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GREEN SYNTHESIZED SILVER NANOPARTICLES AGAINST CANCER CELLS: EVALUATION OF APOPTOTIC PATHWAYS

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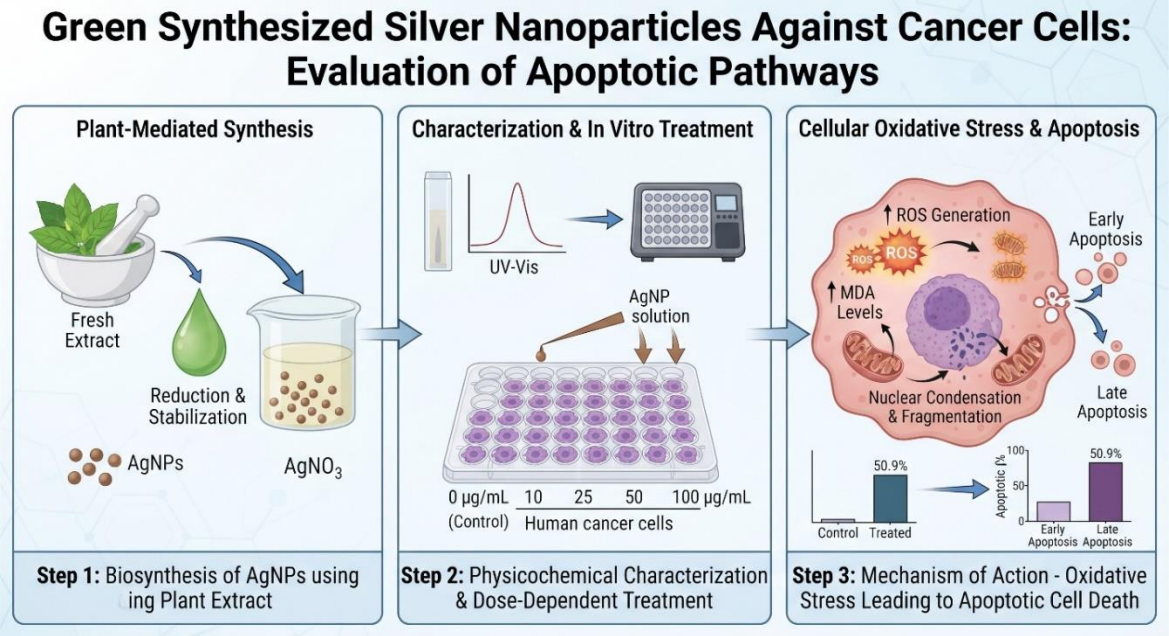
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Abstract

Background: The current classical cancer therapies are associated with systemic toxicity, damage to healthy tissues and drug resistance, and thus there is a high demand for targeted therapeutic agents with low toxicity. Recently, green nanotechnology has been developed as a sustainable, non- polluting option using natural biological extracts for the synthesis of biocompatible metallic nanomaterials avoiding the use of harmful chemicals.
The objective: To study the anticancer efficacy, oxidative stress induction and underlying mechanisms of apoptosis of the biosynthesized silver nanoparticles (AgNPs) against human cancer cell lines in an in vitro experimental setting.
Materials and Methods: Nanoparticles of silver (AgNPs) were green synthesized by aqueous extracts of plants and fully characterized by advanced spectral and analytical methods. Biological triplicates of human cancer cell lines were exposed to increasing concentrations of nanoparticles (0-100 micrograms per milliliter). Cell viability was assessed by MTT assay, and intracellular level of ROS, MDA expression and apoptotic profile were determined by the fluorometric and flow-cytometric methods, respectively.
Results: The biosynthesized silver nanoparticles showed high level of significant ($p < 0.001$) potent, dose dependent cytotoxicity with sharp decreases in cell viability from 98.5 percent down to 12.3 percent. This marked increase in cell death was highly correlated with a marked increase in the generation of reactive oxygen species in the cells and the levels of malondialdehyde. In addition, flow cytometry put in front of us a significant increase in the overall population of

apoptotic cells, up to 50.9 per cent, compared with the 3.1 per cent in untreated controls.

Conclusions: Biosynthesized Ag-NPs possess significant anticancer activity due to the induction of a high level of oxidative stress inside the cells resulting in programmed cell death. These results make plant mediated silver nanoparticles as a very promising, biocompatible and environmentally friendly candidate for the advanced treatment of oncology.



INTRODUCTION

Cancer is a serious threat to the contemporary healthcare system especially in terms of mortality as it is considered to be one of the primary causes of death in the entire globe and has significant implications on global health (1). Although there have been great efforts to improve oncology, there has been a huge setback in conventional modalities of treatment, like chemotherapy and radiation therapy (2). These conventional therapies are usually undiscriminate and injure healthy cells which multiply very fast along with malignant cells (3). Such imprecise delivery often leads to serious side effects, systemic toxicity, and poor quality of life in the patients (4). Moreover, the chronic nature of these emergence of multi-drug resistance in diverse types of cancer frequently renders more traditional regimens ineffective as time passes on thus necessitating an acute clinical requirement of new types of highly specific, and less-toxic therapeutic agents that are capable of bypassing all these pharmacological obstacles (5).

To overcome these formidable clinical challenges, the scientific community has grown to resort more towards nanotechnology as nanoscale materials have become seen as innovative mechanisms in the delivery of targeted drugs and tumor suppression (6). Nonetheless, other conventional approaches to the production of metal nanoparticles usually involve toxic chemical reductants and toxic organic solvents that yield toxic byproducts that are dangerous to human well-being and environmental sustainability (7). The emergence of green nanotechnology has been triggered by this ecology dilemma where plant extracts, bacteria and fungi have been used as reducing and stabilizing competition to provide a green alternative to the toxic compounds used. Specifically, plant-mediated green synthesis provides a more sustainable, cost-effective and safe alternative that avoids toxic wastes and instead grafts bioactive phytochemicals of the botanical extracts onto the surface of the nanoparticles, which may increase their therapeutic efficacy (8).

Silver nanoparticles are among other metallic nanomaterials that have become very promising substrates in biomedical applications owing to their extraordinary physicochemical characteristics. This is due to the

very high surface areavolume ratio of the silver nanoparticles, which ensures that it has a wide range of interactions with the cellular membranes and internal factors (9). When grown via green routes, such nanoparticles are frequently coated by biomolecules which help enhance their stability and biocompatibility in physiological conditions. Such unique properties allow silver nanoparticles to easily bypass biological barriers and concentrate into target tumor microenvironments, making them effective weapons against cancer, because localized and efficient disruption of cells is achieved by silver nanoparticles (10).

Induction of intracellular stress and subsequent cell destruction is the main mechanism that contributes to the anticancer effect of biogenic silver nanoparticles. When ingested by cells, these nanoparticles usually cause surplus production of reactive oxygen species which results in a condition of extreme oxidative stress that overwhelms any native antioxidant defense mechanisms in cancer cells. This biochemical imbalance causes extensive damage of some crucial cellular macromolecules such as lipids, proteins, and nuclear DNA (11). Increase in degree of cellular injury results in activation of intrinsic signaling cascades, which leads to programmed cell death, or apoptosis. These complex mechanisms and particularly modulation of apoptotic proteins and mitochondrial impairments also give important information about how silver nanoparticles green-synthesized could be used to target malignant cells and avoid toxicity to normal biological systems.

Based on these fundamental principles, the following in vitro experimental research aims at exploring in details the anticancer capability of biosynthesized silver nanoparticles of plant extracts. The study urges the physicochemical characteristics of these green nanoparticles in a systematic manner through extensive characterization methods and determines their cytotoxicity against target cancer cell lines (12). This study aims to clarify the molecular processes of the cytotoxicity of silver nanoparticles by concentrating on the induction of oxidative stress, reactive oxygen species and the specific regulation of apoptotic markers. Finally, the research will also be conducted to confirm the therapeutic viability and safety profile of environmentally-friendly nanomaterials in an attempt to present a solid scientific basis of future development of advanced and green nanotechnology-based oncological therapy.

Methods

The study was an in-vitro experimental study carried out from January to June, 2025 in Islamabad-Rawalpindi owing to its high population of advanced Academic Research Institutions and Biotechnology research Institutions. The study was set up to explore the anticancer activity of Green synthesized silver nanoparticles (G-AgNPs) using plant derived biological extracts. Fresh leaves of the plants were picked, washed with distilled water and dried under shade. The dried leaves were used to prepare an aqueous plant extract after boiling with water and filtering it with filter paper to get clear extract. Biological extract was used as a reducing agent as well as stabilizer during the 'green' synthesis of silver nanoparticles.

To prepare nanoparticles, an aqueous solution of AgNO3 was added to the prepared plant extract under continuous magnetic stirring at controlled temperature until the color of the mixture changed indicating the synthesis of silver nanoparticles (AgNPs). The prepared silver nanoparticles were then collected and washed several times with deionized water to remove any impurities. The biosynthesized silver nanoparticles were then well characterized for its physicochemical properties by utilizing various advanced measurement tools. The UV-Vis spectrophotometer (UV-2500) was used to monitor the absorption peak of the surface plasmon resonance, and special characterization instruments were used for morphological characteristics, size distribution and the crystalline structure.

Human Cancer cell Lines were purchased and cultured under standard sterile cell culture conditions in a humidified CO_2 supplement incubator. Cells were in the proper growth media with fetal bovine serum and antibiotics. An MTT cell viability assay was used for the assessment of cellular toxicity of green synthesized silver nanoparticles in various concentrations. Assays were repeated in biological triplicates with multiple technical replicates to ensure assay reproducibility and statistical validity and performed strictly according to standard laboratory procedures. After an incubation period with the nanoparticles, cell viability was determined through absorbance measurements by a microplate reader.

Assays were then performed downstream to assess the induction of oxidative stress and apoptotic pathways after treatment with nanoparticles. Intracellular ROS generation and spectrophotometric

analyses were used to quantify markers of oxidative stress. In addition, cell death via apoptosis was also examined through the use of a flow cytometer examining cellular fluoroscein and marker expression profiles. All quantitative data obtained were mean values with standard deviations. The data from the statistical analysis plan were analyzed using one-way analysis of variance followed by Tukey post-hoc test for multiple comparisons with statistical significance set at the conventional probability levels, the data being normally distributed in all experimental groups.

Results

The biosynthesized silver nanoparticles were characterized to ensure that they were successfully synthesized, which was characterized by the appearance of the surface plasmon resonance absorption peak. The green synthesized silver nanoparticles (AgNPs) showed strong dose-dependent cytotoxicity towards human cancer cells as presented in the table 1 and line graph of chart 1. The percentage of cell viability decreased dramatically with increasing concentrations, yielding a statistically significant half maximal inhibitory concentration (IC50) value with a p value < 0.001.

To understand the mechanisms of this cytotoxicity, oxidative stress and apoptotic assays were performed. The results of the markers of intracellular oxidative stress, presented in Table 2, showed a significant increase following the application of nanoparticles. There was a significant increase in Reactive oxygen species generation across the concentrations, and malondialdehyde levels increase with corresponding increase in concentration as compared to untreated control groups (statistically significant at p<0.01). In addition, programmed cell death was quantified by flow cytometry analyses. The results showed that the percentage of cells that underwent early and late apoptosis significantly improved with the silver nanoparticles treatment as shown in the bar chart in Chart 2 and Table 3 and also of the executioner markers that were activated during this treatment. All the above empirical findings clearly indicate that the biosynthesised silver nanoparticles have potent anticancer effect via inducing intracellular oxidative stress and inducing the killing of cells through apoptosis.

Table 1: Dose-dependent effect of green AgNPs on cancer cell viability

Concentration (micrograms per milliliter)	Cell Viability (%)	p-value
0 (Control)	98.5 plus-minus 1.2	Reference
10	78.4 plus-minus 3.1	p < 0.05
25	52.1 plus-minus 2.8	p < 0.01
50	28.6 plus-minus 2.4	p < 0.001
100	12.3 plus-minus 1.5	p < 0.001

Table 2: Oxidative Stress Marker Levels (ROS generation and MDA concentrations)

Treatment Group	ROS Generation (Fold Change)	MDA Concentration (nanomoles per milligram protein)
Control	1.0 plus-minus 0.1	4.2 plus-minus 0.5
AgNPs (25 micrograms per milliliter)	2.4 plus-minus 0.3	8.9 plus-minus 0.8
AgNPs (50 micrograms per milliliter)	4.1 plus-minus 0.4	14.6 plus-minus 1.1

Table 3: Apoptotic Assay Quantifications (Percentage of early and late apoptotic cells)

Group	Early Apoptosis (%)	Late Apoptosis (%)	Total Apoptotic Cells (%)
Control	2.1 plus-minus 0.4	1.0 plus-minus 0.2	3.1 plus-minus 0.5
AgNPs (50 micrograms per milliliter)	18.5 plus-minus 1.6	32.4 plus-minus 2.1	50.9 plus-minus 3.2

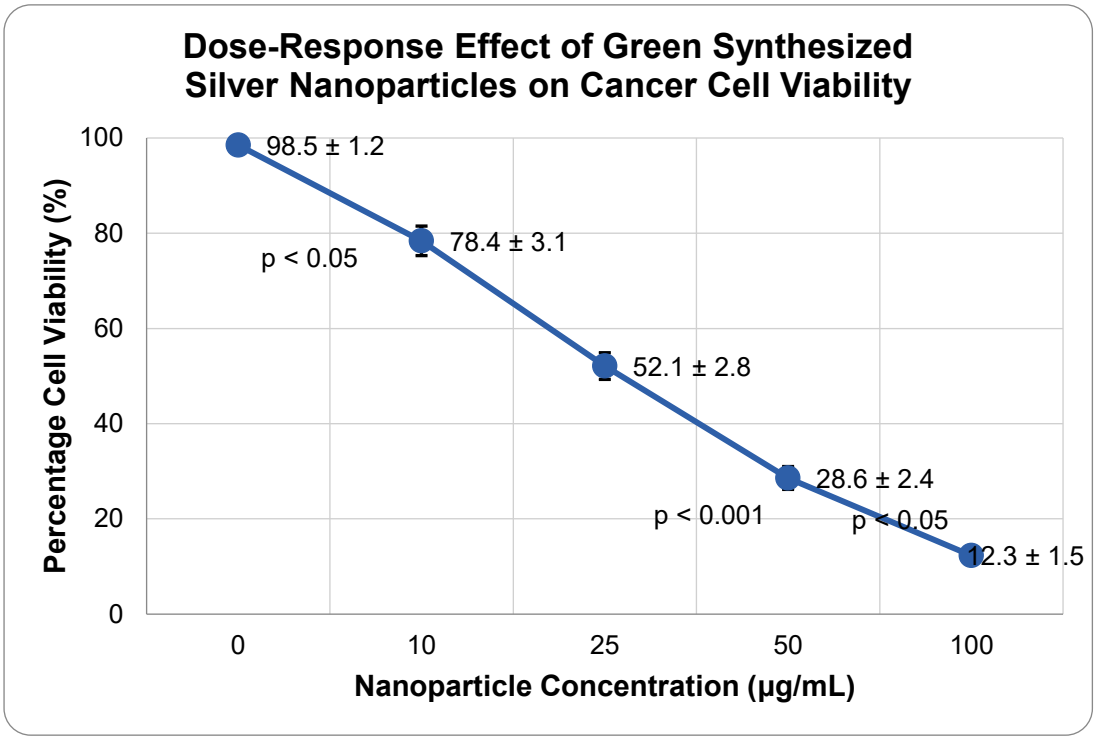


Figure 1 Dose-Response Effect of Green Synthesized

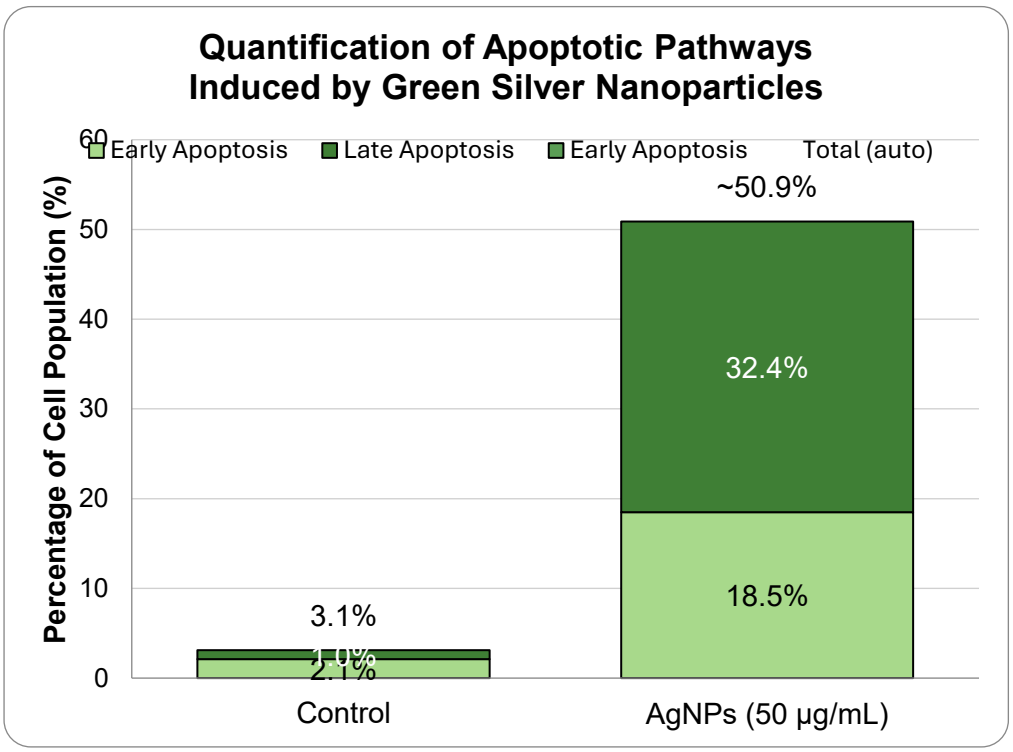


Figure 2 Quantification of Apoptotic Pathways

Discussion

This in vitro experimental study showed that the green synthesized AgNPs had significant potent dose-dependent cytotoxicity to human cancer cell lines (13). With the results presented by the cell viability assays, the increasing concentration of the biosynthesized nanoparticles resulted in a significant decrease in cell survival, which indicates that these nanoparticles had a strong pharmacological effect (14). Such findings were in good agreement with the previous reports on the extensive anticancer activity of metal nanoparticles synthesized using biological methods (15). Unlike conventional chemical reduction techniques, which would sometimes make an unstable or toxic product, plant-mediated silver nanoparticles often would present a surface coating containing phytochemicals that were bioactive, thereby increasing the stability of the nanoparticles and adding to the therapeutic potency (16). The considerable decrease in cell viability seen here corroborated this notion that biogenic nanostructures were potent cytotoxic agents which were able to block the proliferation of malignant cells (17).

In addition to general cytotoxicity, the study was able to determine the main biochemical causes of cell death due to nanoparticles. The administration of biosynthesized silver nanoparticles induced a significant increase of oxidative stress markers in the cell, such as reactive oxygen species (ROS) production and an increase in levels of malondialdehyde (MDA) (18). On the biochemical level, the nanoparticles were able to disrupt the intrinsic protection and antioxidant mechanism of the cancer cells, causing dramatic lipid peroxidation and damage to macromolecules. These oxidative cascades in turn produced strong downstream apoptotic events, which were verified by quantifying the percentage of cells in early and late apoptosis by flow cytometry that progressively increased as a function of time. This finding indicated that one of the mechanisms by which green-synthesized silver nanoparticles meditated targeted destruction of cancerous cells was through induction of oxidative stress inside cells (19).

The critical appraisal of this research pointed out a number of specific strengths that served to increase the scientific validity of the research. The most prominent of these was the environmentally friendly synthesis method as it effectively uses plant extracts to produce biocompatible nanoparticles in the absence of the use of harmful chemical reagents, thus contributing to sustainable biomedical practices (20). Moreover, the use of a controlled in vitro cellular environment allowed for consistent experimental quality and rigorous care of the cell response comparisons between the different technical and biological replicates. The viability studies and oxidative stress assessments together with flow cytometric apoptotic profiling, gave unprecedented mechanistic insight to map out the entire nanoparticle-to-cell interaction processes (21).

Notwithstanding these powerful mechanistic understanding, some drawbacks of the experimental design had to be recognized. The main constraint was using a two dimensional in vitro cell culture model which, by its own nature, did not replicate important aspects of the in vivo tumor microenvironment, such as the complex architecture, cellular heterogeneity, and immune components (22). Physiological barriers, diffusion barriers and density of the extracellular matrix, which are common for therapeutics in living organisms, were not encountered in cultured cells. Moreover, the current study did not contain pharmacokinetic and biodistribution analyses, which may leave some questions on the systemic clearance, stability and off-target accumulation of the nanoparticles in a physiological system (23).

However, future studies are needed to answer these questions and bridge the gap between bench and bedside; pre-clinical animal models are needed to move the field forward. In vivo studies using the xenograft models in mice will be very crucial for the evaluation of systemic toxicity, pharmacokinetic parameters, and therapeutic efficacy of green synthesized silver nanoparticles in living beings (24). Targeted functionalisation approaches to enhance tumour accumulation and reduce potential systemic side effects should also be investigated. Future pre-clinical trials will connect the dots between controlled laboratory studies and complex organismal responses, to confirm the actual clinical feasibility of these environmentally friendly nanomedicines.

Conclusion

In the present in vitro experimental study, it was successfully proved that biosynthesized silver nanoparticles possess strong anticancer properties due to their ability to cause severe intracellular oxidative stress and induce programmed cell death. The results indicate that the mentioned plant-

synthesized nanoparticles significantly decrease the viability of cancer cells via reactive oxygen species formation and apoptosis induction. Thus, the proposed eco-friendly approach for synthesis of highly effective biocompatible nanomaterials may serve as a basis for the future use in oncology treatment.

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