J Nanostruct 14(2): 505-512, Spring 2024

RESEARCH PAPER

Biological Evaluation of Silver Nanoparticles Synthesized from Bitter Orange Peel Extract: Antibacterial and Anticancer Properties on Human Breast Cancer Cell Line

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ARTICLE INFO

Article History:
Received 06 January 2024
Accepted 22 March 2024
Published 01 April 2024

Keywords:
Antibacterial
Anticancer
Green synthesis
Nanoparticles
Silver

ABSTRACT

In recent years, the synthesis of nanoparticles using environmentally friendly and cost-effective methods has become a primary goal for researchers and industries. In this study, silver nanoparticles were synthesized via a green chemistry approach using *bitter orange* peel extract. The synthesized nanoparticles were characterized through UV-Vis, FT-IR, XRD, and TEM analyses. TEM results revealed that the silver nanoparticles had a spherical morphology, with a size of approximately 35-45 nm, showing uniformity and homogeneity. After characterization, the antibacterial activity of the nanoparticles was evaluated against four strains of both Gram-positive and Gram-negative bacteria. MIC results indicated that the synthesized nanoparticles exhibited the highest activity against *Escherichia coli* and *Klebsiella pneumoniae*, with MIC values of 125 and 250 µg/mL, respectively. The antibacterial activity of the nanoparticles was significantly higher than that of the *bitter orange* peel extract alone. Furthermore, the anticancer activity of the nanoparticles was tested against the MCF-7 cancer cell line. The IC50 value of the nanoparticles was found to be 4.28 µg/mL, demonstrating their potential as anticancer agents. Overall, the findings suggest that the synthesized silver nanoparticles have considerable potential for various biological applications.

How to cite this article
Ghoreishi S., Mortazavi-Derazkola S. Biological Evaluation of Silver Nanoparticles Synthesized from Bitter Orange Peel Extract: Antibacterial and Anticancer Properties on Human Breast Cancer Cell Line. J Nanostruct, 2024; 14(2):505-512. DOI: 10.22052/JNS.2024.02.012

INTRODUCTION

The growing interest in the production and use of nanomaterials across a wide range of industries has garnered significant attention in recent years. As the demand for these materials continues to rise, their unique properties and potential applications in fields such as medicine, electronics, energy, and environmental science are becoming increasingly recognized. This surge in interest is driven by the promising innovations nanomaterials offer in enhancing performance, efficiency, and functionality in numerous cutting-edge technologies [1, 2].

Noble metal nanoparticles, especially silver

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nanoparticles (AgNPs), have garnered growing interest in recent years because of their promising therapeutic applications. These nanoparticles are recognized for their diverse biological properties making them highly valuable in medical research and potential treatments [3, 4]. Various studies have demonstrated that the cytotoxic effects of silver nanoparticles (Ag NPs) against a wide range of cancer cells are primarily attributed to the generation of reactive oxygen species (ROS). These ROS lead to oxidative stress, causing damage to nucleic acids and triggering cell death through the apoptosis pathway. This mechanism makes Ag NPs a promising candidate for cancer therapy, as they can selectively induce cytotoxicity in cancerous cells while sparing healthy tissues [5, 6]. Moreover, due to its potent antibacterial properties, silver has been extensively used in the treatment of bacterial infections, particularly those associated with wounds and burns. Its ability to inhibit the growth of a wide range of bacteria makes silver an effective agent for preventing infection and promoting healing in damaged tissues, further highlighting its significance in medical applications [7, 8].

Various chemical and physical methods have been employed for the synthesis of silver nanoparticles (Ag NPs). While these approaches can produce high-quality nanoparticles with precise control over size and shape, they often pose limitations when it comes to biological applications. This has driven researchers to explore more environmentally friendly alternatives to develop biocompatible Ag NPs, aiming for safer and more sustainable solutions in medical and biological fields [9, 10]. Recently, the application of green chemistry principles in the synthesis of biocompatible nanomaterials has attracted considerable attention. These eco-friendly methods allow for the efficient preparation of various metallic and metal oxide nanoparticles by carefully selecting environmentally benign reductants, solvents, and stabilizers. By following green chemistry principles, researchers can produce nanomaterials in a more sustainable and less toxic manner, making them better suited for biological and medical applications [11, 12].

Notably, extracts from a variety of natural sources, including plants, microorganisms, and marine organisms, have been shown to serve as effective reducing agents in the eco-friendly synthesis of metallic nanoparticles, such as silver nanoparticles (Ag NPs). The advantages of using these natural materials are significant, as they help reduce environmental impact, eliminate the need for harmful chemicals, and minimize the generation of hazardous waste. This approach not only enhances the sustainability of nanoparticle production but also aligns with the principles of green chemistry [13, 14]. In addition, nanoparticles synthesized using natural product extracts have frequently exhibited enhanced bioactivity. This improvement is largely attributed to the presence of phytoconstituents, which act as stabilizing ligands on the nanoparticle surfaces. These naturally occurring compounds not only provide stability to the nanoparticles but also contribute additional biological properties, further boosting their therapeutic potential in various biomedical applications [15]. In this framework, Al-Yousef & co-workers reported the article about bee pollen extracts were utilized to eco-friendlily synthesize silver nanoparticles (AgNPs-G), which were characterized through various microscopic and spectroscopic techniques, confirming their spherical morphology. The antibacterial properties of AgNPs-G were evaluated using the Minimum Inhibitory Concentration 50 (MIC50) method against both Gram-positive and Gram-negative bacteria, revealing superior antibacterial activity compared to chemically synthesize silver nanoparticles (AgNPs-C) produced with sodium borohydride. Additionally, the anticancer effects of the Ag NPs were tested on human liver (HepG2) and breast (MCF-7) carcinoma cell lines using the MTT assay, showing significant anticancer activity with high IC50 values in both cell lines [16]. Sadat Shandiz et al. investigated the biological properties of green-synthesized silver nanoparticles (AgNPs) using ethanol extract from *Artemisia tournefortiana Rchb*. Their findings underscore the promising antibacterial and anticancer activities of the phyto-synthesized AgNPs, suggesting their potential as effective agents against pathogenic bacteria and colon cancer [2]. Alaallah & co-workers employed bionanotechnology techniques to create silver nanoparticles (Ag NPs) using *lemon* juice (*Citrus limon*) as a biosynthetic agent, highlighting an economical and environmentally friendly approach. The synthesized AgNPs exhibited strong antioxidant activity, demonstrated through DPPH radical scavenging, total antioxidant capacity, and reducing power assays. Additionally, their

antibacterial activity, evaluated using a well diffusion method, showed greater sensitivity against Gram-positive bacteria compared to Gram-negative bacteria. In vitro studies on MCF-7 breast cancer cell lines revealed a significant cytotoxic effect with an IC50 value of 47 µg/mL. These findings suggest that the biosynthesized AgNPs have promising applications in nanotechnology-based pharmaceuticals and biomedical fields [17].

MATERIALS AND METHODS

Materials

In this experiment, silver nitrate ($AgNO_3$) and sodium hydroxide were purchased from Sigma, along with methanol solution. Throughout the study, double-distilled water was used in all procedures. The MCF-7 cell line was acquired from the Iranian Pasteur Institute. For evaluating antibacterial activity, Mueller-Hinton broth and Mueller-Hinton agar (Merck, Germany) were used. Both Gram-negative bacterial strains, including *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 9997), and Gram-positive strains, namely *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), were sourced from the Pasteur Institute of Iran.

Preparation of bitter orange peel extract

The *bitter orange* peel was collected from the forests of Mazandaran province in Iran. To eliminate contaminants such as dust, the plant was initially washed and then air-dried at room temperature. Once fully dried, it was ground into a fine powder using a milling device. For extraction, 300 g of the powdered plant were immersed in 400 ml of methanol and placed on a shaker for 72 h. The methanol solvent was then removed using a rotary evaporator, resulting in the final red-colored *bitter orange* peel extract.

Green synthesis of silver nanoparticles using bitter orange peel extract

In recent years, the use of simple, cost-effective, and environmentally friendly methods for nanoparticle synthesis has gained significant importance. In this study, silver nanoparticles were synthesized using *bitter orange* peel extract following the method described by Kiani et al., with some modifications [18]. Initially, 51 mg of silver nitrate salt was dissolved in 10 mL of distilled water under vigorous stirring conditions. Following this, 10 mL of *bitter orange* peel extract was added to the silver nitrate solution. The solution changed color from clear to dark brown, indicating the formation of silver nanoparticles. After 1 h, the solution was collected by centrifugation at 10,000 rpm for 30 min and subsequently washed three times with double-distilled water. It is important to note that the concentrations and extraction times reported were optimized during the course of the study.

Antibacterial activity

The antibacterial activity of silver nanoparticles and *bitter orange* peel extract was assessed against four types of Gram-positive and Gram-negative bacterial strains using the broth microdilution method. The bacterial strains used in the study included *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis.* For this purpose, different concentrations of silver nanoparticles and plant extract were added to each well. Then, 100 µl of the Gram-positive and Gram-negative bacterial suspensions at a concentration of 0.5 McFarland were introduced into each well. The plates were placed on a shaker and mixed for 30 s to ensure uniformity. Following this step, the plates were incubated for 24 h at 37°C. After the incubation period, the minimum concentration at which no turbidity, indicating bacterial growth, was observed, was recorded as the minimum inhibitory concentration (MIC).

Anticancer activity

In this study, the anticancer activity of silver nanoparticles synthesized using *bitter orange* peel extract was evaluated against the MCF-7 cell line through the MTT assay. The assessment was performed following the method described by Zare-Bidaki and colleagues [19]. Initially, the MCF-7 cell line was cultured in DMEM medium containing 1% penicillin-streptomycin and 10% FBS. The cells were then incubated in flasks under standard conditions. After incubation, the cells were detached using trypsin, collected by centrifugation, and resuspended. The samples were incubated for 24 h. After this period, the medium was removed, and various concentrations of silver nanoparticles were added to the cells. Subsequently, the culture medium was replaced with fresh MTT solution at a concentration of 0.5 mg/mL. After 3 h of incubation, the MTT solution was discarded, and 100 µL of DMSO was added to each well. Finally, after 15 min, the absorbance

was measured at 570 nm. Cell viability at each concentration was determined by comparing the absorbance of the treated group with that of the control group.

RESULTS AND DISCUSSION

UV-VIS analysis

A fundamental approach for confirming the formation of silver nanoparticles is the appearance of an absorption peak around 420 nm in UV-Vis analysis. Therefore, to assess the formation of silver nanoparticles using *bitter orange* peel extract, UV-Vis spectra were recorded within the wavelength range of 300 to 600 nm. One of the distinctive optical properties of silver nanoparticles is surface plasmon resonance (SPR), which can be visually detected by a color shift of the suspension to dark brown. The intensity of this resonance can also be quantified using UV-Vis measurements. Previous studies have indicated that a higher absorption peak corresponds to a greater concentration of silver nanoparticles. As demonstrated in Fig. 1, the synthesized silver nanoparticles exhibit a strong absorption peak at 424 nm, signifying the reduction of Ag^{+} ions to neutral silver atoms, confirming the successful formation of silver nanoparticles.

XRD analysis

X-ray diffraction (XRD) analysis was conducted to examine the crystal structure and purity of the silver nanoparticles synthesized using *bitter orange* peel extract. The results are presented in Fig. 2. Four prominent peaks were observed at 2θ angles of 38.16°, 44.34°, 62.56°, and 77.18°, corresponding to the crystallographic planes (111), (200), (220), and (311), respectively. These findings are in agreement with the reference data from JCPDS card No. 01-087-0717, confirming the proper synthesis of pure silver nanoparticles with no impurities detected. Similarly, Paulkumar et al. synthesized silver nanoparticles using *Piper nigrum* leaf extract, and their XRD results also showed the same characteristic peaks at similar diffraction angles [20]. Additionally, the Debye-Scherrer equation was employed to estimate the particle size, revealing that the synthesized nanoparticles had an average size of 38.3 nm.

FT-IR

In this study, *bitter orange* peel extract was used for the green synthesis of silver nanoparticles. Therefore, it was essential to analyze the functional groups present in the extract that contribute to the formation of silver nanoparticles using FT-IR analysis. Fig. 3 illustrates the FT-IR spectra of both the *bitter orange* peel extract and the synthesized silver nanoparticles. The extract spectrum shows five distinct peaks at 3435, 2961, 1713, and 1328 related to the hydroxyl stretching vibration, the

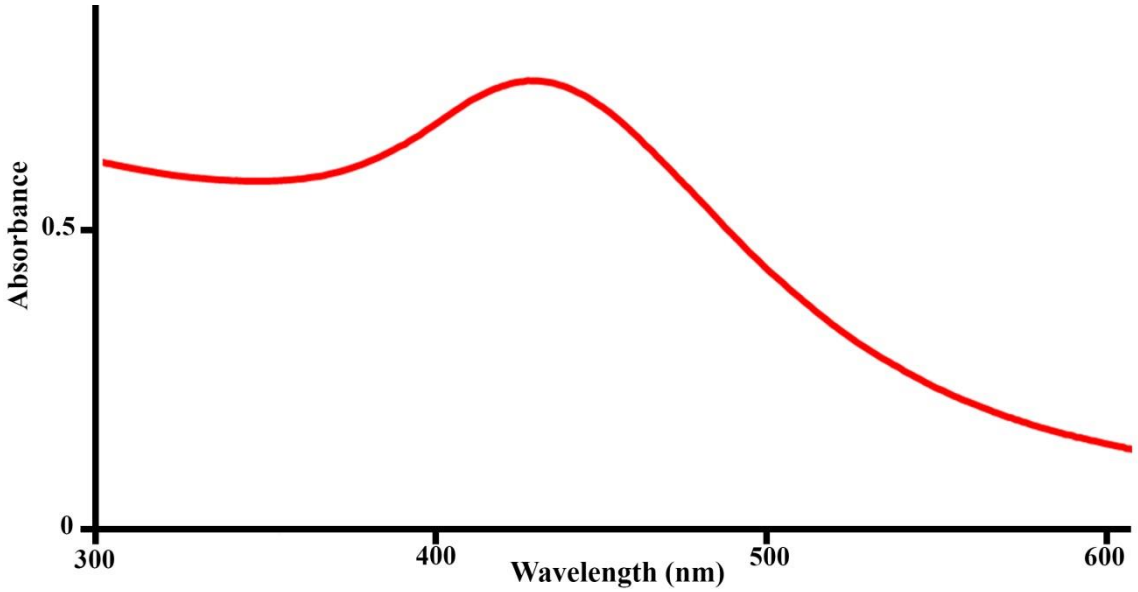

Fig. 1. UV-Vis spectra of green silver nanoparticles using *bitter orange* peel extract.

carbon-hydrogen band, the C=O of carbonyl group, and the stretching C=C aromatic ring, respectively [21]. The FT-IR spectrum of the silver nanoparticles indicates that the peaks are similar to those of the extract, with only differences in intensity, likely due to the deposition of the extract on the nanoparticle surfaces. These functional groups are responsible for reducing silver salts into silver nanoparticles. Similarly, in the study by Zare-Bidaki et al., where silver nanoparticles were synthesized using *Medicago sativa* extract, the FT-IR spectra of the extract and nanoparticles also showed similar peaks, with differences in peak intensity and slight wavelength shifts [21].

TEM analysis

The structure and morphology of the silver nanoparticles synthesized using *bitter orange* peel extract are primarily influenced by their physical and chemical properties. Transmission Electron Microscopy (TEM) is a key technique used to evaluate the structural and morphological characteristics of these synthesized materials. Fig. 4 displays TEM images of silver nanoparticles synthesized using *bitter orange* peel extract. As seen, the nanoparticles exhibit a spherical

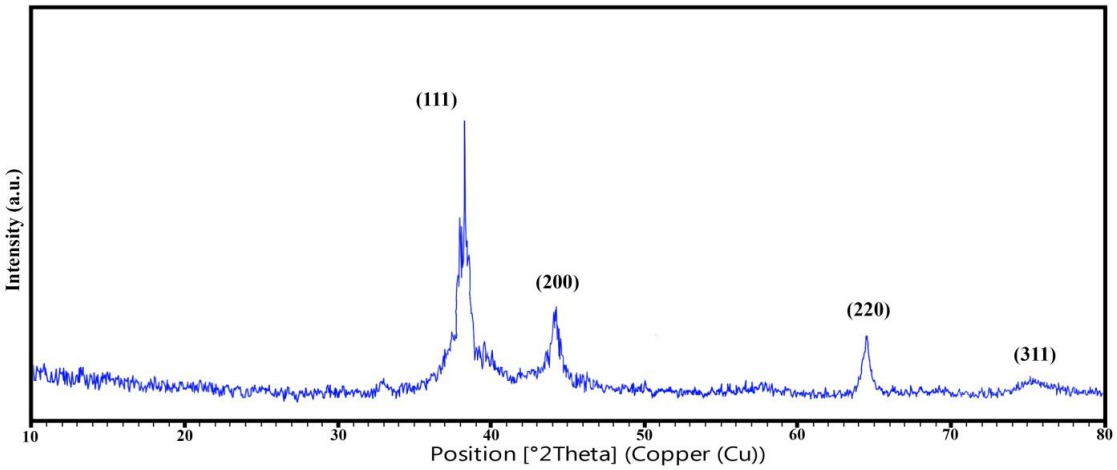

Fig. 2. X-ray diffraction of green silver nanoparticles using *bitter orange* peel extract.

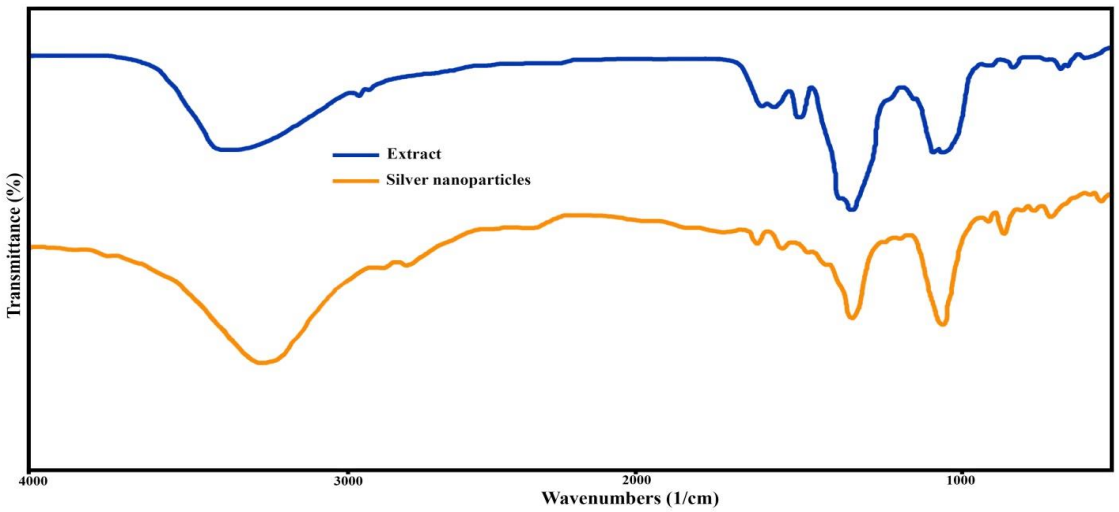

Fig. 3. FT-IR spectrum of *bitter orange* peel extract (a) and biosynthesized silver nanoparticles using *bitter orange* peel extract (b).

morphology and are well-dispersed within the plant extract. The extracts are visible as a gray coating surrounding the surface of the nanoparticles. The synthesized nanoparticles demonstrated high uniformity and homogeneity. A more detailed examination revealed that the particle size ranged from 35 to 45 nm.

Antibacterial activity

This study investigates the antibacterial properties of the *bitter orange* peel extract and silver nanoparticles against both gram-positive and gram-negative bacterial strains. The bacterial strains utilized in this research include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis*. The antibacterial activity results are summarized in Table 1. As indicated by the results, the *bitter orange* peel extract exhibited minimal antibacterial activity against any of the tested strains. In contrast, silver nanoparticles demonstrated a noteworthy antibacterial effectiveness against various strains. The results revealed that the minimum inhibitory concentration (MIC) of silver nanoparticles against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis* were 125, 250, 500, and 500 µg/mL, respectively. Notably, the antibacterial activity

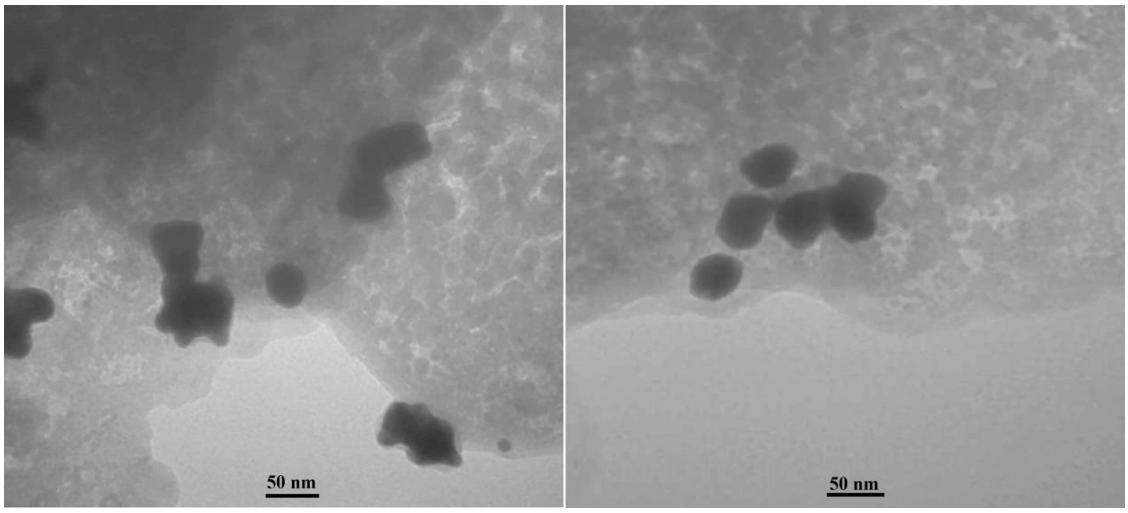

Fig. 4. Transmission electron microscopy images of green silver nanoparticles using *bitter orange* peel extract.

Table 1. MIC and MBC values (µg/mL) of biosynthesis of silver nanoparticles using *bitter orange* peel extract.

Microorganism	*Bitter orange* peel extract		Silver nanoparticles	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Escherichia coli	>4000	>4000	125	250
Klebsiella pneumoniae	>4000	>4000	250	500
Enterococcus faecalis	>4000	>4000	500	2000
Staphylococcus aureus	>4000	>4000	500	1000

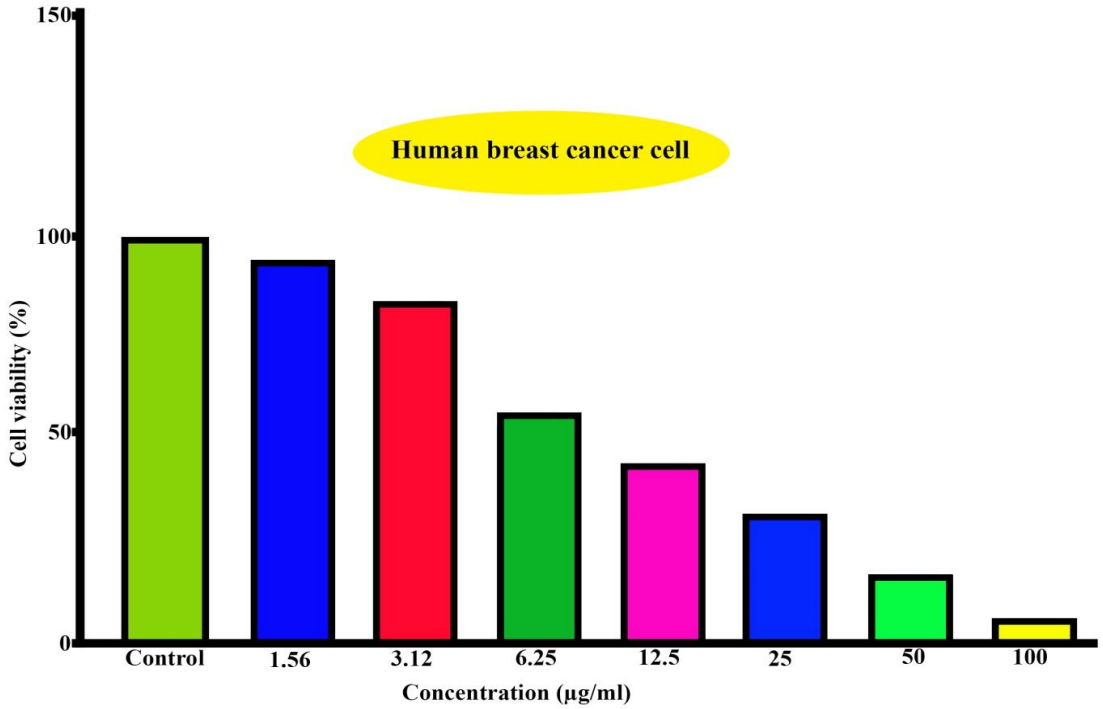

Fig. 5. The results of the MTT assay in human breast cancer cell line with green silver nanoparticles.

was more pronounced against gram-negative strains compared to gram-positive bacteria. One possible explanation for this observation is the thinner peptidoglycan layer of gram-negative bacterial cell walls, which allows for faster and easier penetration. Various mechanisms have been proposed to explain the antibacterial action, yet the precise mechanism remains unclear. A suggested mechanism may unfold as follows: I) Initially, nanoparticles adhere to the bacterial cell wall, releasing metal ions. II) The nanoparticles penetrate the bacterial cell. III) DNA, RNA, and proteins sustain damage. IV) Oxidative stress is generated, ultimately leading to bacterial destruction.

Anti-cancer activity

The anticancer activity of the synthesized silver nanoparticles was evaluated against the MCF-7 cell line, which is a breast cancer cell line, by assessing cancer cell viability through the MTT assay. Concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL of silver nanoparticles synthesized using *bitter orange* peel extract were tested (Fig. 5). As the results indicated, increasing the concentration of silver nanoparticles led to a significant reduction in cancer cell viability, from 93.6% at 1.56 µg/mL to 9.43% at 200 µg/mL. Based on these results, the IC50 value of the silver nanoparticles against the MCF-7 cell line was determined to be 4.28 µg/mL. The findings demonstrated that the synthesized silver nanoparticles possess significant anticancer properties. Various mechanisms have been proposed to explain this effect, one of which involves the interaction between the nanoparticles and cancer cells, resulting in the release of silver ions. These silver ions cause damage to the cells, leading to oxidative stress. The oxidative stress induces cellular damage, triggering pathways that lead to cancer cell apoptosis.

CONCLUSION

In summary, this study demonstrates the successful green synthesis of silver nanoparticles using *bitter orange* peel extract. The nanoparticles were effectively characterized and found to have promising biological properties. They exhibited significant antibacterial and anticancer activities, suggesting their potential for a wide range of applications in biomedicine and related fields. Overall, this research highlights the advantages of

eco-friendly nanoparticle synthesis methods and underscores the potential of silver nanoparticles for future scientific and industrial use. Further investigations could expand on these findings to optimize and broaden their practical applications.

ACKNOWLEDGMENT

This research was conducted in adherence to the Helsinki Declaration and the ethical guidelines established by the institutional research committee. The ethics approval was granted by Birjand University of Medical Sciences under the code IR.BUMS.REC.1399.487.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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