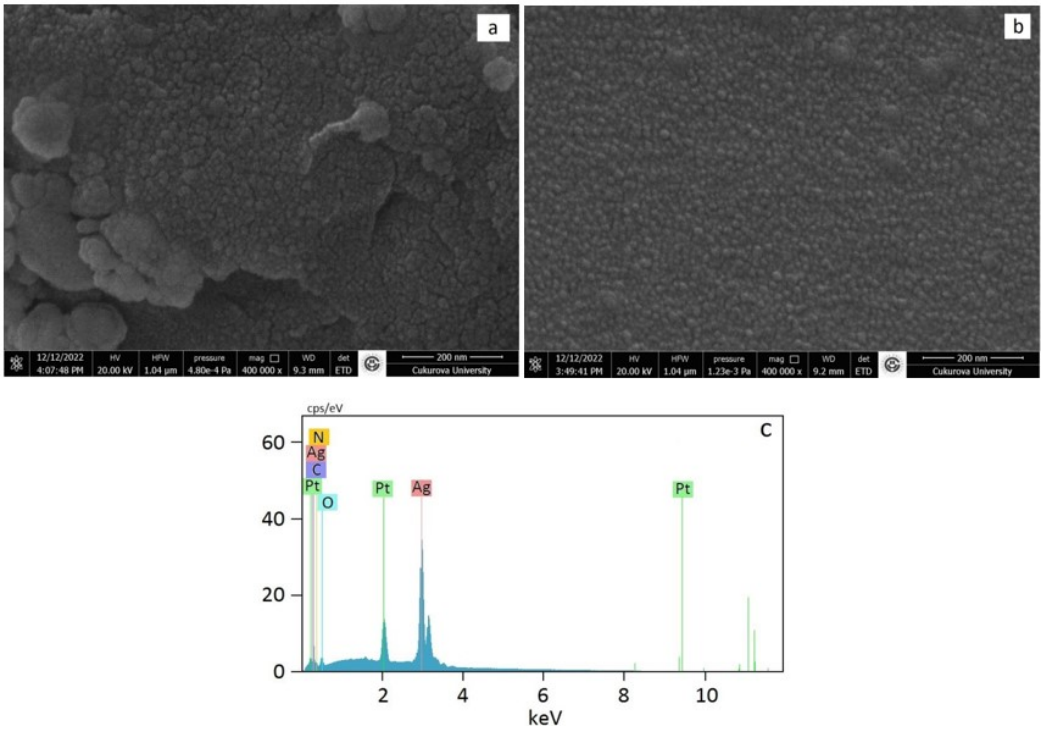

Figure 3. SEM image of AgNPs synthesized with *O papillosa* extract (a), Commercially available AgNPs (b), EDX profile of AgNPs synthesized with *O papillosa* extract.

Table 1. Data of comet assay analysis and statistical results for Genetic Damage Index and Damaged Cell Percentage in human lymphocytes cell treated with AgNPs.

	Donors	Type 0	Type I	Type II	Type III	Type IV	GDI	DCP
Negative Control	**Donor 1**	62	22	13	3	0	57	16
	Donor 2	61	25	10	4	0	57	14
	Donor 3	62	30	6	2	0	48	8
	Donor 4	64	31	4	1	0	42	5
Mean ±S.E.							**51.00 ±7.34**	**10.75 ±5.12**
Green synthesized AgNPs (150 µg/mL)	**Donor 1**	50	26	13	8	3	88	24
	Donor 2	44	39	7	7	3	86	17
	Donor 3	49	41	6	2	2	67	10
	Donor 4	48	33	12	4	3	81	19
Mean ±S.E.							**80.50 ±9.46**	**17.50 ±5.80**
Chemical synthesized AgNPs (150 µg/mL)	**Donor 1**	8	39	29	17	7	176	53
	Donor 2	0	39	40	15	5	184	60
	Donor 3	26	42	19	8	5	124	32
	Donor 4	4	39	35	20	2	177	57
Mean ±S.E.							**165.25 ±27.72*** **	**50.50 ±12.66*** **
Positive control (10mM) (H_2O_2)	**Donor 1**	4	11	13	25	47	300	85
	Donor 2	2	4	8	26	60	338	94
	Donor 3	2	6	8	28	56	330	92
	Donor 4	1	7	14	25	53	322	92
Mean ±S.E.							**322.50 ±16.36*** **	**90.75 ±3.94*** **

***p < 0.001 compared with negative control, GDI: Genetic damage index, DCP: Damaged cell percentage

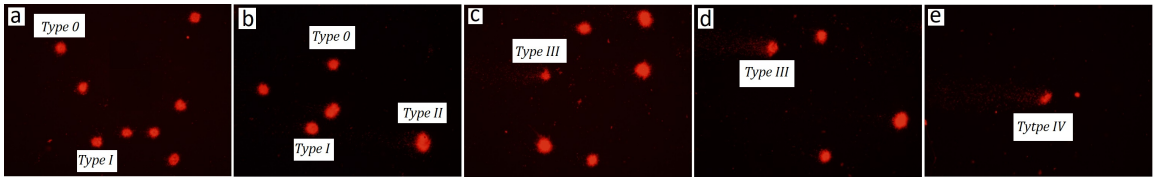

Figure 4. Comet formation of lymphocyte cells observed under fluorescent microscopy. (a:Negative control, b:Green synthesis, c:chemical synthesis, d-e:Positive control)

the negative control. In chemical synthesis, this decrease was observed to be 2.25 times. It was found that there was a significant difference between negative control cultures and cultures treated with AgNPs obtained by green and chemical synthesis compared with the negative control for the number of live cells ($p < 0.001$). The AgNPs obtained by chemical synthesis decreased the number of living cells compared with those obtained from green synthesis. While the number of necrotic cells in negative control cultures is 0.12±0.05, this value increased to 13.55±3.37 and 25.37±14.53, green synthesis and chemical synthesis cultures, respectively. The increase in necrotic cells in cultures exposed to silver nanoparticles obtained by chemical synthesis is 1.92 times higher than that in cultures exposed to silver nanoparticles obtained by chemical synthesis. The AgNPs obtained from both chemical and green synthesis increased the number of necrotic cells. This increase is not statistically significant for the AgNPs obtained by green synthesis, but it is statistically significant for AgNPs obtained by chemical synthesis compared with the negative control ($p < 0.01$) (Table 2).

Table 2. Statistical results for flow cytometry analysis in human lymphocyte treated with AgNPs.

		Examined cells	Early Apoptotic cell (%)	Late Apoptotic cell (%)	Live cell (%)	Necrotic cell (%)
Negative Control	**Donor 1**	2959	0.1	0.1	99.7	0.1
	Donor 2	2905	0	0	99.9	0.1
	Donor 3	3343	0	0	99.8	0.2
	Donor 4	3110	0.1	0	99.8	0.1
Mean ±S.E.		**3079.25±196.07**	**0.05±0.05**	**0.025±0.05**	**99.8±0.08**	**0.12±0.05**
Green synthesized AgNPs	**Donor 1**	3421	22.0	18.7	49.9	9.4
	Donor 2	3396	17.2	16.6	54	12.2
	Donor 3	3638	7.3	9.8	66.8	16.2
	Donor 4	3425	5.9	9.2	68.6	16.4
Mean ±S.E.		**3470±112.73**	**13.1±7.77**	**13.57±4.78****	**59.82±9.27*****	**13.55±3.37**
Chemical synthesized AgNPs	**Donor 1**	3654	32.6	28.2	31.3	7.9
	Donor 2	3562	0.9	15	41.4	42.7
	Donor 3	3732	4.7	13.7	59.9	21.7
	Donor 4	3261	10.2	14.9	45.7	29.2
Mean ±S.E.		**3552.25±206.22**	**12.1±14.18**	**17.95±6.85****	**44.57±11.86*****	**25.37±14.53****
Positive Control (10 mM) H_2O_2	**Donor 1**	2889	4.5	3.4	76.3	15.8
	Donor 2	3697	12	12.4	62.1	13.6
	Donor 3	3453	7.5	11.4	60.2	20.9
	Donor 4	2776	1.8	3.2	72.8	22.1
Mean ±S.E.		**3203.75±442.51**	**6.45±4.37**	**7.6±4.98**	**67.85±7.90*****	**18.1±4.05***

*p < 0.05, **p < 0.01, ***p < 0.001 compared with negative control.

Correlation results between flow cytometry and comet analysis data

Because of the correlation between flow cytometry data, a very high negative correlation was found between live and late apoptotic cell data (r = -0.943). In addition, a high positive correlation was found between early and late apoptotic cell numbers (r = 0.869). There was a moderate negative correlation between live and necrotic cell numbers (0.664). There was a medium-level correlation between live cell and early apoptotic cell numbers (r = -0.720). In addition, a very high positive correlation was found between the genetic damage index and the percentage of damaged cells (0.992).

DISCUSSION

In this study, the genotoxic and apoptotic effects of AgNPs (~ 14-15 nm) obtained using *O. papillosa* and by chemical methods were investigated in human lymphocyte cell cultures. In Comet analysis, an increase in GDI and DCP values was observed in human lymphocyte cell cultures treated with AgNPs obtained from the chemical method. This increase was found to be statistically significant ($p < 0.001$). However, it was determined that the increase in GDI and DCP values of AgNPs obtained from green synthesis in human lymphocyte cell cultures was not statistically significant.

In flow cytometry analysis, it was determined that AgNPs obtained by both methods increased the number of early, late apoptotic, and necrotic cells. The increase in the number of late apoptotic cells was found to be statistically significant ($p < 0.01$). While the increase in the number of necrotic cells was significant ($p < 0.01$) for chemical AgNPs, it was not significant for AgNPs synthesized by the green method. In addition, it was determined that AgNPs caused a decrease in the number of viable cells, and this decrease reached a statistically significant level ($p < 0.001$).

This study is the first to compare the genotoxic and apoptotic effects of AgNPs synthesised by different methods (green and chemical synthesis). Ghosh et al. (2012) investigated the *in vitro* and *in vivo* genotoxic and cytotoxic effects of AgNPs in the 90-180 nm size range using chromosome aberration, comet, and flow cytometry tests. According to the data obtained, it was concluded that AgNPs caused a statistically significant increase in chromosome aberration and comet parameters in mouse bone marrow cells and induced oxidative damage. On the basis of the data obtained from comet test studies in lymphocyte cultures, it was reported that the increase in tail length and reactive oxygen species was dose dependent. Based on the data obtained from comet test studies in plants, it was determined that there was an increase in tail length depending on the dose. The results obtained in our study are consistent with those of Ghosh et al. (2012). Josie et al. (2016) investigated the effects on chromosome aberration, micronucleus induction, repair of double chain fractures, cell renewal potential, and lipid peroxidation caused by different concentrations of AgNPs with sizes varying between 2, 3, 4, 6, and 7 nm in human lymphocytes. As a result of the studies conducted, it was reported that nanoparticles with sizes of 3, 4, 6, and 7 nm cause a clastogenic effect, and nanoparticles with a size of 2 nm cause cell proliferation by increasing insulin-like growth factor concentration. Although the sizes of AgNPs differ between the two studies, they support each other in terms of DNA damage. Jiravova et al. (2016) investigated the genotoxic and cytotoxic effects of AgNPs with a size size of 27 nm in two different mammalian cell cultures (NIH3T3, SVK14). AgNPs were determined to have effects in both cell cultures. However, because NIH3T3 cells are more sensitive to AgNPs, they cause more damage to DNA. They also reported that AgNPs increased apoptotic and necrotic cell numbers. In our study, it is understood from the comet and flow cytometry data that human lymphocytes are sensitive to AgNPs obtained from chemical synthesis. Both studies support each other because they both observe increases in apoptotic and necrotic cell numbers and DNA damage. Dobrzyńska et al. (2014) investigated the genotoxic effects of AgNPs of 20 nm (at doses of 5 mg/kg and 10 mg/kg) and 200 nm (at a dose of 5 mg/kg) sizes in rat bone marrow cells using the comet and micronucleus test methods. The data obtained from the comet test emphasised that AgNPs increased the comet tail length, but this increase was not statistically significant. They reported that the data obtained from the micronucleus test showed genotoxic damage. In our study, AgNPs caused genetic damage in the comet assay. However, we concluded that the increase in this statistically significant value may have resulted from the different cell types and study types between the two studies. Li *et al*. (2012) tested the genotoxic effect of 5-nm AgNPs using Ames (0.15 - 76.8 µg/plate) and TK6 cell cultures (10-30 µg/mL). In the Ames test, they determined that AgNPs did not exhibit a mutagenic effect at doses ranging from 2.4 to 38.4 µg/plate, but they did show an effect at higher doses. In TK6 cell cultures, they reported that AgNPs increased micronucleus frequency at concentrations of 5, 10, 15, 20 µg/mL, with the increase at concentrations of 25 and 30 µg/mL reaching statistical significance compared with the negative control. In our study, demonstrating the genotoxic damage of AgNPs obtained by the chemical method in human lymphocyte cultures is valuable in terms of supporting the data of the two studies. However, the lack of a significant effect on human lymphocytes by AgNPs obtained through green synthesis makes the study unique in terms of showing the advantages of green synthesis. Nakkala et al. (2017) evaluated the serum levels of ALT, AST, LDH, IL-6 and TNF-α to assess the effects of toxicity in an *in vivo* (rat) study of approximately 21-nm AgNPs obtained by green synthesis using *Ficus religiosa* plant for 28 days. On the 29th day following the monitored application, they determined significant increases in these values. They reported that these values completely recovered on the 89th day and determined that AgNPs accumulated in the liver, brain, and lung, respectively, using the ICP-OES technique. In their *in vitro* experimental studies using A549, HeLa, Hep2, COLO 205, and SH-SY5Y cell lines, the authors evaluated the effects of AgNPs on oxidative stress parameters, apoptotic staining techniques, apoptotic cell death, and apoptotic gene expression (caspase 8, 3, 9). It causes apoptotic changes in A549 and Hep2 cells through both extrinsic and intrinsic apoptotic pathways. No changes in biochemical parameters were reported at low concentrations in rats. Considering these data, our study supports the demonstration that AgNPs synthesised by "green synthesis" are suitable for use in the nanomedicine field and can be evaluated as an environmentally friendly material. Rajanahalli et al. (2015) attempted to determine whether two differently coated AgNPs and uncoated AgNPs cause delays in the cell cycle and their effects on the formation of ROS in mouse embryonic stem cells. In our study, AgNPs obtained via chemical synthesis led to genotoxicity of DNA according to comet assay data. In the data from the study by Rajanahalli et al, the result that AgNPs increase ROS in the cell overlaps with the comet data characterising the damage in DNA we obtained in our study. Okafor et al. (2013) tested the cytotoxicity of AgNPs produced using extracts of *Aloe, Magnolia, and Eucalyptus* leaves at concentrations of 2, 4, and 15 ppm on Human Embryonic Kidney 293 cells-HEK293 using automated InQ Plus equipment. They reported that AgNPs with concentrations of 2 and 4 ppm were not toxic to human healthy cells but inhibited bacterial growth.

CONCLUSION

In conclusion, the current study established an easy, economical, and inexpensive eco-friendly protocol for the synthesis of AgNPs using *O. papillosa* leaf extract. UV-Vis spectroscopy, DLS, SEM, and XRD measurements confirmed the formation of green synthesised silver nanoparticles. It was observed that green silver nanoparticles prepared at certain concentrations prevented DNA damage. The results obtained in this work con-

tribute to the increase in knowledge of the effects of AgNPs on mammalian cell systems. The fact that nanoparticles obtained by the green method do not show genotoxic effects in mammalian cell cultures seems to reduce concerns about their use in many industrial and medical areas. The information presented in this study will shed light on nanogenotoxicology, showing that the genotoxic and apoptotic effects of AgNPs are not only related to the size of the nanoparticle but also to the method of obtaining it. The average particle size of the synthesised nanoparticles was measured to be 14 nm with a zeta potential of -20.5 mV. However, further detailed studies are needed to develop biological applications of biosynthesize AgNPs. This study will form the basis for detailed in vivo studies and future projects.

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