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Green Synthesis of Silver Nanoparticles Using *Punica granatum* Peel Extract and Their Cytotoxic Effects on Human Melanoma RPMI-7951 Cells

Iasmina-Alexandra Predescu [1,2,3]✉, Alexandra-Denisa Semenescu [1,2,*]✉ and Dalia Pătraşcu [1,2,3]✉

[1]Faculty of Pharmacy, "Victor Babes" University of Medicine and Pharmacy, 2nd Eftimie Murgu Square, 300041 Timisoara, Romania
[2]Research Centre for Pharmaco-Toxicological Evaluation, Faculty of Pharmacy, "Victor Babeş" University of Medicine and Pharmacy, 2nd Eftimie Murgu Square, 300041 Timişoara, Romania
[3]Doctoral School, "Victor Babeş" University of Medicine and Pharmacy, 2nd Eftimie Murgu Square, 300041 Timişoara, Romania
*email: alexandra.scurtu@umft.ro

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Abstract

Background: Melanoma is the most aggressive skin cancer, and the development of new therapeutic strategies is essential. The synthesis of silver nanoparticles (AgNPs) using *Punica granatum* (PG) peel extract offers a sustainable approach with potential biomedical applications. Methods: The human melanoma cell line RPMI-7951 was used in this study to assess the effects of AgNPs which were produced with ethanolic PG peel extract. The MTT assay was used to measure cytotoxicity, and morphological analysis, Hoechst, and mitochondrial staining were used to assess the cellular response. Results: PG-AgNPs induced a dose-dependent reduction in cell viability, with significant effects observed at concentrations of 25 and 30 μg/mL. Treated cells showed decreased confluency, shrinkage, chromatin condensation, and mitochondrial changes. Conclusions: These findings demonstrate that PG-AgNPs exert cytotoxic effects on melanoma cells and highlight their potential as promising candidates for anticancer therapies, supporting further validation in in vivo models.

Keywords: *Punica granatum; silver nanoparticles; green synthesis; melanoma; cytotoxicity; apoptosis; nanomedicine*

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1. Introduction

In adults, skin is the largest organ, accounting for about one-sixth of body mass. It protects against environmental, chemical, and biological dangers, regulates temperature, and produces antimicrobial substances. Structurally, it is composed of three layers—the epidermis, dermis, and hypodermis—which ensure insulation, absorption, energy storage, and attachment to underlying tissues [1]. Given its crucial role and constant exposure to external factors, the skin is vulnerable to a wide range of disorders. Skin disorders are one of the most common reasons for visits to the doctor, affecting more than 50% of the world's population, and they are a significant public health problem. Skin diseases range from rare conditions such as genodermatoses to common ones such as psoriasis, atopic dermatitis, and skin cancer, and they are one of the leading causes of death globally [2]. Among them, melanoma is the most aggressive form of skin cancer in terms of incidence and mortality worldwide. According to the World Health Organization, incidence is highest in Australia, followed by North America and Europe. The global age-standardized mortality rate is 0.53 per 100,000, ranking 22nd among cancer deaths, with the highest mortality in Oceania and New Zealand. In general, men have been shown to have higher mortality rates compared to women in North America and Oceania. At the same time, this difference is minor in Europe and minimal in Asia [3].

Given its high mortality and rising incidence in some regions, effective treatment strategies for melanoma are essential. Before 2011, the options were surgery, radiotherapy, and chemotherapy. Since then, new treatments have been

approved, such as the first immune checkpoint inhibitor (ipilimumab) and the first small-molecule kinase inhibitor (vemurafenib). However, despite progress in recent years, treatments for melanoma still have limitations, with resistance and side effects being potentially life-threatening in some patients [4].

In recent years, drug delivery systems (DDSs) based on nanotechnology have demonstrated significant potential in advanced melanoma therapy, allowing drugs to be delivered directly to cancer cells. Their size and surface properties allow for precise delivery to melanoma cells, decreasing cytotoxicity while minimizing damage to healthy tissues. These systems also protect drugs from degradation and reduce the doses needed, minimizing their side effects. The investigated nanosystems include lipid-based carriers, inorganic nanoparticles, and natural systems such as exosomes, offering both diagnostic and therapeutic advantages in melanoma management [5]. Given the growing role of nanomaterials in cancer therapy, silver nanoparticles (AgNPs) have gained more attention due to their widespread applications in antimicrobial, biomedical, and environmental fields. Traditional synthesis methods (electrochemical or photochemical reduction, heat evaporation, and biological routes) are often expensive and involve toxic chemicals, which cause environmental and health problems [6]. Recent research increasingly supports green synthesis methods for nanoparticle production, as they minimize poisonous waste, use safe solvents such as water, and avoid chemicals that limit biomedical applications [7]. Green synthesis using plant extracts has emerged as a sustainable alternative because phytochemicals can simultaneously reduce and stabilize nanoparticles, allowing for the rapid and cost-effective production of stable, biocompatible AgNPs, and it is currently one of the most investigated methods in nanoparticle research [6, 8].

Pomegranate (*Punica granatum* L.), a member of the *Punicaceae* family, is a promising plant source for green synthesis. The fruit is full of nutrients and bioactive substances such as flavonoids, phenolic acids, and tannins (ellagitannins, punicalagin), many of which have significant antibacterial and antioxidant qualities. Higher levels of polyphenols are found in the peel, which also exhibits antimutagenic, antibacterial, antiatherogenic, and antioxidant properties. Because of these qualities, pomegranates, especially their peel, are an essential source for the organic synthesis of bioactive AgNPs that can be used in medicine [9, 10]. The dual function of phytochemicals as coating and reducing agents is the mechanism behind the biosynthesis of silver nanoparticles using pomegranate peel extract. While their functional groups bind to the surface of the nanoparticles to prevent aggregation, tannins, anthocyanins, phenolic acids, and flavonoids—particularly punicalagin, ellagic acid, and gallocatechin—donate electrons to reduce Ag^{+} ions to metallic Ag^{0}. The difference in potential between the silver ions and the phytochemicals determines this redox process, which causes them to oxidize and change the color of the extract from pale yellow to brown [11].

In this context, the present study aims to obtain silver nanoparticles (AgNPs) via green synthesis using PG peel extract and to investigate their biological effects on human melanoma cells (RPMI-7951) through cell viability, morphological assessment, Hoechst staining, and mitochondrial .morphology

2. Materials and Methods

2.1. Plant Material

PG fruits were purchased from the western part of Romania (Timişoara, coordinates: 45°44′58″ N latitude, 21°13′38″ W longitude) and identified at the Faculty of Pharmacy, "Victor Babeş" University of Medicine and Pharmacy Timişoara. First, the pomegranate fruits were washed several times with ultrapure water from the Milli-Q® Integral Water Purification System (Merck Millipore, Darmstadt, Germany) to avoid contamination. After thorough washing and drying, the peel was carefully separated, oven-dried at 23 ± 1 °C for 5 days, and ground into a fine powder.

2.2. *Punica granatum* Peel Ethanolic Extract Preparation

For the preparation of the PG ethanolic extract, 50 g of fine powder was mixed with 250 mL of 95% ethanol and left to macerate for 2 weeks. Then, the mixture was ultrasonicated (50% amplitude) for 1 h to extract more polyphenols from the pomegranate peel using a QSonica 700W ultrasonic processor. Finally, the ethanolic extract was filtered through filter paper.

2.3. Green Synthesis of AgNPs from Punica granatum Peel Extract

To obtain PG-AgNPs via the green method, 100 mL of pomegranate peel ethanolic extract was stirred using a magnetic stirrer. At 60 °C, under stirring at 500 rpm, an aqueous solution of 1 M $AgNO_3$ (50 mL) was added to a thin wire, and the mixture was stirred for another two hours. After 15 min, the light red-orange mixture turned reddish-orange to reddish-brown, and after 30 min, it became reddish-black. After two hours, the color of the mixture changed to brown-black,

which confirmed the reduction of the $AgNO_3$ solution to AgNPs. Thus, pomegranate AgNPs synthesized by the green method were obtained.

2.4. Reagents and Instruments

In vitro analyses were performed using reagents and equipment obtained from certified commercial suppliers. From Sigma Aldrich, Merck KGaA (Darmstadt, Germany), the following reagents were purchased: phosphate-buffered saline (PBS) solution and an antibiotic mixture of penicillin/streptomycin. From Roche Holding AG (Basel, Switzerland) the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained and used for cell viability assays. From Thermo Fisher Scientific (Waltham, MA, USA), the nuclear dye Hoechst 33342 and the mitochondrial marker MitoTracker™ Red CMXRos were used for cell staining and visualization; from PAN-Biotech GmbH (Aidenbach, Germany), trypsin–EDTA, fetal bovine serum (FBS), and the culture medium EMEM (Eagle's Minimum Essential Medium) were obtained. For extract preparation, 96% ethanol was acquired from Chemical Company SA (Iasi, Romania).

Regarding instrumentation, all devices and software used were provided by BioTek Instruments Inc. (Winooski, VT, USA), including a Cytation 5 microplate reader, a Lionheart FX automated microscope, and Gen5™ software (version 3.14) used for data acquisition and analysis.

2.5. Cell Culture Protocol

2.5.1. Cell Culture Conditions

The human melanoma cell line RPMI-7951 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic mixture (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.5.2. Study Design

The RPMI-7951 cells were treated with five different concentrations of PG-AgNPs (10, 15, 20, 25, 30 μg/mL) for 24 h. The selected concentration range of PG-AgNPs used in the biological assays was chosen based on preliminary experiments that indicated both efficacy and acceptable cytocompatibility. Moreover, this range is consistent with values reported in the literature for similar silver nanoparticle systems, ensuring comparability and physiological relevance of our findings.

2.6. Cell Viability Assessment by MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability, following the protocol described by Romanu et al. [12]. Experiments were performed after RPMI-7951 cells were exposed for 24 h to PG-AgNPs at concentrations varying from 10 to 30 μg/mL (10, 15, 20, 25, and 30 μg/mL). Cells were cultured in 96-well flat-bottomed plates at a density of 1×10^4 cells/well and treated with the test substance. At the end of the 24 h incubation period, the culture medium was replaced with fresh medium, and 10 μL of MTT kit 1 was added to each well. Cells were then incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. Subsequently, 100 μL of MTT kit two was added, and the plates were left at room temperature for 30 min. The absorbance was measured at wavelengths of 570 nm and 630 nm using a Cytation 5 instrument delivered by BioTek Instruments Inc. (Winooski, VT, USA).

2.7. Bright-Field Cell Morphology Analysis

To evaluate the effects of PG-AgNPs on the cell morphology of RPMI-7951 cells, they were cultivated in 96-well plates (1×10^4 cells/well). To assess the possible morphological changes, images were taken at 20× magnification under bright-field illumination using the Lionheart FX automated microscope and processed with Gen5 Microplate Data Collection software, version 3.14 (from BioTek Instruments Inc., Winooski, VT, USA).

2.8. Hoechst 33342 Nuclear Staining

To further investigate the effect of PG-AgNPs on cell nuclei, the Hoechst staining method was used,following the protocol described by Dan et al. [13]. Cells were cultured in 12-well plates at a density of 1×10^5 cells/well and treated with PG-AgNPs at the highest concentration of 30 μg/mL for 24 h under standard incubation conditions. After this period, protected from light, the culture medium was removed, and the Hoechst solution obtained by 1:2000 dilution in PBS was added to each well. The plate was then incubated for 5–10 min in a dark place. Subsequently, the staining solution was

removed, and the wells were washed three times with PBS. Representative images of the nuclei were captured using the Lionheart FX automated microscope at 20X magnification, and image analysis was performed using Gen5™ Microplate Data Collection and Analysis software (version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

2.9. Mitochondrial Immunofluorescence Staining Using MitoTracker™ Red CMXRos

To assess mitochondrial morphology, RPMI-7951 cells were cultured in 12-well plates at a density of 1×10^5 cells/well and allowed to adhere until optimal confluence was achieved, and the experiments were performed following a previously published protocol [13]. The cells were then treated for 24 h with PG-AgNPs at the highest concentration of 30 μg/mL. The MitoTracker dye was initially dissolved in DMSO at a stock concentration of 1 mM and subsequently diluted in the culture medium (EMEM 10%) to a final concentration of 300 nM. Following a 30 to 45 min incubation period with the staining solution under standard culture conditions, the cells were thoroughly washed with PBS to remove any remaining dye. For evaluation, images were captured using a Lionheart FX automated fluorescence microscope at 20× magnification, and the data were analyzed with Gen5™ Microplate Data Collection and Analysis software (version 3.14, BioTek Instruments Inc., Winooski, VT, USA).

2.10. Statistical Analysis

All results are presented as mean ± standard deviation (SD). Data distribution was confirmed to be normal using the Shapiro–Wilk test. Therefore, a one-way ANOVA followed by Dunnett's multiple comparisons test was applied for statistical analysis. The analysis was performed using GraphPad Prism version 10.2.3 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Statistical significance was indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results and Discussions

Melanoma is a malignant tumor arising from melanocytes. Although it accounts for only a small proportion of skin cancers, it is the leading cause of mortality in this group because of its aggressive progression and high metastatic potential [14]. Although chemotherapeutic agents such as antimetabolites, terpenoids, and alkaloids remain essential in cancer therapy, their use is limited by their toxicity and drug resistance. These challenges emphasize the need for safer and more effective anticancer alternatives [15]. Due to their numerous biological activities, such as antioxidant, antiproliferative, anti-inflammatory, antibacterial, antifungal, and antitumor properties, silver nanoparticles (AgNPs) have attracted much attention as a promising treatment, especially for melanoma therapy [16]. AgNPs are metallic nanoparticles that have shown promise in preventing tumor cell growth. The release of Ag^+ ions, which produce ROS, alters mitochondrial transmembrane potential and triggers cell death pathways, thereby linking it to their toxicity. Cytotoxicity in cells is influenced by physicochemical characteristics, including size and metal content; smaller particles usually exhibit higher toxicity [17]. Plant extracts offer a sustainable and environmentally friendly substitute for traditional chemical treatments in nanoparticle synthesis, enabling large-scale production without the use of hazardous reagents, high pressure, coatings, or high energy [18].

In the present study, AgNPs were obtained by a green method using ethanolic extract from PG peel in a 1:2 ratio with $AgNO_3$ solution at 60 °C. Then, the cytotoxic effect of AgNPs on the RPMI-7951 human melanoma cell line was analyzed using an MTT assay, morphology analysis, Hoechst, and Mitotracker staining.

3.1. Pomegranate-Derived AgNPs Induced a Moderate, Dose-Dependent Decrease in RPMI-7951 Melanoma Cell Viability

The MTT assay was employed to assess the cytotoxic potential of PG-AgNPs on human melanoma RPMI-7951 cells at concentrations of 10, 15, 20, 25, and 30 μg/mL (**Figure 1**). A dose-dependent decrease in cell viability was observed following exposure to the nanoparticles. At the lowest tested concentration (10 μg/mL), cell viability was 95.06% compared to the control group, gradually decreasing to 88.85% at 15 μg/mL, 84.38% at 20 μg/mL, 81.06% at 25 μg/mL, and reaching 79.43% at 30 μg/mL. These findings indicate a moderate cytotoxic effect of AgNPs on melanoma cells, indicating a precise dose–response relationship across the tested concentration range.

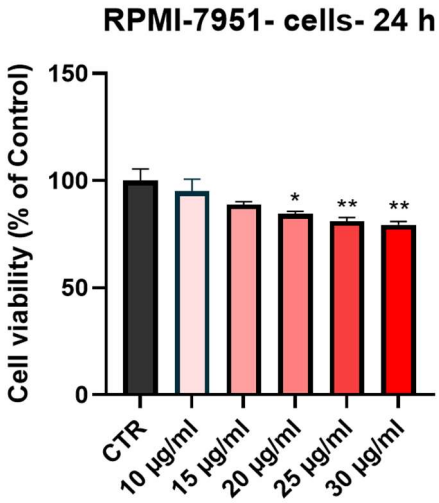

Figure 1. Evaluation of the in vitro cell viability after 24 h of treatment with PG-AgNPs at concentrations of 10, 15, 20, 25, and 30 μg/mL using the human melanoma cell line RPMI-7951. Results are expressed as percentages (%) of cell viability normalized to the control group (untreated cells). All data are expressed as mean values ± SD from three independent experiments performed in triplicate. Statistical analysis was performed by a one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons. Statistically significant differences from the control were noted as follows: * $p < 0.05$; ** $p < 0.01$.

In line with these results, Fernandes et al. demonstrated that green-derived (with pomegranate extract) silver nanoparticles exhibited low cytotoxicity on mouse fibroblast cells (L929), maintaining a viability of about 80% at 50 μg/mL. In contrast, conventional nanoparticles were found to be toxic at 6.25 μg/mL, with viability below 20%. This offers a promising perspective for the use of green synthesis nanoparticles in biomedical applications due to their better profile [19]. Moreover, similar concentration-dependent decreases in cell viability have been reported in other cell lines, including cervix adenocarcinoma (HeLa), larynx carcinoma (HEp-2), liver carcinoma (Hep-G2), prostate adenocarcinoma (PC3), and kidney epithelial cells (Vero), confirming the generalized nature of this biological response [20]. According to another study by Badawi et al., solid lipid nanoparticles or PG formulations exhibit more potent cytotoxicity against cancer cell lines such as human mammary carcinoma (MCF-7), human prostate carcinoma (PC-3), and human hepatocellular carcinoma (HepG2); their nanoencapsulation further enhances this selectivity [21]. In line with these findings, the present results on RPMI-7951 melanoma cells also demonstrated dose-dependent reductions in viability and morphological alterations, supporting the anticancer potential of pomegranate-based nanostructures.

3.2. Pomegranate-Derived AgNPs Induced Morphological Changes in RPMI-7951 Cells

Investigating the impact of natural compounds on human cells and epithelial structures is essential, as morphological evaluation provides insight into cellular responses and potential therapeutic applications [22]. To further investigate the cytotoxic effects observed in the MTT assay, the impact of PG-AgNPs on the morphology of RPMI-7951 human melanoma cells was evaluated after 24 h of treatment at concentrations of 10, 15, 20, 25, and 30 μg/mL (**Figure 2**). A progressive, dose-dependent reduction in cell confluency was noted, with pronounced morphological alterations at higher concentrations, particularly at 25 and 30 μg/mL.

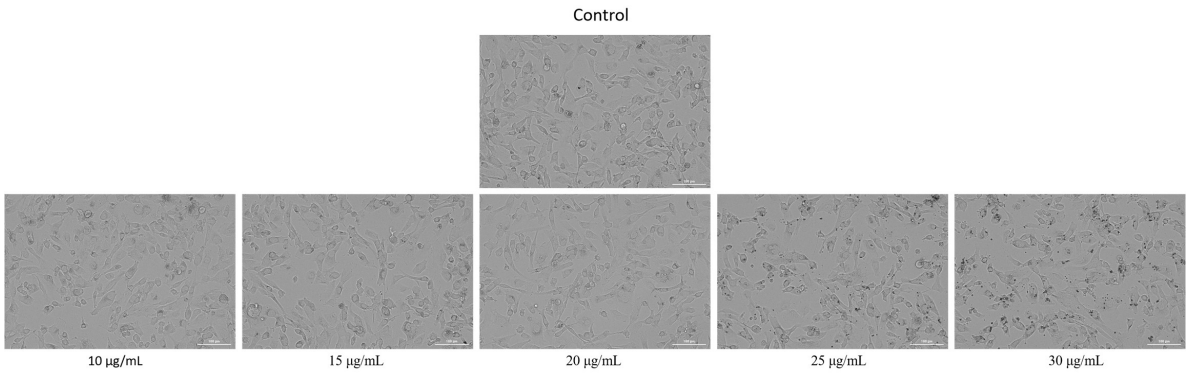

Figure 2. The images presented illustrate the morphological aspects of RPMI-7951 cells 24 h after treatment with PG-AgNPs at concentrations of 10, 15, 20, 25, and 30 μg/mL. The experiment was performed in triplicate. The scale bar represents 100 μm.

3.3. Pomegranate-Derived AgNPs Induced Nuclear Apoptotic-Like Alterations in RPMI-7951 Cells

To gain a more detailed understanding of the mechanism of action of PG-AgNPs, the nuclear morphology of RPMI-7951 cells was assessed using a staining method. Hoechst staining is a fluorescent method used to observe apoptotic nuclear changes, such as chromatin condensation and fragmentation. It is widely applied for in vitro studies due to its DNA specificity and low toxicity [23]. According to **Figure 3**, after 24 h of treatment with the highest tested concentration (30 μg/mL), mild changes in nuclear morphology—highlighted by yellow arrows—were observed compared to the untreated control. The nuclei appeared slightly rounded and reduced in number, and in some cells, chromatin condensation was noticeable, a typical feature associated with apoptotic-like changes.

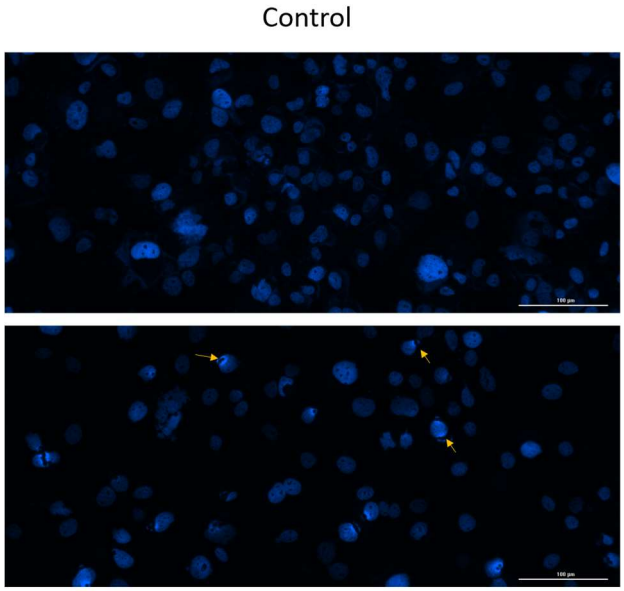

Figure 3. Representative fluorescence images showing nuclear morphological changes in RPMI-7951 cells after 24 h of treatment with PG-AgNPs at a concentration of 30 μg/mL. Yellow arrows indicate alterations in nuclear appearance. Scale bar indicates 100 μm. The experiment was performed in triplicate.

3.4. Pomegranate-Derived AgNPs Disrupted Mitochondrial Morphology and Integrity in RPMI-7951 Cells

To assess the effect of PG-AgNPs on mitochondrial morphology, RPMI-7951 cells were treated for 24 h with the highest tested concentration (30 μg/mL), followed by immunofluorescence staining with MitoTracker (**Figure 4**). Compared to untreated control cells, the treatment induced mild morphological alterations at the mitochondrial level, including mitochondrial condensation (indicated by yellow arrows) and a decrease in mitochondrial confluency.

Previous studies have demonstrated that AgNPs can induce cytotoxic effects through molecular pathways such as reactive oxygen species (ROS) generation, mitochondrial dysfunction, and the activation of caspase-dependent apoptosis [24, 25, 26]. The nuclear condensation and mitochondrial alterations observed in our study on the RPMI-7951 cells are therefore in line with these reported mechanisms, suggesting that a similar mechanism may underlie the effects of PG-AgNPs in this model.

One of the limitations of this study is the lack of detailed physicochemical characterization of PG-AgNPs (e.g., particle size distribution, morphology, and stability), which will be addressed in future research to enhance reproducibility and interpretation of the results.

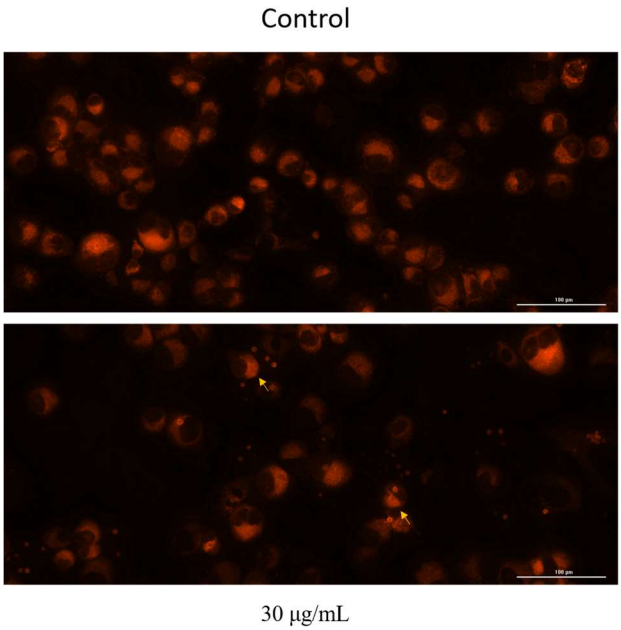

Figure 4. Fluorescence microscopy images showing mitochondrial morphological alterations in RPMI-7951 cells following 24 h exposure to PG-AgNPs at a concentration of 30 μg/mL. The scale bar represents 100 μm. The experiment was conducted in triplicate. m. The yellow arrows indicate changes at the mitochondrial level.

4. Conclusions

The results highlight the cytotoxic potential of PG-AgNPs on the human melanoma cell line RPMI-7951. The MTT assay demonstrated a significant dose-dependent reduction in cell viability, especially at concentrations of 25 and 30 μg/mL, indicating a moderate cytotoxic effect. Morphological analysis demonstrated characteristic cellular changes, such as decreased confluency, cell shrinkage, and the presence of cell fragments, predominantly observed at higher concentrations. Hoechst staining indicated nuclear changes characteristic of an apoptotic-like process, including chromatin condensation and a reduction in atomic size. In parallel, mitochondrial analysis performed by immunofluorescent staining with MitoTracker revealed structural alterations, including mitochondrial condensation and reduced integrity. These findings support further in vivo investigations and mechanistic studies to validate the biomedical potential of PG-AgNPs as eco-friendly anticancer agents.

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Author contributions

Conceptualization, I.-A.P. and A.-D.S.; methodology, I.-A.P.; formal analysis, D.P.; investigation, I.-A.P. and A.-D.S.; resources, A.-D.S.; writing—original draft preparation, I.-A.P. and D.P.; writing—review and editing, A.-D.S.; visualization, D.P.; supervision, A.-D.S. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

Data supporting these findings are available within the article or upon request.

Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

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