

ORIGINAL RESEARCH ARTICLE

Combination therapy of cisplatin and green silver nanoparticles enhances cytotoxicity and apoptosis in breast cancer cells

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Abstract

Cisplatin is one of the first-line drugs for the treatment of breast cancer and is known for its ability to disrupt cancer cell DNA. However, cisplatin chemotherapy carries side effects and the risk of drug resistance. A strategy to improve its anti-cancer efficacy while reducing its negative effects on health is to leverage the synergistic potential of natural molecules with cisplatin. In this study, we explored combination therapy using cisplatin along with biosynthesized silver nanoparticles (Ag NPs) to enhance apoptosis induction in MCF-7 breast cancer cells while reducing cisplatin resistance. The biosynthesized Ag NPs, derived from *Acacia Luciana* flower extracts, possess active molecules that effectively inhibit MCF-7 cells. Concurrent administration of cisplatin and Ag NPs resulted in a notable decrease in the IC₅₀ value – approximately 22 – 26 times lower compared to individual treatments of free Ag NPs and cisplatin, respectively. Furthermore, the combination therapy significantly increased the *BAK1/BCLX* ratio by 162-fold compared to the control, while the cisplatin alone failed to activate intrinsic apoptosis pathway. In addition, the expression level of the *CASP3* gene, indicative of the extrinsic pathway of apoptosis, increased approximately 273 times compared to free cisplatin (*CASP3* expression = 3.5). Notably, the combination therapy also reduced the expression of the *AKT1* gene, associated with cell survival and treatment resistance, when compared to free cisplatin (1.87 vs. 4.488). In conclusion, our findings proved that combination therapy effectively enhances apoptosis induction by cisplatin while reducing drug resistance.

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1. Introduction

Cancer encompasses a group of diseases characterized by uncontrolled cell growth and proliferation. Cancer cells possess the ability to grow abnormally, infiltrate nearby healthy tissues, and metastasize to distant sites through blood or lymphatic vessels, while benign tumors remain localized.¹ Breast cancer is increasingly prevalent in developed countries, with recent statistical studies revealing a rapid increase in global incidence, accompanied by an alarming 80% rise in mortality rates, raising significant concerns.²

Hormone therapy is the preferred treatment for metastatic breast cancer, but resistance toward this form of therapy would gradually develop among the treated patients over time, necessitating the use of chemical drugs for treatment.³ Among the various types of organic and inorganic drugs utilized in cancer treatment, platinum (Pt) compounds hold significant importance. Notably, cisplatin represents the first examined metal compound known for its ability to intercalate within the DNA of cancer cells.⁴ However, resistance to chemotherapy drugs often develops during the course of treatment. Tumors may exhibit intrinsic resistance to drugs either before chemotherapy or acquire resistance during treatment with drugs.⁵ Several factors contribute to treatment resistance, including genetic and epigenetic changes, interference in drug delivery to cells, weak drug absorption, increased drug metabolism or excretion, decreased blood drug levels, and decreased drug diffusion from the blood to tumors.⁶

In addition, cisplatin induces toxicity and side effects in various organs, including hepatotoxicity, nephrotoxicity, neurotoxicity, and cardiotoxicity.⁷ One of the promising methods to reduce the toxicity caused by cisplatin and eliminate drug resistance is the combined treatment of cisplatin with natural products.⁸ Several studies have confirmed the protective effects of natural products against cisplatin-induced side effects in various organs.⁹ In addition, natural products aid in the treatment of cisplatin-resistant tumors by modulating specific signaling pathways that induce apoptosis.¹⁰ Combination therapies involving cisplatin and a range of natural compounds, such as terpenoids,¹¹ Vitamin C,¹² naphthoquinones,¹³ phenylpropanoids,¹⁴ polysaccharides,¹⁵ alkaloids,¹⁶ saponins,¹⁷ and flavonoids,¹⁸ have been explored, with their results documented in various research articles.

Previous research has established the significant role of plant compounds in the green synthesis of silver nanoparticles (Ag NPs) and their effectiveness in inhibiting cancer cell growth.¹⁹ In our previous work, we utilized *Acacia Luciana* for the synthesis of Ag NPs and revealed the presence of active biomolecules such as hydrocarbons, sesquiterpenes, monoterpenes, and oxygenated diterpenes, which have demonstrated the potential to enhance apoptosis-related gene expression.²⁰ Building upon this foundation, we aim to investigate the cytotoxic effects of simultaneously administered biosynthesized Ag NPs with cisplatin on the MCF-7 breast cancer cell line. In addition, we aim to evaluate the effect of combination therapy on the expression of *BAK1*, *BCLX*, *CASP3*, and *AKT1* genes to quantify the rate of apoptosis enhancement and cisplatin resistance reduction.

2. Methods

The method for biosynthesis and characterization of Ag NPs from *Acacia Luciana* flower extract is available in our previous work.²⁰

2.1. Evaluating cell viability using MTT assay

The MCF-7 cell line, obtained from the Pasteur Institute, Iran, serves as a suitable model for laboratory studies of breast cancers due to its special characteristics.²¹ Similar to other epithelial cells, the MCF-7 cell line is cultured as a monolayer and requires a complete culture medium containing 45 mL RPMI, 5 mL fetal bovine serum, and 50 μ L penicillin/streptomycin. The culture medium was periodically changed based on the number of cells and the rate of cell proliferation until the desired cell count was reached. The cells are maintained in optimal conditions within an incubator set to 5% CO₂ and 37°C. Once cells occupy 75 – 80% of the flask bottom, they are transferred to 96-well plates (1.6×10^4 cells per well). After 24 h, the adhesion of cells to the bottom of the plate was confirmed using an inverted microscope. Then, the culture media was discarded, and 200 μ L of fresh complete culture medium was added into the control wells. The remaining wells were filled with a culture medium containing either free cisplatin, free Ag NPs, or a cisplatin/Ag NPs mixture. The selected concentrations of each were chosen equally, six concentrations from 0.25 – 5 mg/mL of Ag NPs, six concentrations from 0.25 – 5 μ M of cisplatin, and six concentrations of the mixture of two compounds (0.125 – 5). Following 48 and 72 h of compound exposure, the cell supernatant was removed, and 200 μ L of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well for incubation for an additional 4 h. Then, the mixture of culture medium and MTT dye was removed from the cells and replaced with 200 μ L of DMSO per well to dissolve the formazan crystals. Absorbance was measured using a microplate reader at a wavelength of 570 nm.

2.2. Examining gene expression using real-time polymerase chain reaction (PCR)

MCF-7 cells were cultured in four separate flasks until reaching a density of 1×10^6 cells. Subsequently, the following treatments were applied: 4.17 mg/mL of Ag NPs was added to the first flask, 3.25 μ M of cisplatin to the second flask, a mixture of 0.178 mg/mL of Ag NPs, and 0.178 μ M of cisplatin to the third flask, while the fourth flask served as the control treated with only culture medium. Treatment concentrations were selected based on IC₅₀ values. The cells were exposed to the compounds for 24 h, after which the supernatant was removed from the flasks and the cells were washed with PBS. Trypsin was

used to detach the cells from the bottom of the flask, and finally, the cells were collected by centrifugation and the supernatant was completely removed. RNA isolation was performed using the High Pure RNA Isolation Kit from Roche, Switzerland. Subsequently, cDNA was synthesized using the Easy™ cDNA Synthesis Kit from Pars Tous Company, Iran. The details of both stages, along with the thermal cycling conditions for the real-time PCR quantitative assays targeting *BAK1*, *BCLX*, *CASP3*, and *AKT1* genes, are fully documented in previous works.^{22,23}

3. Results and discussion

3.1. Evaluating cell viability using MTT assay

In our previous work,²⁰ we discovered that biosynthesized Ag NPs using *Acacia Luciana* flower extracts, with a favorable size of approximately 50 nm, effectively reduced the growth of MCF-7 breast cancer cells. Specifically, we obtained an IC_{50} value of 4.37 mg/mL for Ag NPs after 72 h of treatment. Therefore, we reduced the concentration range to observe better effects. On the other hand, various sources have reported the IC_{50} value of cisplatin after exposure to MCF-7 cells to be approximately 5 – 10 μ M, depending on the treatment concentration range.^{24,25}

Therefore, considering the importance of combination therapy to improve the efficacy of cisplatin and eliminate drug resistance, we decided to explore a mixture of Ag NPs and cisplatin for MCF-7 treatment. As illustrated in Figure 1A, Ag NPs effectively inhibit the growth of MCF-7 breast cancer cells, with concentrations of 2.5 and 5 mg/mL reducing viable cells below 50%. Correspondingly, cisplatin demonstrated its anticipated efficacy in reducing the number of MCF-7 cancer cells, particularly at an approximate concentration of 5 μ M (Figure 1B). In other words, both Ag NPs and cisplatin with concentrations below 2.5 (mg/mL or μ M) did not exhibit significant inhibitory effects after 48 and 72 h exposure (refer to the arrows indicating IC_{50} in Figure 1).

As observed, the selected dose of cisplatin in this research was ≤ 5 μ M, aimed at determining the weakness of cisplatin in growth inhibition. Notably, the maximum confirmed concentration of cisplatin is 15 μ M.²⁶ Therefore, at higher concentrations, cisplatin accumulation within cells decreases, along with the formation of platinum-DNA adducts, resulting in reduced cytotoxicity and heightened resistance to cisplatin.²⁷ Numerous articles have confirmed that varying concentrations of cisplatin yield no significant

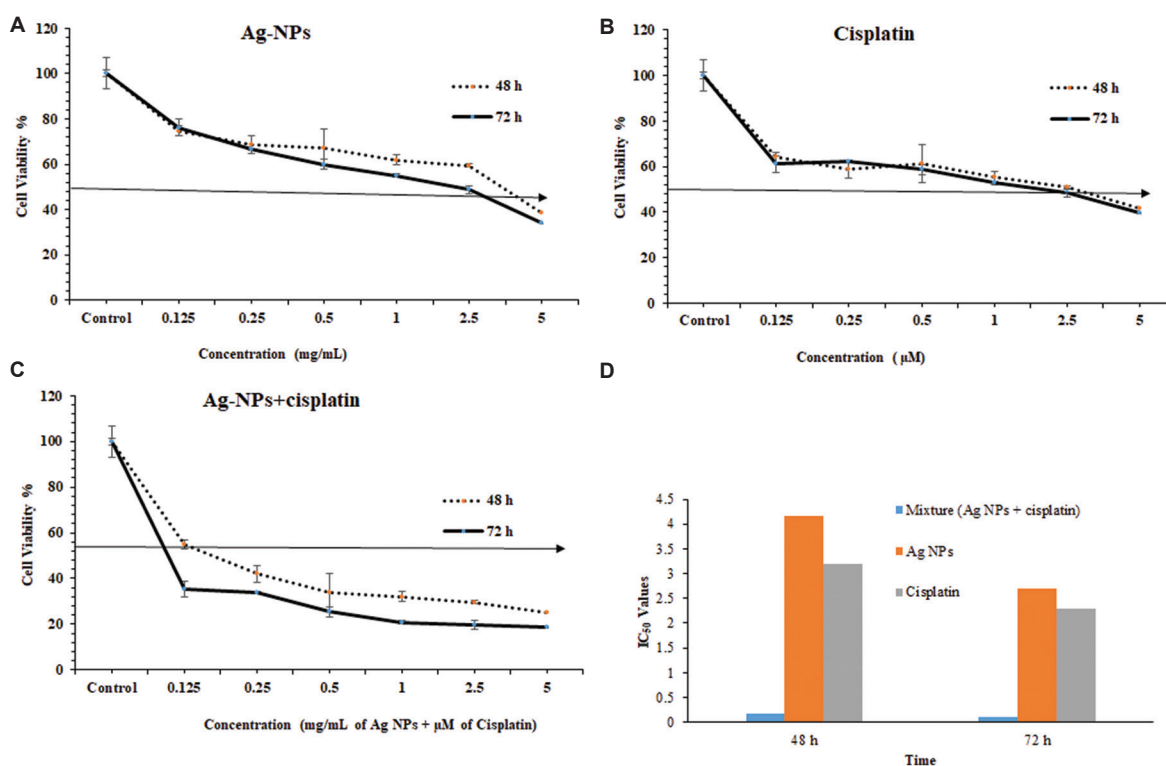


Figure 1. MCF-7 cell viability test using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 48 and 72 h of exposure to: (A) 0.125 – 5 mg/mL of Ag NPs; (B) 0.125 – 5 μ M of cisplatin; and (C) combination therapy with six mixtures of Ag NPs and cisplatin (in equal proportions to their free compounds). Panel (D) illustrates the IC_{50} values of compounds after 48 and 72 h of treatment.

Abbreviation: Ag NPs: Silver nanoparticles.

difference in MCF-7 cell growth inhibition. Typically, the resulting graph exhibits a very low slope, indicating that cisplatin, at concentrations higher than LD_{50} , completely inhibits MCF-7 growth without altering cell viability percentages.^{28,29}

Various studies have indicated that the optimal response of cancer cells to chemotherapy agents typically results in 60 – 70% cell death, with a maximum of 25% cell survival. This response reflects a predetermined limitation attributed to resistance factors and is considered one of the main side effects of cancer cell chemotherapy. Several studies have demonstrated that the presence of resistant cancer stem cells during chemotherapy is the reason, which also applies to cisplatin therapy.³⁰ This underscores the rationale behind our chosen concentration range (0.125 – 5 μ M) for several reasons. First, in any case, 25% cell survival is established, and 100% of deaths are never observed. Second, choosing the lowest possible concentration ensures the distinct effect of combination therapy, thereby yielding statistically comparative results.

When the two compounds are utilized in combination, the growth inhibition is significantly increased, as evidenced in [Figure 1C](#), where all concentrations succeeded in inhibiting the growth of MCF-7 cancer cells at both 48 and 72 h. This underscores the capacity of biosynthesized Ag NPs with *Acacia Luciana* flower extract to increase the sensitivity of MCF-7 cells to cisplatin, eventually increasing its antitumor effect. The reason for this claim is a 22 – 26-fold reduction in the IC_{50} value of the combination therapy compared to the IC_{50} values of Ag NPs and cisplatin individually after 72 h exposure, which is evident in [Figure 1D](#). In a similar study carried out by Fattah *et al.*,³¹ it was demonstrated that the combined treatment of cisplatin with piperine, an alkaloid found in black pepper, exhibited synergism and increased inhibition of cell viability in the MCF-7 breast cancer cell line compared to cisplatin or piperine alone.

3.2. Examining the genes involved in apoptosis and resistance using real-time PCR

In several studies, it has been proven that cisplatin increases the expression of the anti-apoptotic *BCLX* gene in MCF-7 cells, contributing to treatment resistance.³² In addition, it has been confirmed that the stimulation of *BAK1* gene expression by cisplatin requires auxiliary factors for apoptosis induction within the cell.^{33,34} Given the documented positive effects of biosynthesized Ag NPs with *Acacia Luciana* flower extract on causing apoptosis through the mitochondrial pathway and increasing the *BAK1/BCLX* ratio, we decided to investigate its combined effects with cisplatin on apoptosis.

In the previous study, it was confirmed that Ag NPs could increase the ratio of *BAK1/BCLX*, which expresses the intrinsic pathway of apoptosis (mitochondrial pathway), by 101.46 times compared to the control.²⁰ Therefore, in this present study, we aim to explore the extrinsic pathway of apoptosis by examining the expression of the *CASP3* gene. On the other hand, since the *PI3K/AKT* pathway and the *BCLX* gene cooperate to promote cell survival, examining the amount of *AKT1* gene expression can reveal the cell's resistance to death.³⁵

As depicted in [Table 1](#), cisplatin treatment only increased the expression of the *CASP3* gene, approximately 3.5 times greater than the control, aligning with results published by Henkels and Turchi.³⁶ Conversely, cisplatin led to an elevation of *AKT1* gene expression, indicating the development of resistance in MCF-7 cells after just 24 h of treatment ([Figure 2](#)). Another indicator of treatment resistance in MCF-7 breast cancer cells was the increase in the expression of the anti-apoptotic *BCLX* gene post-cisplatin treatment, which increased by 1.44 times compared to the control.

The combination therapy of cisplatin with Ag NPs increased the ratio of *BAK1/BCLX* by 162 times compared to the control, indicating activation of the intrinsic pathway of mitochondria-dependent apoptosis. Notably,

Table 1. Expression level of genes involved in apoptosis and drug resistance in MCF-7 cells when treated with Ag NPs, cisplatin, and combination therapy using Ag NPs and cisplatin

Compounds	Genes	CT (mean)	Δ CT	$\Delta\Delta$ CT	RQ
Control	<i>BAK1</i>	26.1379	7.0518	0	1
	<i>BCLX</i>	15.5125	-3.5735	0	1
	<i>CASP3</i>	30.9565	11.8704	0	1
	<i>AKT1</i>	17.5871	-1.4989	0	1
	<i>GAPDH</i>	19.086			
Ag NPs	<i>BAK1</i>	30.7307	2.1705	-4.8813	29.47
	<i>BCLX</i>	26.8873	-1.6729	1.9006	0.268
	<i>CASP3</i>	35.4621	6.9019	-4.9685	31.31
	<i>AKT1</i>	32.7324	4.1722	5.6711	0.0196
	<i>GAPDH</i>	28.5602			
Cisplatin	<i>BAK1</i>	24.8419	6.3832	-0.6686	1.589
	<i>BCLX</i>	14.3555	-4.1032	-0.5297	1.444
	<i>CASP3</i>	28.5332	10.0745	-1.7959	3.472
	<i>AKT1</i>	14.7936	-3.6651	-2.1662	4.488
	<i>GAPDH</i>	18.4587			
Combination therapy	<i>BAK1</i>	32.4156	3.4249	-3.627	12.3544
	<i>BCLX</i>	29.133	0.1414	3.7149	0.0762
	<i>CASP3</i>	32.7691	3.7775	-8.093	273.0432
	<i>AKT1</i>	26.5894	-2.4022	-0.9033	1.87
	<i>GAPDH</i>	28.9916			

Note: Gene expression levels were presented using relative quantification (RQ), which is equal to $2^{-\Delta\Delta CT}$.
Abbreviation: CT: Cycle threshold.

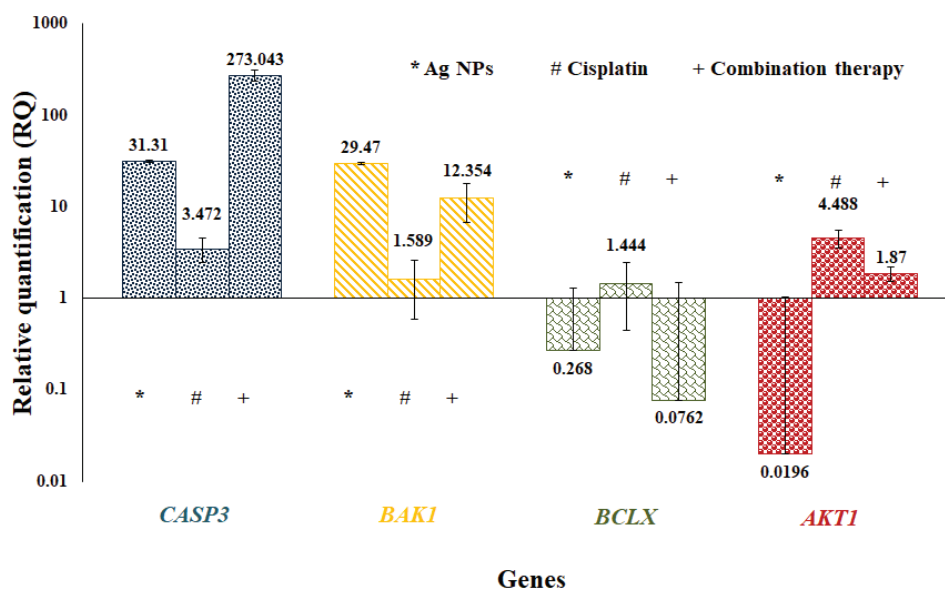


Figure 2. Bar graph of expression of genes related to apoptosis and resistance obtained by real-time PCR method in MCF-7 cells.

Notes: Graphs marked with * correspond to Ag NPs, graphs marked with # correspond to cisplatin, and graphs marked with + correspond to combination therapy using cisplatin and Ag NPs.

Abbreviations: Ag NPs: Silver nanoparticles; PCR: Polymerase chain reaction.

the synergistic effect of cisplatin and Ag NPs increased the expression of the *CASP3* gene, signifying the involvement of the extrinsic pathway of apoptosis. As shown in Figure 2, combination therapy increased the *CASP3* gene expression by 273 times compared to the control, while free Ag NPs and free cisplatin increased *CASP3* expression by 31 and 3.5 times, respectively.

Cisplatin and other Pt-based drugs can penetrate the cell membrane by passive diffusion, endocytosis, and other mechanisms based on transporters, such as copper transporter 1 (CTR1).³⁷ In a previous study, it was demonstrated that when mice models are fed a diet containing silver chloride (AgCl) before cisplatin treatment, Ag is selectively absorbed by the liver and then excreted through bile exchange.³⁸ The study revealed that mice exposed to Ag exhibited a delay in the absorption of Pt by their organs during the first 30 min, compared to the control group. In other words, the presence of Ag causes a delay in the action of copper transporters, indicating a potential association between the uptake of cisplatin and the CTR1 transporter. At first glance, this might raise concerns that the combination therapy of cisplatin with Ag NPs could have a negative effect or even induce resistance to cisplatin treatment in MCF-7 cancer cells.

In this case, the reduction of *AKT1* gene expression during combination therapy compared to treatment with free cisplatin presents a promising indication that the inclusion of green Ag NPs synthesized with *Acacia*

Luciana flower extracts not only lacks negative effects on cisplatin but also fosters synergy, leading to a reduction in *AKT1* gene expression, which serves as an indicator of cell resistance. Notably, the *AKT1* expression levels decreased from 4.5 with free cisplatin to 1.87 with combination therapy, underscoring the synergy of cisplatin and Ag NPs to reduce the treatment resistance in MCF-7 cells. The reduction of treatment resistance is also proven by the reduction of anti-apoptotic *BCLX* gene expression using combination therapy compared to cisplatin treatment alone. Therefore, it can be concluded that the conversion of Ag ions to Ag NPs reduces the inhibitory effects on CTR1, which can be due to the nature of nanoparticles or the presence of biomolecules on the surface of Ag NPs. However, it is possible that the route of entry of cisplatin in the presence of Ag NPs changes and is unrelated to CTR1, but further research is required to confirm this.

4. Conclusion

Considering the side effects and drug resistance associated with cisplatin, utilizing combination therapy with natural substances holds promise for improving its performance. To explore this potential, we employed biosynthesized Ag NPs derived from *Acacia Luciana* flower extracts in conjunction with cisplatin for treating MCF-7 breast cancer cells. The MTT test results demonstrated that combination therapy achieved unprecedented IC₅₀ values close to 0.178 in breast cancer cells exposed to cisplatin. Furthermore, the combination therapy increased the

expression of the genes of the mitochondrial pathway of apoptosis (intrinsic pathway) and increased the expression of the genes of the extrinsic pathway of apoptosis, while concurrently decreasing genes implicated in treatment resistance. Therefore, combination therapy offers optimism for reducing the dosage of cisplatin while simultaneously enhancing its efficacy.

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Conflict of interest

The author declares that he has no competing interests.

Author contributions

This is a single-authored article.

Ethics approval and consent to participate

This research was approved by the Institutional Review Board at Zabol University of Medical Sciences under the code of ethics (IR.ZBMU.REC.1400.002).

Consent for publication

Not applicable

Availability of data

All data and findings from this study will be made available by the corresponding author on request.

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