

A Potent and Safer Anticancer and Antibacterial *Taxus*-Based Green Synthesized Silver Nanoparticle

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Purpose: Paclitaxel is a generic drug produced based on Taxol which is an extract of *Taxus* tree, well known for its anticancer and antibacterial effects. This study was aimed at building up an agent with the antibacterial and anticancer benefits of both the silver ions and Taxol, together with less cytotoxic effects.

Materials and Methods: Colloidal silver nanoparticles (AgNPs) were synthesized by reducing aqueous AgNO₃ with aqueous *Taxus* leaf extract at nonphotomediated conditions, without any catalyst, template or surfactant. The AgNP production was confirmed by ultraviolet-visible (UV-VIS) spectroscopy, scanning electron microscopy (SEM), X-ray diffraction (XRD) and Fourier-transform infrared (FTI) spectroscopy. The MTT assay for human breast cancer cells as well as the DAPI fluorescent staining microscopy tested the biocompatibility and anticancer effects of AgNPs, silver nitrate, and Taxol. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) techniques were performed to determine the shape and size of the nanoparticles. MTT assay showed the best inhibitory concentration of AgNPs on cancer cells. The antibacterial activity of the three case study materials was tested for gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) using well diffusion test.

Results: This work proposes more anticancer effects for AgNP made by *Taxus brevifolia* extract, comparing Taxol solution. IC₅₀ was observed as 3.1 mM for Taxol while 1.5 mM for new AgNP. Moreover, *Taxus* showed no antibacterial effects while the new AgNP showed a dose-dependent biocompatibility along with slightly more antibacterial effects (MIC: 1.6 and 6.6mM for gram-positive and -negative bacteria, respectively) comparing with silver nitrate solution (MIC: 1.5 and 6.2 mM for gram-positive and -negative bacteria, respectively).

Conclusion: The production of herbal-mediated silver nanoparticles may be an efficient substitution for the silver nitrate-based medicines with less side effects.

Keywords: *Taxus* tree extract, green synthesis, silver nanoparticle, antibacterial, anticancer

Introduction

Over the past few decades, researchers have paid attention to plant extracts as the treatment for various diseases, such as some bacterial infections and cancer.¹⁻⁶ Taxol is an extract of the *Taxus* tree (Scientific name: *Taxus brevifolia*, Family: *Taxaceae*), which has been shown to have anticancer effects.^{4,6,7} The American National Cancer Institute (NCI) began the first studies of the antimicrobial properties of Taxol in the late 1950s which is still a popular research topic.^{8,9} Taxol has been shown to have suppressive effects on a variety of breast, skin and ovarian cancers by preventing the de-polymerization of tubulins. A generic drug named “paclitaxel” has produced based on Taxol which has a registered trade name as Taxol(R) BMS [Bristol-Myers Squibb].

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The other aspect of the current study was the synthesis of silver nanoparticles (AgNP). Recently, phyto-nanotechnology has proposed a new “green” method for the synthesis of nanoparticles which are eco-friendly, stable, rapid, simple, and cost-effective.^{10,11} Although the “Green synthesis” has a disadvantage of the slower kinetics; however, they offer a couple of advantages like better handwork and overgrowth control, and stabilization. These benefits have led to no need for high temperature and pressure as well as toxic chemicals.^{12–15}

The most important use of AgNP is in the medical industry, such as topical ointment to prevent infection in open wounds. Moreover, the antibacterial effect of the AgNP depends on their size, the effect of AgNP decreases when its size is increased.^{16,17}

Materials and Methods

The leaves, trunks, and shells of *Taxus brevifolia* were collected from the southern mountains of Ziarat village of Gorgan, Iran. Silver nitrate (AgNO₃) [Sigma-Aldrich] was applied as an initial source of silver ions as well as an active case study material. Traditional medicine of Taxol (Chemotaxel 30mg/5mL – Paclitaxel injection USP) was prepared as a standard solution for the HPLC experiments.

In order to test the antibacterial activity of AgNP, *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC 1399) and *Pseudomonas aeruginosa* (ATCC 1430) bacteria were prepared. The human breast cancer cell line MCF-7 was purchased from Pasteur Institute – Cell Bank.

Making Three Extracts from Various Parts of *Taxus brevifolia*

Several samples of the leaves, trunk, and shells were collected from the *Taxus brevifolia* tree in order to determine the maximum extractable amount of Taxol in different parts of the tree. The samples were collected and packed in clean drying paper to dry at room temperature. All dried samples were powdered. An equal mass of 20 grams of each dry powdered samples (leaves, trunk, and shells) was boiled separately in 100 mL of double-distilled water. The boiled mixtures were heated in a completely closed container at 40 °C for 24 hours. The heated materials were filtered through a Whatman No.1 filter paper centrifuged at 5000 rpm for 5 min and a clear solution of the extracts was stored at 4 °C for further experiments.

High-Performance Liquid Chromatography (HPLC) Experiment

HPLC determined the best extract regarding the highest concentration of Taxol. Different extracts of the *Taxus brevifolia* tree leaves, stem, and crust were compared to the standard Taxol solution (Chemotaxel 30mg/5mL - Paclitaxel injection USP) using a reverse phase HPLC method (Agilent 1200). 2 µL filtered extract (100.0 µg/mL) applied in a C18 column (Agilent ZORBOX XDB-C18, 250 mm × 4.6 mm) and UV detection was performed at 227 nm as described previously.¹⁸ The mobile phase used in this study was a solution containing acetonitrile:water (65:35, v/v) with an adjusted pH of 4.5, according to the protocols.¹⁸

Synthesis of Silver Nanoparticles Using *T. brevifolia* Extract

Based on HPLC experiments, *T. brevifolia* leaves extract were only used for the synthesis of nanoparticles. Two milliliters of the *T. brevifolia* leaves extract was mixed with 2 mL of silver nitrate 12.5 mM (0.21 gram/10mL) and was incubated at 37 °C for one hour. The formation of nanoparticles and the reduction of blue silver ion using the extract of the plant was determined by the color change of the mixture to a yellow-brown (Figure 1).^{19,20}

Characterization of the Green Synthesized Silver Nanoparticles

It has been reported that the size and shape of nanoparticles mainly depend on different factors like concentration of leaf extract, salt solution, pH, temperature and time.^{18,19} The obtained AgNPs were washed by centrifuging at 5000g for 45 min, re-suspended in deionized double-distilled water, and washing repeatedly for two times. These were characterized by ultraviolet-visible spectroscopy (UV-VIS), scanning electron microscopy (SEM), X-ray diffraction spectroscopy (XRD) and Fourier-transform infrared spectroscopy (FTIR) as described previously.^{21–24} In the current work, synthesized AgNPs were measured using UV-Vis spectrophotometry at a range of 350–700 nm (the Agilent 8453 UV-Vis Spectrophotometer, America).

Determination of Antibacterial Activity of Silver Nanoparticles

The synthesized AgNPs were examined for antimicrobial activity by a standard agar diffusion method against different bacterial cultures, *S. aureus*, *E. coli*, and *P. aeruginosa*. An

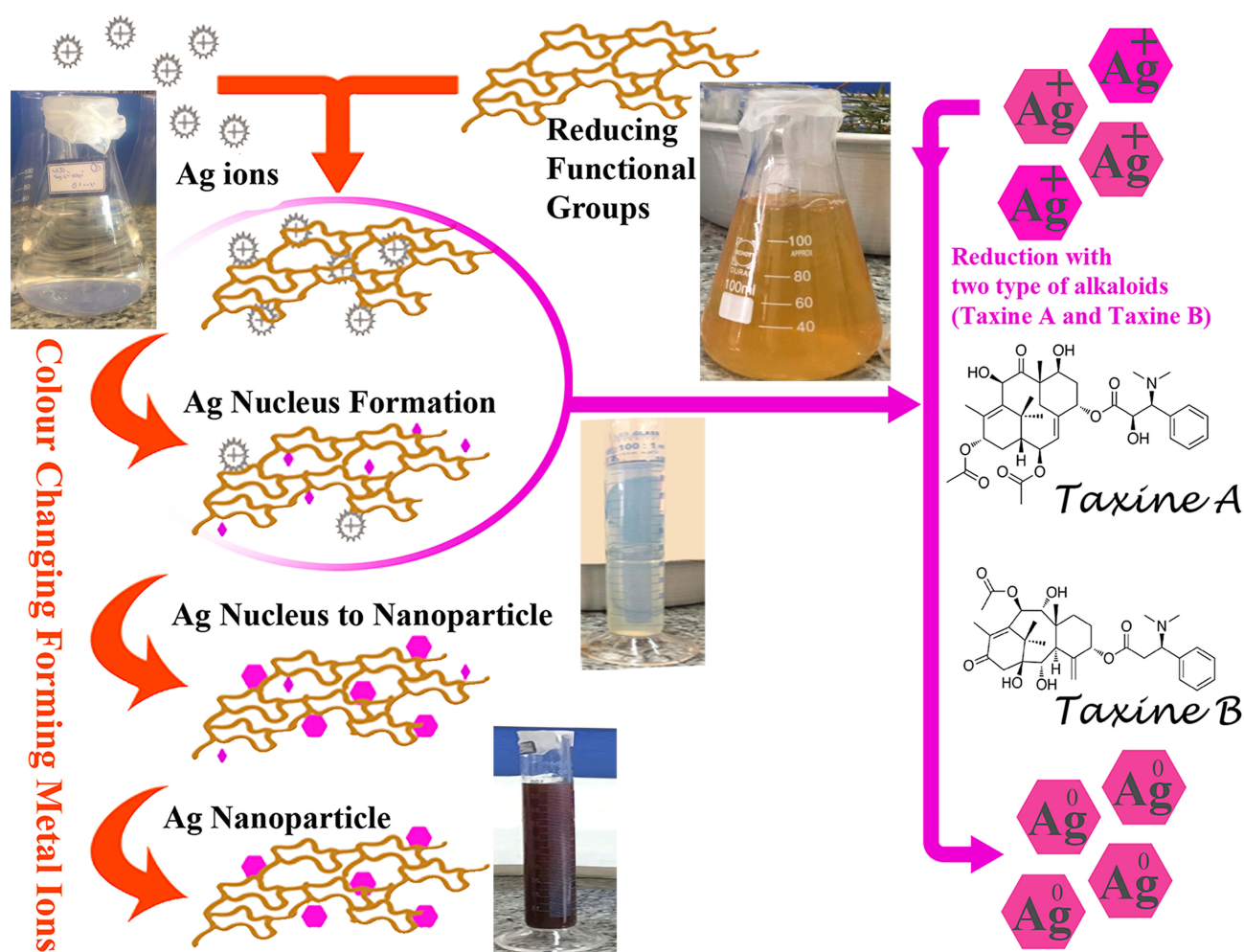


Figure 1 Silver nanoparticle synthesis using *Taxus brevifolia* extract.

equal number of initial bacterial culture with 0.5 McFarland concentrations was sub-cultured on LB agar. An equal number of initial bacterial culture with 0.5 McFarland concentrations was sub-cultured on LB agar.

All three case study solutions including pure *T. brevifolia* extract, silver nitrate, and AgNP were diluted individually to obtain seven serial concentrations of titers as follows: (1/1): 50 mM, (1/2): 25 mM, (1/4): 12.5 mM, (1/8): 6.25 mM, (1/16): 3.1 mM, (1/32): 1.5 mM and (1/64): 0.75 mM. Several sets of three selected bacterial culture plates were provided. Equal volumes of 100 μ L/well of each dilution were applied to a set of three selected bacterial cultures as described above. Same sets of three bacterial culture plates were considered without any additive material as well as with cephalexin standard antibiotic disks as negative and positive controls, respectively. All plates were evaluated after 24 hours incubation at 37°C. The inhibition zone of the bacteria was accurately

measured by the caliper and the mean diameter of the bacterial inhibition zone. The comparison with positive and negative control was considered as an indicator of antibacterial activity evaluation.²⁵ Each assay was repeated three times as described previously.^{26,27}

Minimum Inhibitory Concentration (MIC) Test

The minimum concentration of bacterial growth inhibitory was determined. Same as the previous step, serial dilutions of each case study solutions were prepared. Using a 96-well plate, a set of 7 wells were considered separately for each study materials containing 100 μ L of the serial diluents as described above. One hundred microliters of brain-heart-infusion (BHI) broth solution and 100 μ L of active bacterial suspension 1.5×10^6 cfu/mL were added to each well. The same procedure was performed for all three bacterial species. Positive and negative controls were

considered for each run of the experiments. Positive control wells included the relevant bacteria with BHI solution and negative control included the case study solution (extract, silver nitrate or AgNP) with BHI solution. The plate was incubated for 24 hours at 37°C. The turbidity of the wells, which was due to the growth of the bacteria, was investigated by reading their OD. The first dilution with no turbidity (lack of growth) was recorded as the minimum deterrent concentration.^{27,28} To reduce the experimental errors, all of the above experiment was repeated four times.

Assessment of Anticancer Activity by MTT Assay

General anti-cancer agents, likewise chemotherapeutic medications or Taxol can suppress cell proliferation; therefore, the viability assay of these agents is expected to show almost the same results on any high proliferating cell line despite the cancerous or normal source of the cells. Consequently, researchers only determine the effects of such remedies on the specific tissue cancer cell lines.^{2,4}

According to the previous step in the present study, 7 dilutions of case study solutions were prepared. The human breast cancer cell line MCF-7 was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. Equal numbers of the cells were passaged in 96-well plates (8×10³ cells/well) containing 100 µL of the medium for 24 hours. One hundred microliters of the different concentrations of the three case study solutions were dispersed in each well and incubated for 24 hours at 37°C with 5% CO₂. Fresh medium (100 µL) containing 0.5 mg/mL of MTT was replaced by the previous media in each well. The growth of the cells was quantified by the ability of the live cells to reduce the MTT Pink dye to a pale violet formazan product. After 4 hours, the formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a microplate reader. Effect of AgNP as the percentage of control absorbance of reduced dye was measured at 570 nm as well.

Results

Nanoparticle Production HPLC Experiment to Confirmation of Extracts

HPLC experiment was applied for determining Taxol in the extracts. The standard solution of Taxol showed an absorption of 350 mAU at 24–25 minutes after injection

(Figure 2A). No peak was observed after injection of the extracts of the crust (Figure 2B) and the stem (Figure 2C) at this time. However, a significant peak of 350 mAU absorption was observed at 24–25 minutes after injection of the leaf extract of yew tree (Figure 2D and E). Therefore, the leaf extract only which was confirmed to have the proper amount of Taxol was applied for the rest of the present study.

In the present study, we applied the green method for producing the AgNP as described in the material and method section. Using ultraviolet-visible spectroscopy (UV-Vis) method, researchers have reported 410 nm wavelength absorption for the AgNP. Our results show the highest SPR absorption band around 410 nm wavelength region (Figure 3A) which confirmed the formation of AgNP.^{21,29}

Surface morphology of the AgNP was studied applying scanning electron microscopy (SEM) with an acceleration voltage of 15kV and 1,04 µm resolution (VEGA-TESCAN SEM analyzer) to reveal the size and shape of the produced particles. The SEM analysis displayed the accumulation of the nanoparticles with a size of 15nm. SEM micrographs indicated that the products have been made of inflorescence circular nanoparticles (Figure 3B).

Figure 3C shows the experimental X-ray powder diffraction (XRD) pattern of the prepared AgNP. The diffraction peaks at 38.25, 46.21, 68.32 and 77.34 degrees can be assigned to hexagonal metallic silver corresponding to the (111), (200), (220) and (311) (JCPDS file, no. 04–0783) facets of the silver nanoparticle, respectively. The crystallite size was calculated from the width of the XRD peaks, assuming that they were free-form and non-uniform strains, using the Scherrer formula:

$$D = (0.94 \lambda) / (\beta \cos \theta)$$

where D is the average crystallite amplitude size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle.²³ Details of the data are given in the supplementary Table S1. The particle size was obtained for three long peaks (depending on the beta and FWHM of each courier) respectively: 7.37, 15.94 and 15.941. The average particle size of the synthesized silver NPs in this work was recorded as 13 nm using the XRD method.

The nature of phytochemicals responsible for the reduction of AgNP and *T. brevifolia* extract was studied by Fourier-transform infrared (FTIR) spectrometry (Rayleigh WQF-510A spectrometer). The benefit of the FTIR study is the identification of the various functional

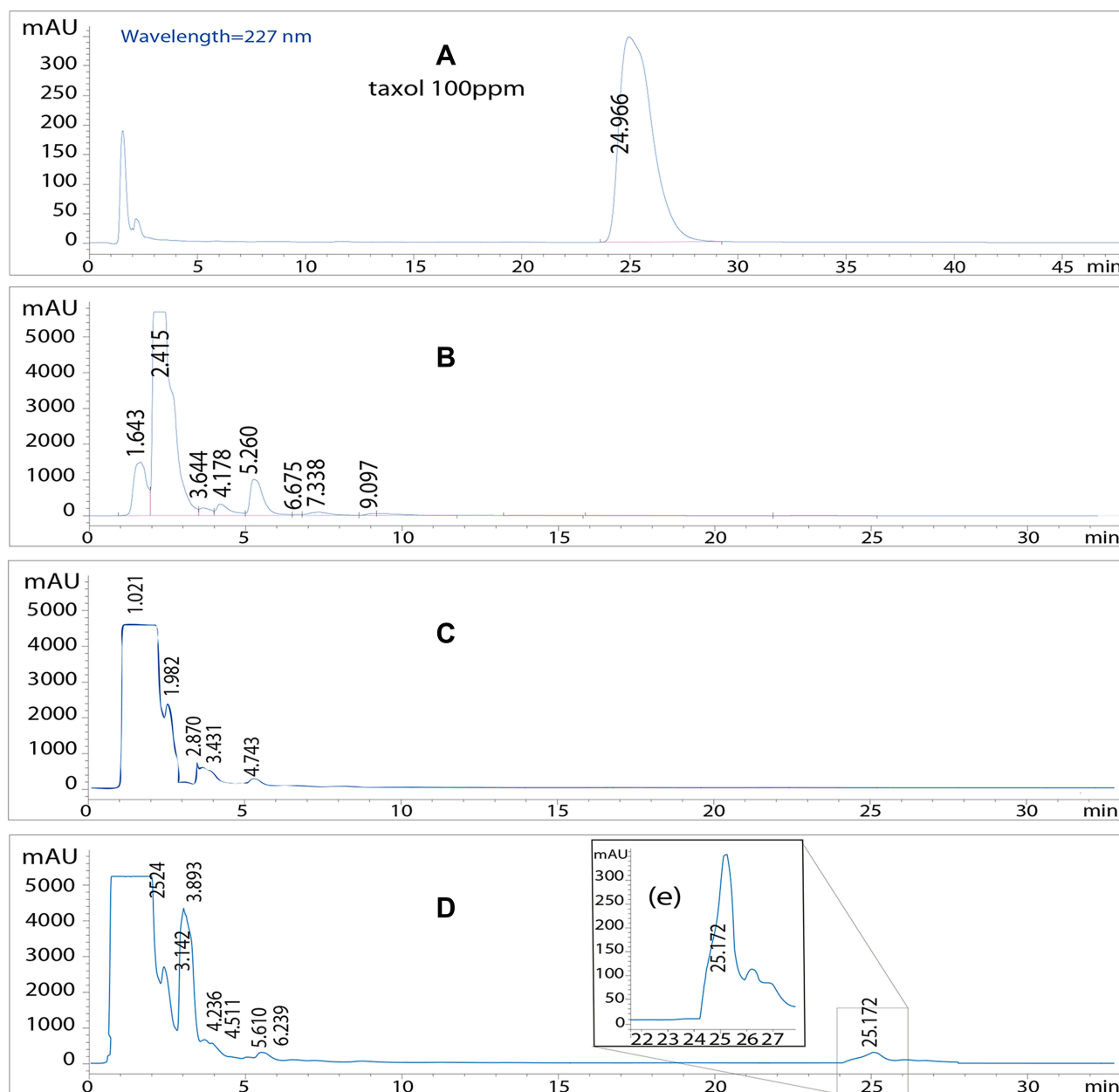


Figure 2 HPLC test for determining Taxol in the *Taxus brevifolia* extracts. **(A)** Standard solution of Taxol, 350 mAU peak at 25 minutes after injection. **(B)** Extracts of the crust, no peak at 25 minutes post injection. **(C)** Extracts of the stem, no peak at 25 minutes post injection. **(D)** Extracts of the leaves. **(E)** 350 mAU peak at 25 minutes after injection (leaf extract).

groups for capping and efficient stabilization of the synthesized metallic nanoparticles. The spectra for characterizing the AgNP were recorded in the range of 4000–400 cm^{-1} using the KBr pellet method.³⁰

The FTIR spectra bands of the *T. brevifolia* extract-mediated AgNP of this work are represented in Figure 3D. The observed bands at 3418 and 2920 cm^{-1} can be assigned to the –OH and aldehyde C–H stretching vibrations, respectively.¹¹ The band located at 2355 cm^{-1} indicates the N–H stretching/C55O stretching vibrations.³¹

The band at 1635 cm^{-1} corresponds to amide I, which arises due to carbonyl stretching vibrations in proteins.³² The characteristic bands observed at 1380 and 1079 cm^{-1} are attributed to the C55O and C–O stretching vibrations, respectively.^{31,32} The results demonstrate that the alkaloids may be adsorbed on the surface of metal nanoparticles by a possible interaction via carbonyl groups or π -electrons. The presence of biomass over the surface of AgNP may cause steric or electrostatic barriers, which effectively prevented the accumulation of nanoparticles.

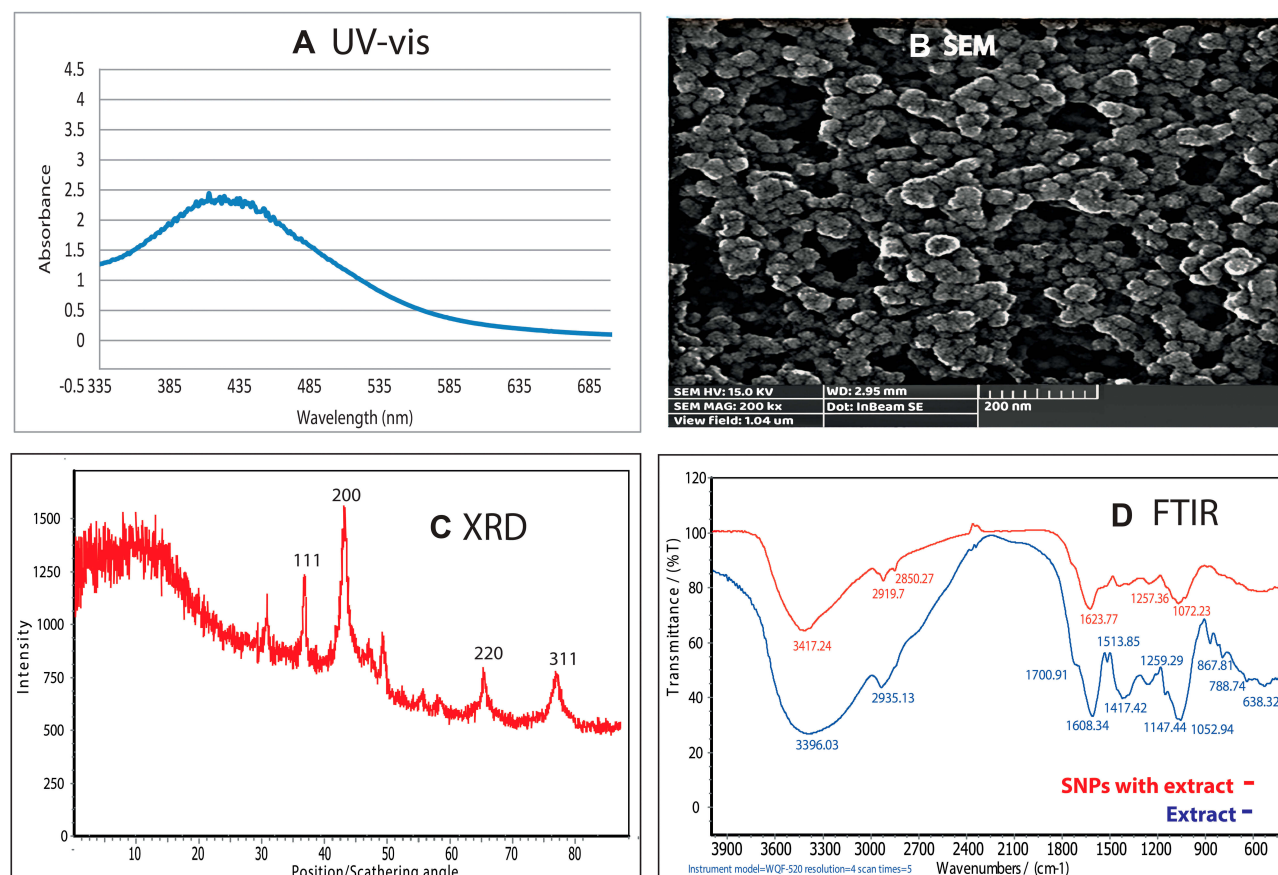


Figure 3 Confirmation methods. **(A)** UV-vis spectroscopy of silver nanoparticles synthesized by *Taxus brevifolia* extract. **(B)** SEM images of silver nanoparticles synthesized by *Taxus brevifolia* extract. **(C)** X-ray diffractogram of silver nanoparticles synthesized by *Taxus brevifolia* extract. **(D)** FT-IR spectra of *Taxus brevifolia* extract and silver nanoparticles synthesized by *Taxus brevifolia* extract.

Antibacterial Effect of Nanoparticle Against Three Bacteria

Silver NP solution (Figure 4A) and silver nitrate solution (Figure 4B) had a closed inhibition effect on germ-positive and gram-negative bacteria. However, the *Taxus* extract solutions did not show antimicrobial effects on three case study bacteria. MIC test results show no antibacterial activity for the pure *T. brevifolia* extract, where the AgNP showed even slightly more antibacterial effects (MIC: 6.6 mM) comparing the silver nitrate solution (MIC: 6.2 mM) on *Staphylococcus aureus*. Average MICs were recorded as 1.5 mM for *E. coli*, and 1.5 mM for *Pseudomonas* applying silver nitrate and AgNP (Table S2).

Assessment of Anticancer Activity by MTT Assay and DAPI Staining

Silver nitrate solution was observed to have more cytotoxic activity compared to the pure *T. brevifolia* extracts

and synthesized AgNP. *T. brevifolia* extract is a known anti-cancer remedy by a mechanism of cell division prevention likewise chemotherapeutic reagents. Obviously, the cells with higher proliferation are more affected by such a remedy. The anticancer activity of new synthesized AgNP tends to be more effective in more concentrated solutions. In the 25 mM of silver nanoparticle, about 78% of cancer cells are dead. Extract in the half dilution state showed the most anti-cancer activity where 72% of cancer cells die. All dilutions of silver nitrate solution exhibited closed anticancer activities with an 82–86% of cancer cells' mortality rate (Figure 5). Breast cell proliferation inhibitory activity of *T. brevifolia* extract, silver nitrate, and silver NPs demonstrated in the supplementary Table S3. DAPI staining is a method to detect cell death in which the dye can infiltrate the nucleus of the dead cells only. DAPI staining revealed a high rate of apoptosis of the breast cancer cells following the treatment with the nanoparticles (Figure 6).

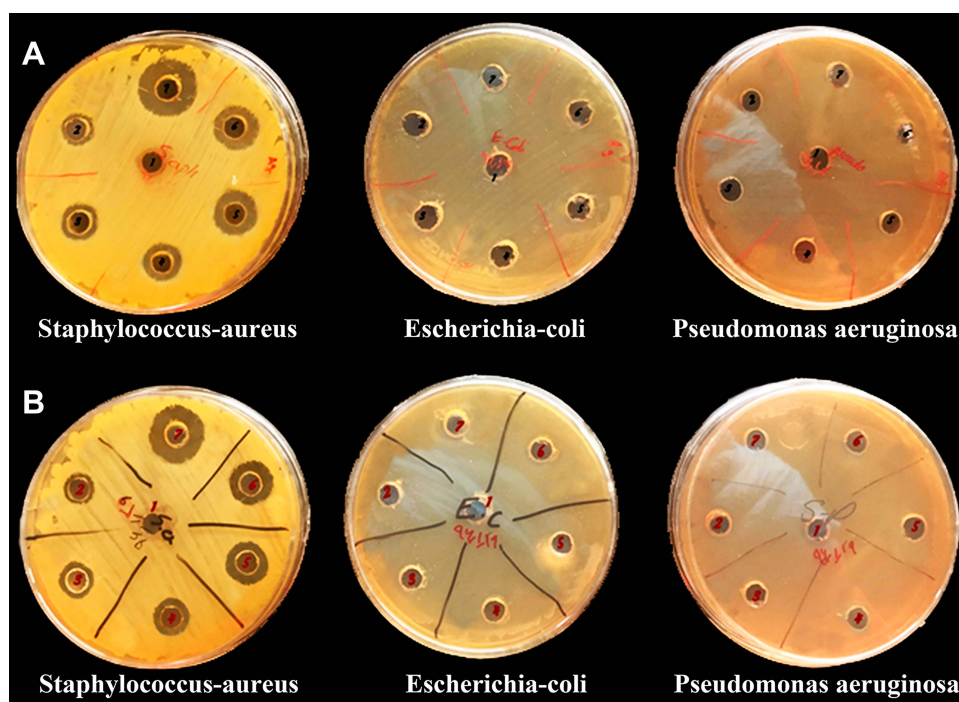


Figure 4 The well test. (A) Effects of the silver nanoparticle on three types of bacteria. (B) Effects of silver nitrate on three types of bacteria.

TEM Imaging and DLS Confirmation

The transmission electron microscope (TEM) image and dynamic light scattering (DLS) techniques were applied to determining the shape and size of the nanoparticles. TEM measurements were performed on a Zeiss model EM900 instrument operated at 80 kV accelerating voltage. The green method synthesized AgNP were prepared for TEM measurements by placing a drop over carbon-coated copper

grids and allowing the solvent to be evaporated. In the present study, the morphology of the AgNP, which was observed through the TEM micrograph, was hexagonal (Figure 7A). This hexagonal shape for the green method synthesized AgNP has been reported previously using the sunlight as reducing agent as well.³³ The TEM micrograph revealed a range of 5 to 25nm for the produced particles. This range was confirmed using the DLS technique too (Figure 7B).

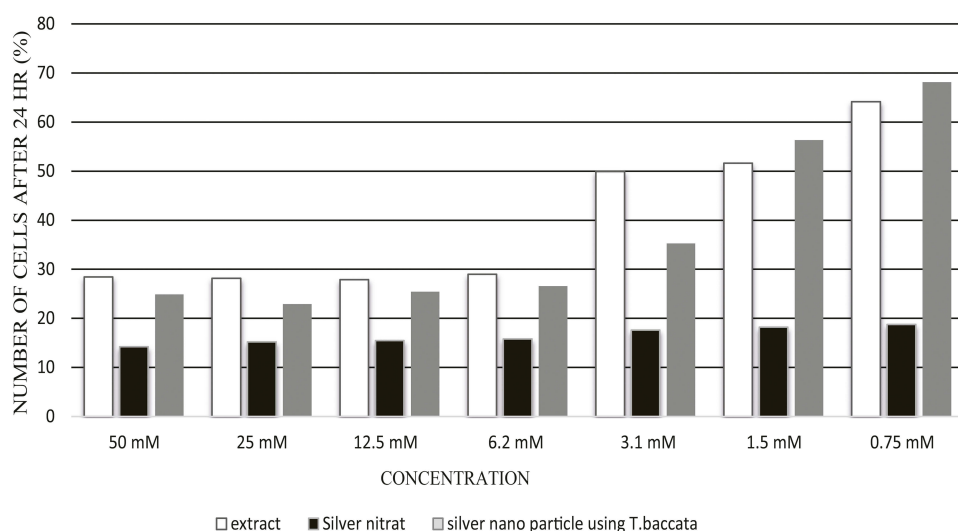


Figure 5 Comparing anticancer effect of new AgNP, Taxol and silver nitrate solution using MTT assay on the breast cancer cells.

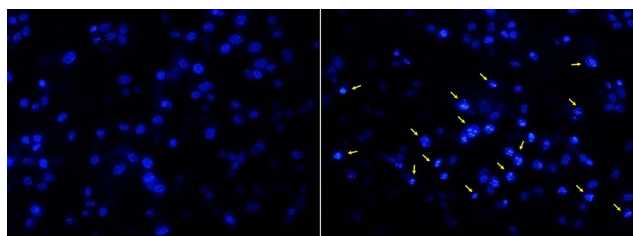


Figure 6 DAPI staining approved the apoptotic effects of the silver nanoparticles on the breast cancer cells regarding the infiltration of the nucleus by the dye (arrows).

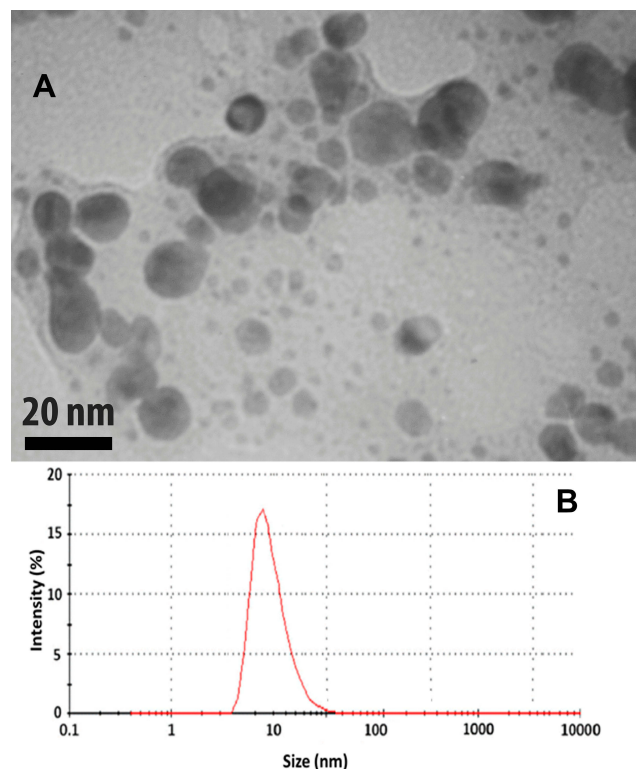


Figure 7 Determination of the shape and size of the silver nanoparticles synthesized by *Taxus brevifolia* extract. (A) Transmission electron microscope (TEM). (B) Dynamic light scattering (DLS).

Discussion

The *Taxus* tree has been proven to contain an anticancerous substance called Taxol. In the present study, three different samples from leaves, trunk, and shells of *T. brevifolia* tree were used to prepare the extracts. HPLC experiments revealed more Taxol content in the *T. brevifolia* leaves extract.

Consequently, AgNPs were synthesized by *T. brevifolia* leaves extract using the green method synthesis. Antimicrobial and anticancer effects of *T. brevifolia* leaves extract, silver nitrate and AgNP were compared. In conclusion, the synthesis of AgNP using *T. brevifolia* leaves

extract showed good results for biomedical applications. An absorption peak at 410 nm in the UV-vis spectrum proved the formation of silver NPs. Moldovan also reported a 407 nm absorption peak in UV-vis spectrometry for the silver nanoparticle which is synthesized with *European black elderberry* extract by the same method.³³ The obtained results from UV-vis spectrophotometry, as well as SEM, and XRD of AgNP confirmed the efficiency of *T. brevifolia* leaves extract in the synthesis of hexagonal AgNP. Same methods have been applied to confirm the synthesis of AgNPs.³⁴ Taruna et al reported spherical silver NPs and Sinha & Paul cubic silver NPs synthesized with green methods.^{18,35} The FTIR spectrum of the silver NPs and the extract has shown reduction functional groups for silver ion reduction. The functional groups for reducing metal ions in any tree or plant are different. The detected functional groups are alkaloids such as Taxanes A and Taxanes B in the extract of *T. brevifolia* leaves, as well as hydroxyflavones and catechins in the extract of papaya fruit,³⁶ and molecules with hydroxylamine factor in the seed extract of *Punica granatum*.³⁷

Metal-based nanoparticles have many free electrons that move by conduction and balance bands which are caused by surface plasmon resonance (SPR) after the UV light collision to them. The spectrum of this resonance records the vibrations of the free electrons of the nanoparticle.^{38,39} Khodadadi et al also have described the same method to confirm the formation of silver NPs with *Achillea millefolium* L. extract using the green method nanoparticle synthesis.⁴⁰ In the present study, the results from high-throughput techniques such as UV-visible spectroscopy, FTIR, SEM, and XRD measurements indicated the successful formation of Silver NPs.

The antibacterial activity experiment revealed the highest inhibition for *S. aureus* bacteria, which was recorded as 20mm at 50mM, 18mm at 25mM, 16mm at 12.5mM, 14mm at 6.25mM, 13mm at 3.1mM, and 12mm at 1.5 mM concentration of AgNP and silver nitrate solution. No inhibitory effect observed around the wells at 0.75 mM concentration of AgNP and silver nitrate solution. Furthermore, there was no bacterial inhibition by AgNP and silver nitrate solution in *E.coli* and *P. aeruginosa* plates. The *T. brevifolia* extract did not show any antibacterial effects.

Previous works reviewed several mechanisms of AgNP antibacterial effects, in which the release of silver ions from the nanoparticle surface is an essential step in all mechanisms.^{41,42} The MIC test in this study revealed

a slightly more antibacterial effect of the silver nanoparticle. This MIC similarity can indicate that there are no ion-releasing suppression effects on AgNP produced by adding *T. brevifolia* extract.

Anticancer activity of *T. brevifolia* extract, silver nitrate, and AgNP was investigated. *T. brevifolia* extract is a known anti-cancer remedy. The anticancer activity of the new synthesized AgNP tends to be more effective in more concentrated solutions. MTT assay showed that bio-synthesized Silver NPs had a cytotoxic effect on human breast cancer cell line MCF-7 like silver nitrate solution. According to the recorded results, one of the AgNP solutions which had the most anticancer activity was selected (25 mM). DAPI staining confirmed the apoptotic effects of AgNP on the breast cancer cells in terms of the infiltration of the nucleus by the dye. The TEM images of this solution confirmed the presence of nanoparticles with a size range of 5–25 nm.

Although AgNPs are well known as an antimicrobial agent,^{43,44} however, several scientists have described applications like an anticancer,^{7,45} biosensor,⁴⁶ and water microbial filters⁴⁷ (Supplementary Table S4). The antimicrobial effect of silver NPs may be due to either (i) the formation of holes in the cell wall, which ultimately leads to leakage of the cellular cytoplasmic content or (ii) the silver ions change the ribosome and inhibits the expression of the enzymes and thiol including proteins essential for the generation of ATP and DNA thus resulting in cell death.^{48,49} Furthermore, researchers reported the interaction of silver NPs with HIV-1 by binding to gp120 glycoprotein knobs. This type of interaction of silver NPs specifically inhibits the binding of the virus to host cells.⁴⁸ The plasmonic properties of silver NPs belong to the shape, size and dielectric medium that surrounds those.⁵⁰ Therefore, this dependency can conduct the applicability of silver NPs in biosensing.⁴⁶ They suggest a size range of 12–50 nm with a Neutral Surface charge. The average particle size of the synthesized silver NPs in the present study was recorded as 13 nm using an XRD method with a range of 5–25 nm according to the TEM imaging.

Prohibiting the growth of harmful microorganisms by improving or covering the surfaces with antimicrobial agents has received serious consideration for application in biomedical devices and health, as well as in the food and hygiene industries.⁴⁷ This application should possess adequate antibacterial benefits along with lower toxicity for humans. Silver nitrate solution shows a severe dose-independent cell toxicity in our results (Figure 5). Silver

nanoparticle seems to have lower toxicity and residues in water, food, as well as less systemic absorption from topical remedies comparing silver ion-containing solutions such as silver nitrate.^{51–53}

Conclusion

As an overall view, the present study proposed more anticancer, as well as antibacterial effects for AgNP made by *Taxus brevifolia* extract, comparing Taxol solution. Moreover, it revealed a dose-dependent biocompatibility along with slightly more antibacterial effects for AgNP compared with silver nitrate solution. The production of herbal-mediated silver nanoparticles may be an efficient substitution for the silver nitrate-based medicines with fewer side effects.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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