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Investigating the Antioxidant and Apoptosis Inducing Effects of Biologically Synthesized Silver Nanoparticles Against Lymphoma Cells *in Vitro*

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Abstract

The current study aimed to synthesize silver nanoparticles (Ag NPs) in a safe and eco-friendly biological method using green tea extract, characterize them using UV-vis spectroscopy, FTIR, XRD, FESEM, and AFM tools, then investigate their antioxidant potentials against DPPH and their cytotoxicity toward lymphoma cells using MTT and ethidium bromide/propidium iodide dual staining assays, and their genotoxic capacities by assessing their effects on the gene expression of *p53*, *caspase3*, *bcl-2*, and *bax* genes by qPCR and DNA fragmentation. Results confirmed the synthesis of silver nanoparticles; the UV-vis results showed a peak of absorbance at 414 nm; FTIR analysis showed absorbance belonging to the functional groups of green tea; XRD assay results confirmed the crystallinity of Ag NPs; and AFM revealed a mean diameter of around 24.37 nm. Finally, FESEM showed that the shape of Ag NPs was that of spherical aggregated nanoparticles. The synthesized nanoparticles showed antioxidant activity and cytotoxic effects against lymphoma cells, with an IC50 = 1.138 µg/ml. Cell staining results showed alternations in the shape of treated cells due to apoptosis. Ag NPs affected gene expression as they increased proapoptotic genes *p53*, *caspase3*, and *bax* by 3.3, 2, and 4.25fold, respectively, while decreasing antiapoptotic gene expression *bcl-2* by 0.59fold. We concluded that the biological way is an efficient way to synthesize silver nanoparticles, and the synthesized NPs have antioxidant potential and are cytotoxic and genotoxic toward lymphoma cells; thus, they may be utilized in related biomedical applications.

Keywords: Apoptosis, Bio nanotechnology, Cancer biology, silver nanoparticles

التحري عن التأثيرات المضادة للأكسدة والمحفزة للموت الخلوي المبرمج لدقائق الفضة النانوية المصنعة بيولوجياً ضد خلايا سرطان اللمف خارج الجسم الحي

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الخلاصة :

هدفت الدراسة الحالية لتحضير دقائق الفضة النانوية بطريقة بيولوجية امنة وصديقة للبيئة باستعمال مستخلص أوراق الشاي الأخضر ثم توصيفها بتقنيات (UV–vis spectroscopy, FTIR, XRD, AFM, FESEM) ثم التحري عن فعاليتها المضادة للأكسدة بواسطة اختبار DPPH وسميتها الخلوية تجاه خلايا سرطان اللمف باختباري MTT والتصبيغ الخلوي وأخيراً التحري عن سميتها الجينية باختبار تأثيرها على جينات *p53*, *caspase3*, *bcl–2*, *bax* بواسطة تقنية qPCR واختبار تجزأ المادة الوراثية. أكدت نتائج التوصيف نجاح تحضير دقائق الفضة، اذ اعطى اختبار UV–vis قمة امتصاصية عند الطول الموجي 414nm, فيما بينت نتائج تحليل FTIR وجود امتصاصية تعود للمواد الفعالة في الشاي الأخضر، أظهرت نتائج فحص AFM ان متوسط قطر دقائق الفضة 24.37nm, واخيراً بين فحص FESEM أن شكل الدقائق كان كروي متجمع. أظهرت الدقائق المصنعة فعالية مضادة للأكسدة وسمية تجاه الخلايا السرطانية وكانت قيمة التركيز المثبط ل 50% من النمو 1.138 µg/ml وبينت نتائج التصبيغ الخلوي التغيرات الشكلية الحاصلة في الخلايا المعاملة نتيجة لحصول الموت الخلوي المبرمج. أثرت الدقائق المصنعة على التعبير الجيني في الخلايا المعاملة حيث زادت من التعبير الجيني للجينات المحفزة للموت الخلوي المبرمج *p53* بمقدار 3.3ضعفاً و*caspase3* بمقدار ضعفين و*bax* بمقدار 4.25 ضعفاً فيما قللت من التعبير الجيني للجين المثبط للموت الخلوي المبرمج *bcl–2* بمقدار 0.59ضعفاً وحفزت بشكل واضح على تجزأ المادة الوراثية. استنتجنا من الدراسة الحالية كفاءة الطريقة البيولوجية في تحضير الفضة النانوية وامتلاك الدقائق المصنعة خواصاً مضادة للأكسدة وسمية خلوية وجينية لخلايا سرطان اللمف مما يتيح المجال لاستعمالها في التطبيقات الحيوية–الطبية ذات العلاقة.

1. Introduction

Cancer is one of the leading causes of death globally; it is the third leading cause of death after heart disease and stroke [1]. In 2020 alone, cancer is believed to be responsible for 10 million deaths [2]. Furthermore, WHO predicted this number would increase up to threefold by 2040 [3]. Commonly used treatments for cancer include surgery, chemotherapy, radiation, and immunotherapy, or a combination of them [4,5]. Those treatment approaches have several drawbacks, like poor bioavailability, non-specificity, damaging healthy cells, toxicity, and unwanted side effects [6–8]. The cancers of the lymphatic system are called lymphomas. Lymphomas may broadly be classified as either Hodgkin's (HL) or non-Hodgkin's (NHL) [9]. NHL accounts for around 90% of all lymphoma cases [10].

Nanotechnology deals with the production and applications of systems and structures by manipulating atoms and molecules at nanoscale levels [11]. Utilizing nanotechnology in the treatment of cancer led to the introduction of nanomedicine, which includes dealing with the use of nanostructures in the diagnosis and treatment of cancer [12]. Silver nanoparticles can be synthesized by different routes, all of which can be put into three main categories: physical methods, chemical methods, and biological methods [13]. Physical and chemical approaches involve the use of toxic chemicals that may lead to hazardous consequences like toxic, carcinogenic, and environmentally harmful effects [14]. The biological approach, or green chemical approach, deals with the usage of biological entities, parts of them, or their products as reducing and stabilizing factors in the nanoparticle synthesis process, thus offering a clean, reliable, and eco-friendly method for approaching [15].

Programmed cell death, or apoptosis, is a key mechanism for homeostasis; it is responsible for eliminating aged cells as well as damaged cells, virally infected cells, and cancer cells [16]. The main apoptosis pathways are the intrinsic, also known as the mitochondrial pathway, and the extrinsic, also known as the death receptor pathway. Apoptosis can be triggered by various events like DNA damage, mitochondrial membrane damage, increased reactive oxygen species, and cell cycle arrest. This may activate various genes and proteins that orchestrate the process of cell death, like caspases 8, 9, and 3, and tumor suppressor genes like p53. Cancer cells evade

apoptosis and continue to proliferate even when they lose their genomic and cellular integrity [17]. One of the leading approaches to treating cancers like leukemia is to induce apoptosis in them [18, 19]. Various studies pointed out Ag NP's potential as an anticancer agent mainly through two mechanisms: the first is by increasing reactive oxygen species production, which results in cellular damage and causes the upregulation of apoptotic genes like caspase 8, 9, 3, p53, bax, and bak; conversely, it downregulates antiapoptotic genes like bcl-2 and bcl-XL [20, 21]. The second mechanism involves acting as an anti-angiogenic agent. Angiogenesis is the formation of new blood vessels from the already existing ones around the tumor cells to increase the blood supply and thus nutrients. It is induced by vesicular endothelial factor (VEGF). Ag NPs are potent inhibitors of VEGF [22, 23].

Since silver nanoparticles have various applications in biomedical fields, the current study aimed to synthesize Ag NPs in an environmentally friendly way with minimal chemical and physical involvement and to investigate their antioxidant and antiproliferative capacities for a better understanding of their potential in the cancer research field and other related fields.

2. Materials and methods

Methods

2.1 Preparing Green Tea extract

The dried green tea leaves were ground using an electric grinder. 2 grams of the grinded leaves were mixed with 100 ml of deionized distilled water and heated at 65 °C for 30 minutes with continued and steady stirring. The mixture was left to cool to room temperature. Then, it was filtered using Whatman filter paper no. 1 and kept at 4 °C until used.

2.2 Synthesis of silver nanoparticles

Silver nitrate (AgNO3) was purchased from ThermoFisher (Kandel, Germany). The biological synthesis of silver nanoparticles was done by adding 1.5 ml of green tea extract dropwise to 50 ml of 0.1 M AgNO3 with continuous stirring for 30 minutes at room temperature. After that, the mixture was kept in a dark condition for another 30 minutes to allow the reduction and capping processes to complete. The synthesized nanoparticles were separated from the other reaction components by centrifugation and washed twice with ddH2O, then dried and collected in a sealed dark vial and kept in a dry, dark place until needed.

2.3 Characterization of silver nanoparticles

The formation of Ag NPs was confirmed by using UV-vis spectroscopic analysis (Shimatzu 1900 UV-vis spectroscope, Japan), where a wavelength between 200 and 800 nm was scanned. To identify the functional groups bound to the surface of the nanoparticles, an FTIR analysis was done (Shimatzu 8400 FTIR spectrometer, Japan). The crystallinity state of the NPs was detected using an XRD analysis (Haoyuyan DX-27 X-ray diffractor, China). To determine the shape and size of the synthesized nanoparticles, field emission scanning electron microscopy (FESEM) (Fei Inspect F50, Netherlands) and atomic force microscopy (AFM) (Angstrom Advanced AA2000 atomic force microscope, USA) assays were used.

2.4 DPPH scavenging assay

The scavenging activity of Ag NPs was assessed using the DPPH radical scavenging assay as described by [24]. Briefly, 0.5 ml of serial dilutions of Ag NPs (50, 100, and 150 g/ml) were added to tubes, and then 0.3 ml of DPPH and 3 ml of DMSO were added to each concentration. Samples were mixed by vortex and then left at room temperature for one hour in dark conditions. The colorimetric changes from dark purple to light yellow were measured

spectrophotometrically at 517 nm. Ascorbic acid was used as a positive control. The scavenging activity was measured as follows:

$$\text{scavenging activity}(\%) = \frac{\text{absorbance of negative conrol} - \text{absorbance of sample}}{\text{absorbance of negative control}} \text{x}100$$

2.5 Culture maintenance

Large-cell immunoblastic lymphoma cells (SR cell line) were purchased from the American Type Culture Collection (Virginia, USA), and RPMI and fetal bovine serum (FBS) were purchased from Capricorn Scientific (Ebsdorfergrund, Germany). Lymphoma cells were maintained in RPMI 1460 medium supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 units/ml penicillin. Trypsin-EDTA was used to passage the cells; cells were reseeded at 50% confluence twice a week and incubated at 37 °C.

2.6 Viability assay (MTT assay)

The cytotoxicity of Ag NPs against non-Hodgkin's lymphoma cells was determined using an MTT assay. MTT stain was purchased from Bio-World (Dublin, USA). The procedure was done according to [25]. Briefly, lymphoma cells were seeded at 1×104 cells/well and incubated for 24 hours until a confluent monolayer was achieved. Cells were treated with a series of dilutions of Ag NPs (100, 50, 25.12.5, 6.25, and 3.12 µg/ml), then incubated for 24 hours at 37 °C, 5% CO2. After the incubation, the medium was removed and 28 µL of a 2 mg/ml MTT solution was added. Cells were incubated for 1.5 hours at 37 °C. The MTT solution was removed, and any remaining formazan crystals were dissolved by adding DMSO. The cells were incubated for 15 minutes with gentle shaking. Cell viability was determined using a microplate reader at 492 nm. The MTT assay was performed in triplicate. The viability and cytotoxicity were calculated, and the IC50 was estimated.

2.7 Acridine orange/Propidium iodide (AO/PI) dual staining

To measure and visualize Ag NP-induced cell death in lymphoma cells, an AO/PI assay was used. The procedure of [26] was adopted with some modifications. Briefly, cells were cultured at a rate of 7×103 cells per well in a 96-well plate. Cells were treated with the IC50 of Ag NPs, calculated from the MTT assay previously, and then incubated for 24 hours at 37 °C, 5% CO2. After that, 50 µl of the AO/PI mixture was added to the treated well and kept for 30 seconds at room temperature. The stain was removed, and the images were taken using a Lecia fluorescent microscope at 10X magnification.

2.8 RNA extraction, cDNA synthesis and gene expression evaluation

Total RNA from both control (untreated lymphoma cells) and lymphoma cells treated with 1.138 µg/ml Ag NPs for 24 hours was extracted. The extraction was performed using Transzol Up plus RNA Kit® referring to the manufacturer's instructions (TransGen Biotech Co., Ltd., Beijing, China). Complimentary DNA synthesis was performed by EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix, referring to the manufacturer's instructions (TransGen Biotech Co., Ltd., Beijing, China). A real-time PCR reaction was carried out using Rotor-Gene Q (Qiagen, Germany). Primer sequences of the P53, caspase3, bax, and bcl genes were retrieved from [27]. Each reaction mix consisted of 10 µl of EasyTaq® PCR SuperMix (TransGen Biotech Co., Ltd., Beijing, China), 2 µl of primers, 2 µl of cDNA, and 6 µl of ddH2O. The PCR program consisted of 1 cycle of one initial denaturation at 94 °C for 10 minutes followed by 40 cycles of reaction, a denaturation step at 94 °C for 45 seconds, an annealing step (55° for bax, 60° for P53 and Caspase3, and 65° for bcl-2) for 40 seconds, an extension step at 72 °C for 45 seconds, and one hold step at the end of the reaction at 72° for 6 minutes. Each reaction was performed three times, results were calculated by the slandered

curve method with β-actin used as an internal control, and data were displayed as fold change using the Livak method [28].

2.9 DNA ladder assay

The genomic DNA of both control (untreated lymphoma cells) and lymphoma cells treated with 1.138 µg/ml Ag NPs for 24 hours was extracted using the ReliaPrep™ Blood gDNA Miniprep System (Promega-USA) following the instructions of the manufacturer. The extracted DNA was separated with agarose gel electrophoresis; the agarose gel concentration was 1.5%, and the run time was 1 hour at 100 V [29].

2.10 Statistical analysis

GraphPad Prism8 software (California, USA) was used to carry out the results analysis. Results were expressed as mean ± standard deviation; to compare two groups, a student t-test was used. An analysis of variance (ANOVA) was done to find out the significance of the results; statistical significance was defined as $p \leq 0.05$.

3. Results and discussion

3.1 Synthesis and characterization of Ag NPs

Upon adding and mixing green tea extract with silver nitrate solution, a color change was observed from colorless to pale red. The color darkened gradually with the complete addition of the 1.5 ml of green tea and mixing to become a dark brown color. Color change was considered an indication of Ag NP formation (Figure 1a). The UV-vis assay results showed a peak of absorbance at 414 nm (Figure 1-b), which is a characteristic wavelength for Ag NPs. The absorbance curve indicated that Ag NPs were somehow uniform in shape and well dispersed throughout the solution.

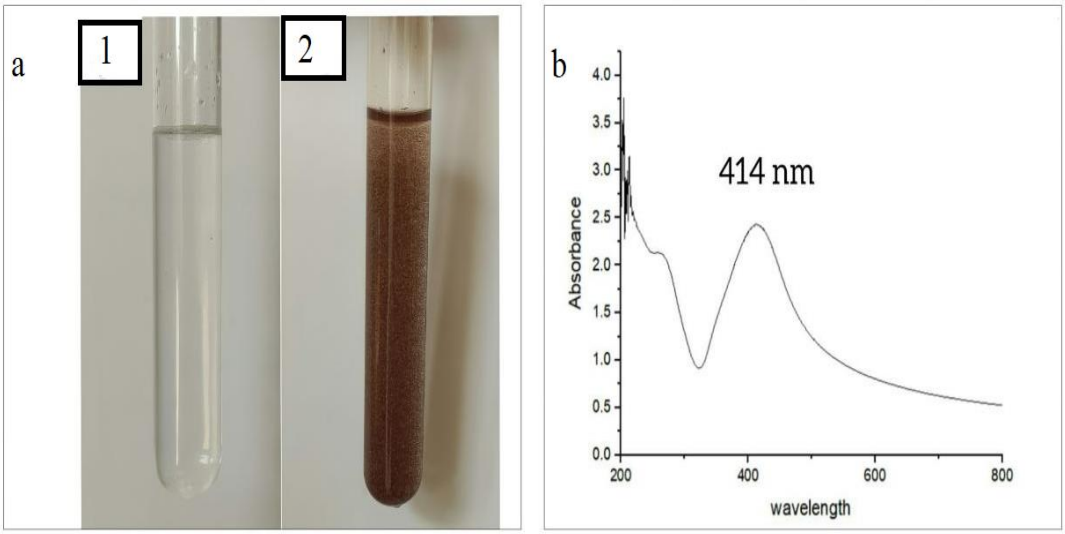

Figure 1: Synthesis of Ag NPs by green tea extract showing: **a1**: color of 0.1mM silver nitrate solution **a2**: color change after adding green tea extract, **b**: UV-vis spectrum of the synthesized Ag NPs (200-800 nm).

FTIR results showed peaks at 3259 cm1 correspond to O-H stretching vibration, 2360 cm1 and 2114 cm1 correspond to N-H stretching, 1631 cm1 correspond to C=C stretch, 1543 cm1 corresponds to N-O asymmetric stretch, and 1338 cm1 corresponds to C-O-H vibration (Figure 2-a). These values belong to the polyphenolic compounds of the green tea extract, which confirms that the active compounds in the extract are responsible for the reduction and capping of silver ions. The XRD results confirmed the crystalline nature of Ag NPs as they showed

peaks in the 2θ= 37.96, 44.15, 64.27, and 77.33; those values match Bragg's reflection of [111], [200], [220], and [311] (Figure 2-b), which are considered characteristic peaks for crystalline silver NPs. The size of Ag NPs was determined by AFM. (Figure 2-c) represents a two-dimensional image of Ag NPs; it reveals the generally monodispersed nature of NPs with a mean diameter of 24.37 nm; 90% of nanoparticles were below 35 nm in diameter. (Figure 2-d) shows the topology of NPs' surfaces in 3D mode. The shape of the synthesized nanoparticles was observed with FESEM. Results showed the NPs were mostly spherical, with some oval and irregular-shaped particles. The NPs were mostly aggregated, with diameters ranging between 15 and 37 nm (Figure 2e, f).

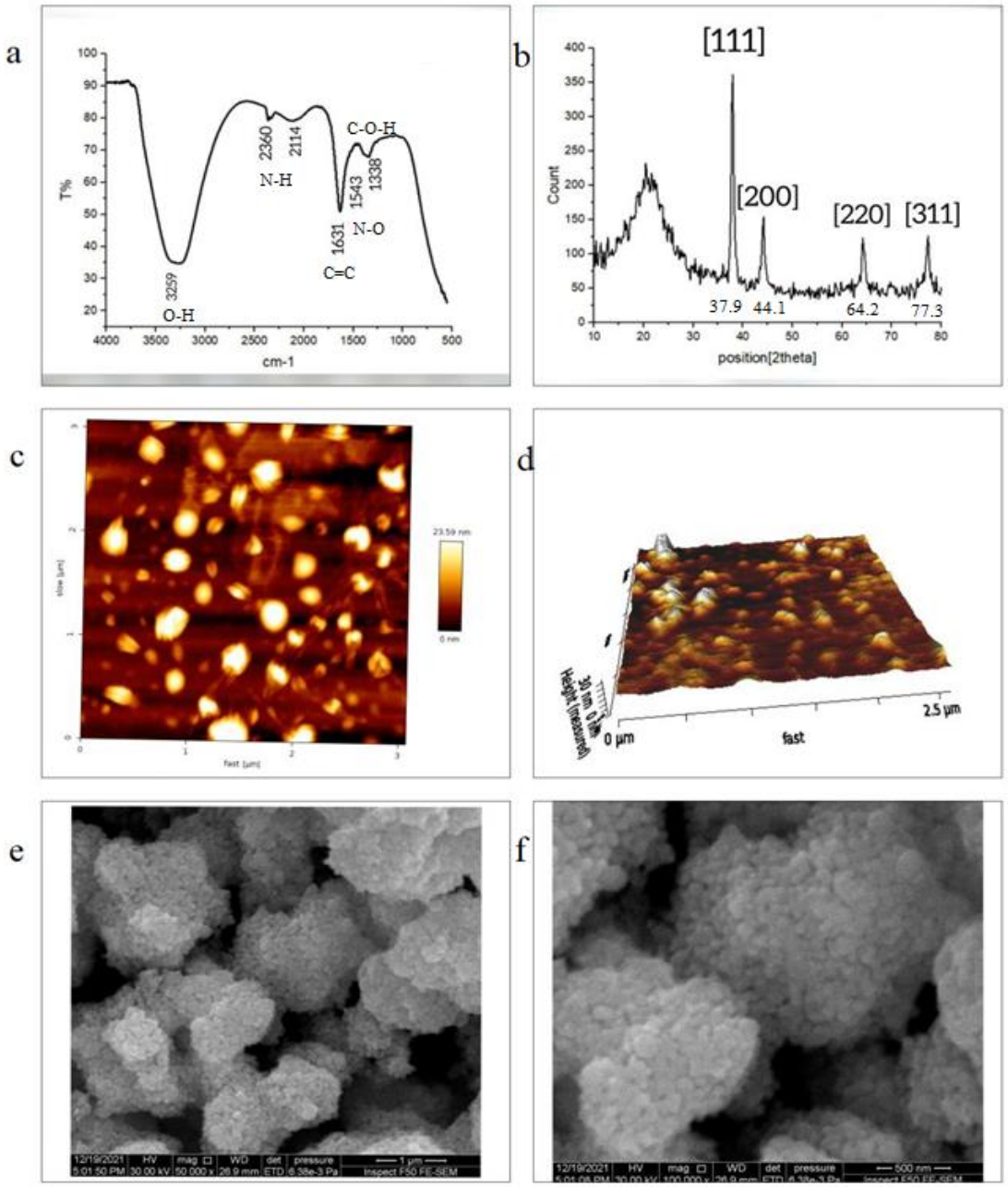

Figure 2: Characterization of Ag NPs showing: **a**: FTIR analysis, **b**: XRD analysis, **c**: 2D AFM image, **d**: 3D AFM image, **e**: FESEM micrograph at 1µm scale, **f**: FESEM micrograph at 500nm scale

3.2 Antioxidants capacity of Ag NPs (DPPH assay)

The free radical 2,2-diphenyl-1-picrylhydrazyl, or DPPH, is a stable free radical that is used to assess the antioxidant capacity of different materials; in other words, it measures a material's electron transfer ability. The synthesized Ag NPs showed promising antioxidant scavenging activity, which manifested in a dose-dependent manner as it increased with increasing the NPs' concentration. The maximum antioxidant capacity of 83.71% at a concentration of 150 µg/ml was, however, still lower than the ascorbic acid capacity of 87.86% at 25 µg/ml. Figure 3 shows the antioxidant capacity of different concentrations of Ag NPs, with ascorbic acid capacity as a positive control.

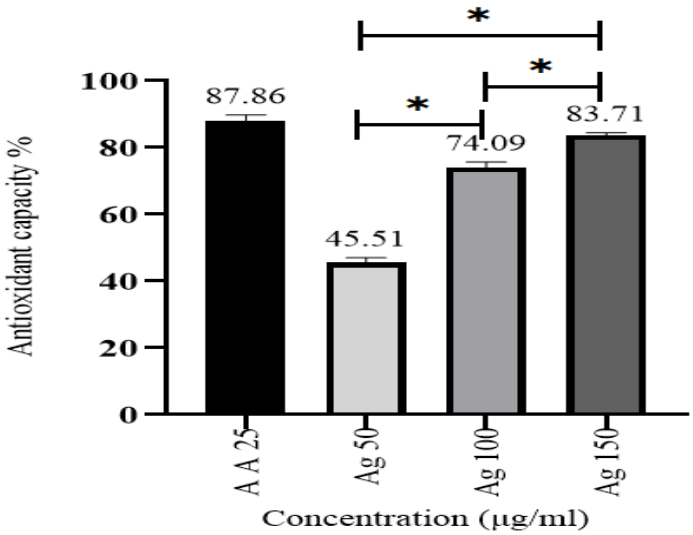

Figure 3: Free radicals scavenging activity of biogenic Ag NPs against DPPH. *: $P<0.05$, AA: ascorbic acid.

The high antioxidant capacity shown in this study may be attributed to both the nanosized Ag properties, such as high reactivity and a large surface/volume ratio, as well as the bioactive compounds on the nanoparticle's surface from the reducing/capping processes with green tea, such as flavonoids and phenols, as they are considered to have a powerful natural antioxidant capacity [30, 31]. These results coincide with the previous results reported by other researchers regarding the biologically synthesized Ag NPs [32–34].

3.3 Assessing Ag NPs cytotoxicity toward lymphoma cells

The MTT assay is used to measure the metabolic activity of treated and untreated cells to estimate their viability. It is based on the ability of NADPH-dependent enzymes to reduce MTT stain into its insoluble form, formazan, in viable cells. Results revealed an evident cytotoxic potential of silver NPs against cancer cells (Figure 4-a); the cytotoxic effect is dose-dependent as it increases as the dose goes up. Inhibition concentration 50 (IC50) was found to be 1.138µg/ml (Figure 4 b, c). Studies have shown that Ag NPs are highly toxic because they are between 10 and 100 nm in size [35]. The main proposed mechanism that can explain the cytotoxic effects of Ag NPs includes the induction of reactive oxygen species; this increase causes a decrease in glutathione levels, leading to an oxidative stress status in the cell that ultimately disrupts calcium regulation and DNA integrity and causes apoptosis [36, 37]. This mechanism has been approved in several in vitro models [38]. The current study results support the documented evidence of spherically shaped biosynthesized NPs' inhibitory activity against several types of cancer cell lines, like colon cancer cell lines [21, 39] and lung cancer cell lines [40–42]. human gastric adenocarcinoma [43]. The Ic50 of biosynthesized Ag NPs varied from

1–300 µg/ml mainly in a dose-dependent manner. Different Ic50 values may vary due to the differences in the method used to prepare Ag NPs, the type of reducing factor(s), nanoparticle characteristics, incubation period, and the cancer cell line they tested against, as well as other experimental conditions [44].

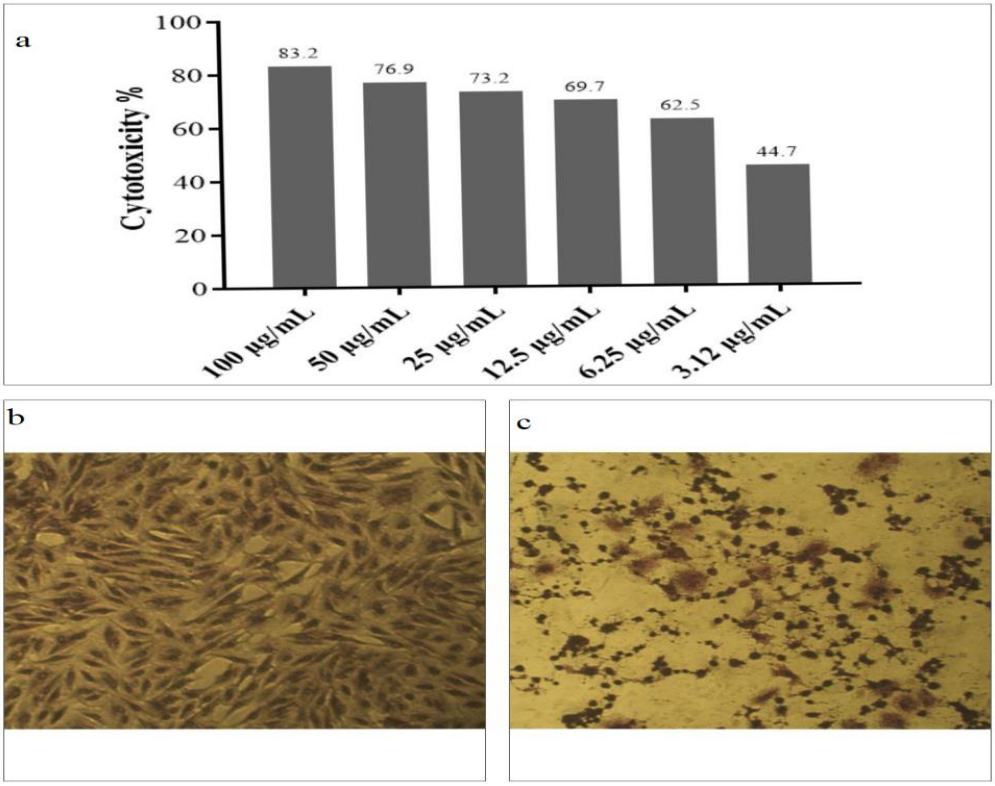

Figure 4: MTT assay results showing: **a**: The cytotoxic effects of biogenic Ag NPs against lymphoma cells in six concentrations, **b**: Untreated lymphoma cells (control) after 24 hours incubation at 37̊C, 5%CO2, **c**: Lymphoma cells treated with 100µg/ml of Ag NPs after 24 hours of incubation at 37̊C, 5%CO2.

3.4 Silver nanoparticles induces apoptosis in lymphoma cells

Acridine orange/propidium iodide dual staining was used to detect the cellular and nuclear changes in lymphoma cells treated with 1.138 µg/ml of Ag NPs for 24 hours. Acridine orange stain can penetrate both living and dead cells and thus stains all cells, giving a green fluorescence, while propidium iodide stains only dead cells that have lost their membrane selective permeability, giving a red fluorescence. Staining results showed that the treated cells shrank with condensed chromatin and appeared to lose their cell-cell communication. All those observations point to the fact that the Ag NPs induced apoptosis in the treated lymphoma, as they represent the classical apoptosis signs [45, 46]. Conversely, untreated cells appeared in normal shape and density with bright green nuclei without any of the apoptosis marks mentioned above (Figure 5). Similar observations were mentioned in previous studies regarding the apoptosis-inducing activities of biogenic silver nanoparticles. One study synthesized Ag NPs from Potentilla fulgens extract and examined their anticancer effects against breast cancer, represented by the MCF-7 cell line, and glioma, represented by the U-87 cell line. Ag NPs showed inhibitory activity against both cell lines, with higher toxicity toward breast cancer cells [47]. In another study of biologically synthesized Ag NPs using the extract of Lycium Chinese and studying their anticancer activities, they found that it causes chromatin condensation and other apoptotic cell signs in cells of breast adenocarcinoma and hepatocellular

carcinoma [48]. The ability of biogenic Ag NPs to induce apoptosis in different cells was mentioned in other studies [49, 50].

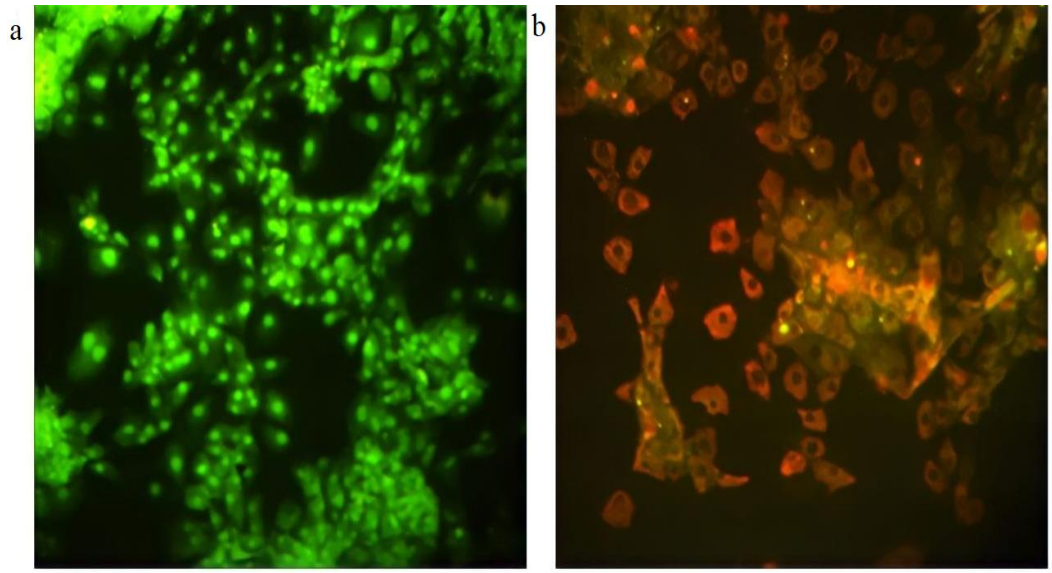

Figure 5: Ethidium bromide/Propidium iodide dual staining of lymphoma cells after 24 hours of incubation at 37̊C, 5%CO2 showing: a: Untreated lymphoma cells (control), b: Lymphoma cells treated with 1.138µg/ml of Ag NPs.

3.5 Ag NPs upregulate the gene expression of *p53*, *caspase3*, *bax* in treated lymphoma cells

The change in gene expression of the selected genetic markers p53, caspase3, bax, and bcl-2 was observed by qPCR after treating lymphoma cells with 1.138 µg/ml for 24 hours. Results showed an upregulation in gene expression of proapoptotic genes p53, caspase3, and bax by 3.3, 2, and 4.25fold, respectively. Conversely, a downregulation of the antiapoptotic gene bcl-2 by 0.59fold was observed, and the change in gene expression was significant ($p = 0.0427$), as shown in Figure 6. The mechanism by which silver NPs could affect gene expression may follow two scenarios: the first involves the increase of oxidative stress caused by Ag NPs, which may downregulate the AKT enzyme family, causing the proapoptotic kinase p38 to increase, ultimately leading to a total P53 increase. Thus, Ag NPs may induce apoptosis via the p53 signaling pathway [51]. The second one relates to their effect on mitochondrial membrane integrity, which leads to upregulation of bax and downregulation of bcl-2; consequently, mitochondrial enzymes like cytochrome c may be released into the cytosol; all those events lead to caspase3 initiation and cause apoptosis in a caspase-dependent manner [52]. Close results were reported regarding the ability of biogenic Ag NPs to increase the expression of pro-apoptotic genes and decrease the expression of anti-apoptotic genes [53].

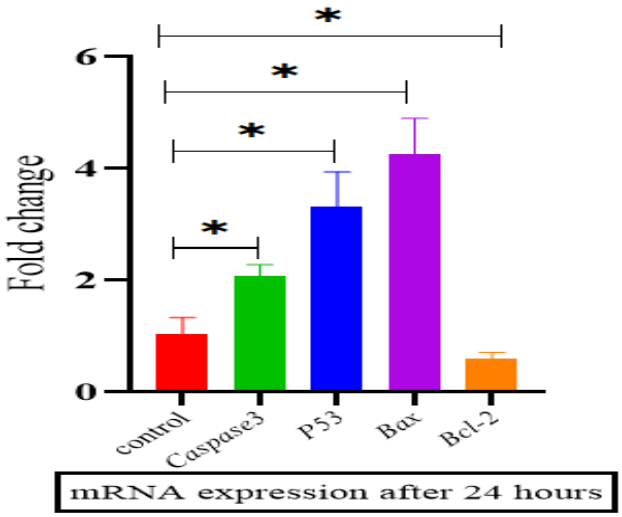

Figure 6: qPCR results showing the change in *p53*, *caspase3*, *bax* and *bcl2* expression as fold change in lymphoma cells treated with 1.138µg/ml of Ag NPs after 24 hours of incubation at 37̊C, 5%CO2, (p= 0.0427).

3.6 DNA fragmentation

A key feature of apoptosis is the fragmentation of the DNA, which results from the action of endogenous endonucleases, especially caspase-3-activated DNase. This process turns the DNA into small pieces of about 180 pb and multiples [54]. The results of the ladder assay clearly point out the fragmentation caused by Ag NPs, as the treated cell's DNA appeared in a smeared and ladder-like shape while the control cell's DNA appeared intact and in one piece (Figure 7). A recent study that synthesized silver NPs using *Peltophorum pterocarpum* extract and investigated its ability to induce DNA fragmentation in various cell lines of lung, breast, and liver cancers showed similar results [55].

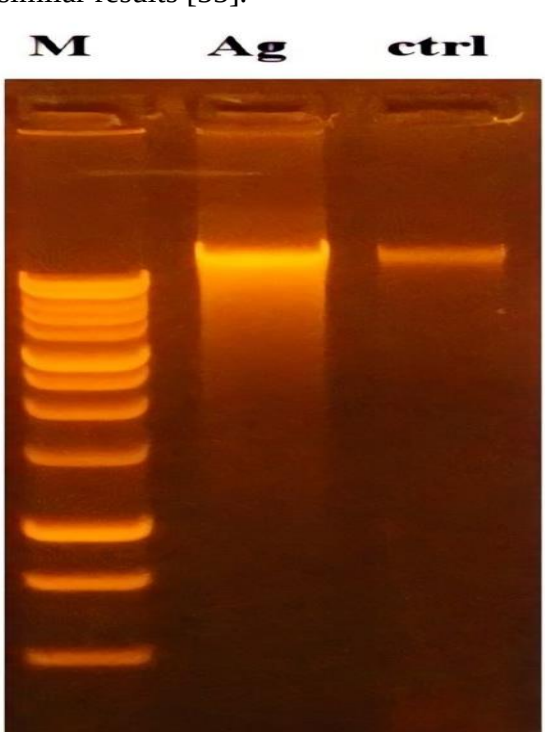

Figure 7: DNA ladder assay results showing lymphoma cells genomic DNA after 24 hours of incubation at 37̊C, 5%CO2. M: 1Kb Ladder, Ag: lymphoma cells treated with 1.138µg/ml of Ag NPs, ctrl: untreated lymphoma cells.

4. Conclusions

The plant-mediated synthesis method proved itself to be a safe, easy, and environmentally friendly way to prepare silver nanoparticles with small sizes and good crystallinity; furthermore, the prepared NPs proved their antioxidant potential and antiproliferative capacities against non-Hodgkin's lymphoma cells, making them of possible interest in biomedical applications.

5. Acknowledgment

6. Conflict of interest

The authors declare no conflict of interest

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