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Research Article

Facile and Eco-Friendly Fabrication of Silver Nanoparticles Using *Nyctanthes arbor-tristis* Leaf Extract to Study Antibiofilm and Anticancer Properties against *Candida albicans*

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The synthesis of silver nanoparticles has been gaining more attention in recent years due to their small size and high stability. For this study, silver nanoparticles were biosynthesized from leaf extract of the medicinal plant (*N. arbor-tristis*). Vitally, the shrub with tremendous medicinal usage was diversely observed in South Asia and South East Asia. The synthesized silver nanoparticles were characterized by color visualization, ultraviolet-visible spectrophotometry (UV-Vis), Fourier-transform infrared spectroscopy (FTIR), field emission-scanning electron microscopy (FESEM), energy-dispersive X-ray spectroscopy (EDX), and dynamic light scattering (DLS) technique. A sharp peak at 427 nm for biosynthesized nanoparticles was obtained using UV-Vis, which represents surface plasmon resonance. Thus, characterization techniques showed the green synthesis of AgNPs leads to the fabrication of spherical shape particles with a size of 67 nm. Furthermore, AgNPs were subjected to antibiofilm studies against *Candida albicans* and it was observed that 0.5 $\mu g\,mL^{-1}$ of AgNPs significantly reduced 50% of biofilm formation. These biosynthesized nanoparticles also showed a considerable reduction in viability of HeLa cells at 0.5 $\mu g\,mL^{-1}$. The morphological changes induced by AgNPs were observed by AO/EB staining. The toxic effect of AgNPs was studied using brine shrimp as a model system. Therefore, it is envisaged that further investigation with these AgNPs can replace toxic chemicals, assist in the development of biomedical implants that can prevent biofilm formation, and avoid infections due to *C. albicans*.

1. Introduction

Candida albicans is part of the common microflora in humans, and any slight alteration in the environment or immune suppression leads to the transition from commensal into pathogen [1]. *C. albicans* is an opportunistic fungal pathogen that can establish infection in almost all the organs of the human body. The pathogenesis of *C. albicans* is due to the production of several virulence factors; among them, biofilm formation plays an important role during infection [2]. *C. albicans* forms biofilm on indwelling devices and catheters that are in contact with host tissues. Thus, *C. albicans* complicates the therapy by penetrating deep into the tissues, virtually spreading to other superficial sites, and establishing the infection [3]. *C. albicans* is reported to be the one among the species of Candida that can result in indwelling material mediated bloodstream infections [4]. *C. albicans* biofilm has shown resistance to antifungal drugs such as echinocandins that are currently used for the treatment of invasive infection [5]. However, the development of new antifungal drugs is an escalating problem due to the antifungal resistance, toxicity, and paucity in the selection of the targets. Together, all these factors are responsible for clinical failure and lead to mortality in patients with invasive disease. Therefore, there is a need to identify new antibiofilm agents against *C. albicans* mediated infection [6].

Nanoparticles studies have remarkable recognition worldwide because of their size, shape, and surface morphology, which can play exclusive implications on controlling the physiochemical and biological properties of nanoparticles [7, 8]. Thus, nanoparticles application as nanomaterials has grown tremendously in different fields of biological science and medicine and has paved a way to design new devices and technocommercial products [9]. The synthesis of nanoparticles using a greener approach is gaining an advantage over the other methods since they are eco-friendly and prevent biological and environmental issues [10]. Biosynthesis of nanoparticles from medicinal plants has gained importance because they are easily available and extensively prescribed and practiced for the treatment of several diseases [11–13]. Plants are the major source of secondary metabolites, which has several biological activities. Different parts of the plants were used for synthesis of nanoparticles. Flavonoids, nonflavonoids, phenolic chemicals, lipids, and glycosides are occasionally found in plants. Thus, usage of plant materials for biosynthesis of metal particles can improve the efficacy of the biological activity. Leaves being a major source of the plants biological process were explored vastly for nanoparticle synthesis. The leaves of *Lotus lalambensis* and AgNPs synthesized have shown protective effect against oral candidiasis [14]. The synthesis of AgNPs from leaf extracts of *Semecarpus anacardium*, *Glochidion lanceolarium*, and *Bridelia retusa* has shown antibacterial and antibiofilm activity against human pathogens. *Nyctanthes arbor-tristis* belongs to the group of medicinal shrubs with a common name "night Jasmine" or represented as 'Pavalamalli' in Tamil. This shrub is very famous for its medicinal property and is dynamically used in most Asian countries for various ailments in mankind [15]. Almost every part of this shrub is used for treatment; for example, the leaf juice is used as purgative and to deworm infants and children. Leaves of *N. arbor* are used for treating high fever, rheumatism, and liver disorders. The bark of the shrub is used as antivenom. Besides, this shrub was also studied for its antibacterial, antifungal, antiviral, antiallergic, antihistaminic, anticancer, immunotoxic, and ulcerogenic properties [16–19]. Recently, Betulinic acid from the leaves of *N. arbor* was reported for having *in vitro* anticancer property [20]. Crude herbal plant extracts have been used to cure a variety of conditions since ancient times, including osteoarthritis, ulcers, cancer, heart disease, bone fractures, and diabetes. Extracts with antioxidative, antibacterial, and anti-inflammatory properties, in particular, might help to avoid difficulties and delays in tissue repair and regeneration [21].

Hence, in the current study, silver nanoparticles (AgNPs) from *N. arbor-tristis* leaf extract were synthesized and investigated against *C. albicans* biofilm. Consider using *Nyctanthes arbor-tristis* leaf extract to cure a variety of diseases as an effective and safe alternative to chemical medications that have no side effects [22].

2. Experimental

2.1. Preparation of the Plant Extract. Leaves of the medicinal plant *N. arbor-tristis* were collected from Karaikudi, Tamil Nadu, India. The collected leaves were rinsed twice with deionized water to remove any dust particles and dried at room temperature (RT). Then, dried-out leaves were prepared as fine particles using a mixer grinder. About 10 g of the fine powder was added to 250 mL of deionized water and boiled for 30 min and left at RT. After complete cooling, extract was filtered using ordinary filter paper and stored in a vial at RT for further use.

2.2. Green Synthesis of Silver Nanoparticles. For the synthesis of sliver nanoparticles (AgNPs), 90 mL of silver nitrate (HiMedia, Mumbai, India) ($AgNO_3$ 1 mM) solution was mixed (added as drop) with 10 ml of the plant extract taken in a 250 ml conical flask. The reaction mixture was kept in continuous shaking conditions until the color of the extract changes. The changes in the reaction color within 15 minutes were recorded. The deep brown color of the solution without any further change will indicate that the silver ions have been reduced completely by the extract.

2.3. Characterization of AgNPs. The rapid synthesis of silver nanoparticles was characterized using UV-Vis spectroscopy (Cary-60, Agilent, USA). The spectra of the sample and control were recorded periodically for a wavelength range of 300 nm–600 nm at 2 nm resolution. The aqueous silver nanoparticles solution was dried and stored at 4°C for further study. The functional group of AgNPs was obtained using Fourier-Transform Infrared Spectroscopy (FTIR) (PerkinElmer Spectrum-One, USA) with a resolution of 2 cm^{-1} at a diffuse reflectance mode. The characteristics features of AgNPs such as morphology and size were studied

using field emission-scanning microscopy (FESEM) with EDAX (Supra-55, Carl Zeiss, Germany) at an accelerating voltage of 20.00 kV.

2.4. Culture Preparation. *C. albicans* (ATCC: 90028) were maintained in Sabouraud Dextrose Agar (SDA). Before all the assays, the overnight culture was harvested by centrifugation (10,000 rpm, 5 min). The supernatant was discarded followed by the suspension of the pellet with Sabouraud Dextrose Broth (SDB), containing 10^6 cells mL^{-1} in the suspension, and used for further assay.

2.5. Biofilm Inhibition Assay. The formation of biofilm was allowed in polystyrene microtiter plates (24-well) for 48 h followed by removal of media from the well and washing gently with Phosphate buffer saline (PBS) to remove the planktonic cells. The wells in the microtiter plates were stained with crystal violet stain (0.4%) to determine the biofilm inhibitory effect of AgNPs using a spectrophotometer at 570 nm [19].

2.6. Light and FESEM. The biofilm morphological changes were studied using different microscopic techniques. *C. albicans* cells grown on a glass slide (1 × 1 cm) were kept inside 12-well microtiter plates and incubated for 48h at 37°C. A test well was supplemented with AgNPs and the well without AgNPs was referred to as the control. The control and treated glass slides were washed with PBS and stained with crystal violet (0.4%) for 5 min. Later, the slides were destained, dried, and observed under a light microscope (Lieca, DM2000LED) at 400 × magnification. For FESEM analysis, experimental slides were removed from the microtiter plates after incubation and washed with PBS and immersed into 2% glutaraldehyde solution for about 3 h. Immediately, the slides were with PBS and fixed with series of ethanol 25%, 50%, and finally 100% and left for air drying. The slides were then sputtered with gold and examined under FESEM (Hitachi S-3000H, Japan).

2.7. Cell Culture Preparation and Condition. For the present study, the HeLa cervical cancer cell line was obtained from NCCS (National Centre for Cell Sciences), Pune, India. Eagle's minimum essential medium was added with 10% fetal bovine serum (HiMedia) and 20 μg/ml gentamicin to culture the HeLa cells. The cells were maintained under 97% humidity in a biological incubator at 37°C with 5% CO_2 and monitored routinely. Cells after reaching 80% confluence were used for other experiments.

2.8. Cytotoxicity Studies Using HeLa Cells. Through colorimetric reduction assay, the cytotoxic effect of the biosynthesized AgNPs against HeLa cells was measured. The soluble MTT will be metabolized by the mitochondrial enzymes of the viable HeLa cells into an insoluble colored tetrazolium salt [23]. Before the experiments, the HeLa cells (10^5 cells mL^{-1}) were dispensed into wells of the 96- well plates and kept under incubation for 24 h at 37°C. Test wells were added with HeLa cells and different concentrations of AgNPs (0.25 μg, 0.5 μg, and 1 μg/mL^{-1}). The plates were incubated (5% of CO_2 at 37°C) again for 24 h. To evaluate the cell survival in the presence of AgNPs, 10 μL of MTT solution (10 mg mL^{-1} in PBS) was added to control and test wells and the plates were kept in dark condition at RT for about 4 h. Finally, 100 μL of DMSO was added to each well replacing the media from each well. The absorbance (OD_{570} nm) was determined with a microtiter plate reader and values were recorded (PerkinElmer, EnSpire, USA).

2.9. Acridine Orange/Ethidium Bromide (AO/EB) Staining. AO/EB staining techniques were used to study the morphological changes in the HeLa cells in the presence and absence of AgNPs (0.5 μg/mL^{-1}) [24]. Control and treated setup of HeLa cells were prepared as mentioned above. HeLa cells were stained with a final concentration of 100 μg mL^{-1} of AO/EB stain and the plates were left as such for 5 min at RT. HeLa cells were washed with deionized water twice and visualized under a fluorescence microscope (EVOS-fl digital).

2.10. Toxicity Assay Using Brine Shrimp. For studying the toxic effect of AgNPs, 4 g of cysts was mixed with 200 mL of seawater and kept at 30°C for 48 h light conditions. After 48 h, the cyst hatched into the *Artemia* stage which was used for further studies. For this experiment, into each well of the 12-well microtiter plates, 2 mL of sterilized seawater was added and later 10 *Artemias* were transferred into each well. The infection wells containing *Artemia* were coincubated with 10^6 cells mL^{-1} of *C. albicans*. Test wells containing *C. albicans* coincubated with *Artemia* were supplemented with AgNPs. *Artemia* with seawater alone was considered as a control well. The rate of survival of *Artemia* in the presence and absence of AgNPs was recorded for 24–48 h at 30°C.

2.11. Statistical Analysis. Biological triplicates were conducted for all experiments and the results were shown in bar diagrams as the representation of standard deviation. The data were statistically compared and the difference was significantly defined using one-way ANOVA with $P < 0.05$ recorded as significant.

3. Results

3.1. Characterization of AgNPs. The synthesis of AgNPs from plant extract was confirmed visually with color change in 15 min from greenish-brown to dark-brown color (Figure 1(a)). From the UV-Vis spectroscopy, a strong spectroscopic resonance peak was observed at 427 nm, which is the signature peak of AgNPs (Figure 1(b)) that represents the fabrication of AgNPs. The functional groups of biosynthesized AgNPs were also analyzed using FTIR (Figure 1(c)). FTIR spectrum of AgNPs showed major

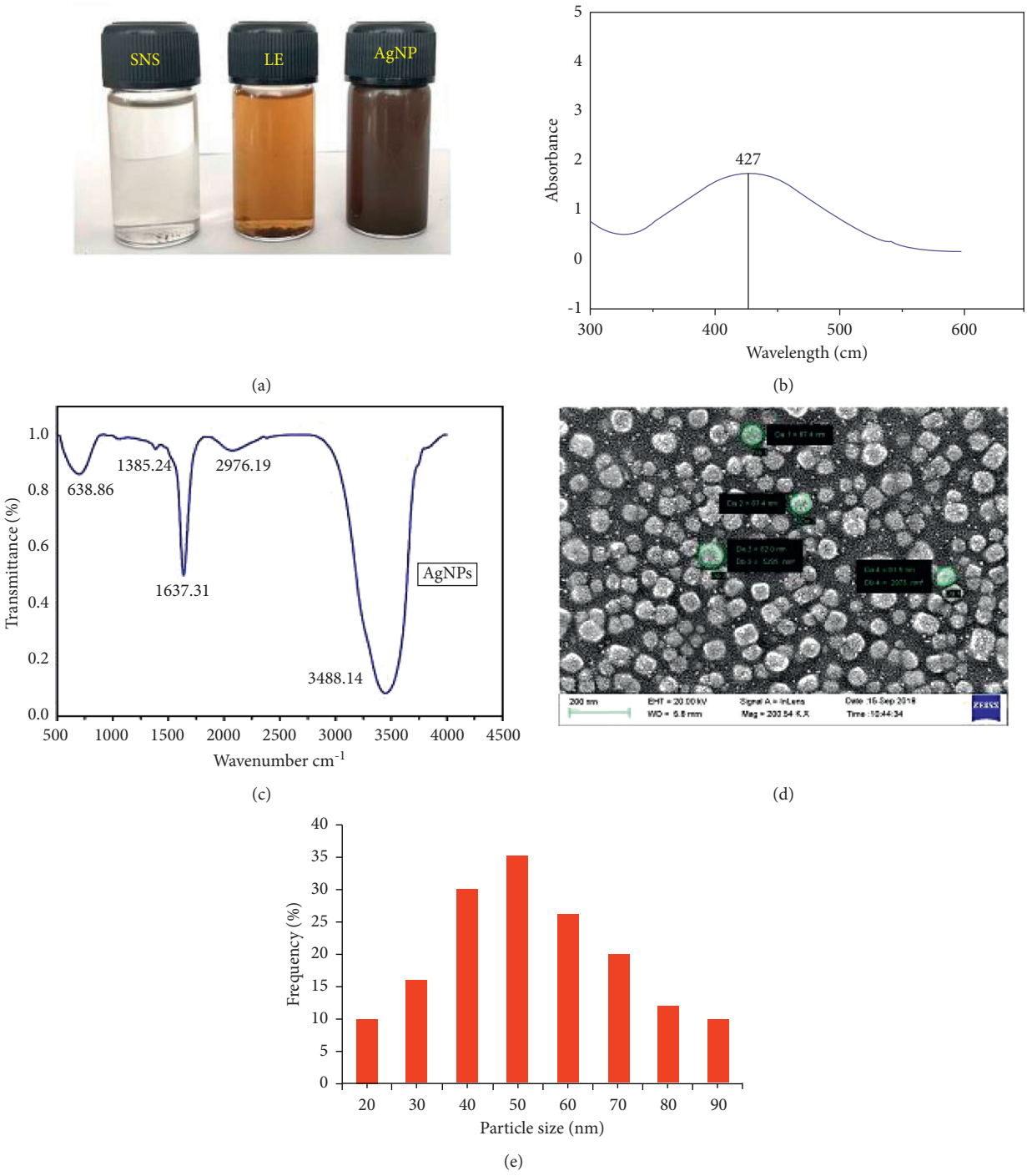


Figure 1: Biosynthesis and characterization of silver nanoparticles (AgNPs) by using the leaf extract of *N. arbor-tristis*. (a) The color change during AgNPs synthesis (silver nitrate solution: SNS, leaf extract: LE, silver nanoparticles: AgNPs). (b) UV-Vis spectrum shows the surface plasmon resonance reduction by leaf extract at 427 nm due to silver nanoparticles synthesis. (c) FTIR spectrum of AgNPs synthesis. (d) SEM micrograph of silver nanoparticles showing spherical morphology. (e) Particle size distribution histograms of AgNPs.

absorption peak at 698 cm^{-1}, 1385 cm^{-1}, 1637.31 cm^{-1}, 2075.10 cm^{-1}, and 3488.14 cm^{-1}. The peak represents the stretching vibrations of C-H bend (698 cm^{-1}), C-F (1385 cm^{-1}), C=C stretch (1637 cm^{-1}), and O-H intramolecular hydrogen bonds (3488.14 cm^{-1}). The broad signal at 3488.14 cm^{-1} may also correspond to stretching of the O-H or the H-bonds present in the polyphenolic group, polysaccharides, or from the amino acids. The signal at 1637 cm^{-1} represents the stretching of the (NH) = O group in either of the macromolecules [25]. From FESEM, the

morphology and size of AgNPs were determined and with the average size of 67 nm and spherical shape (Figure 1(d)) and particle size distribution were revealed in Figure 1(e). Further, the elemental silver in the biosynthesized AgNPs was examined using EDX analysis and a peak at 3 KeV represents the silver ions as the main component, indicating the fabrication of *N. arbor-tristis* leaf extract into AgNPs (Supplementary Figure S1).

3.2. Antibiofilm Effect of AgNPs against C. albicans Biofilm. Antibiofilm activity of AgNPs was determined using crystal violet assay, which showed a progressive inhibition of biofilm formed by *C. albicans*. AgNPs at 1 μg mL^{-1} concentration showed 81% of biofilm inhibition as shown in (Figure 2). However, AgNPs at 0.5 μg mL^{-1} concentration showing 50% biofilm inhibition were determined to be biofilm inhibitory concentrations (BIC). From light microscopic images, it was observed that the *C. albicans* formed a structured arrangement of yeast cells in the biofilm (Figure 3(a)). Figures 3(b)–3(d) represent that in the presence of AgNPs there is a sequential reduction in the adhesion of *C. albicans* to the matrix. From the SEM image, it was confirmed that *C. albicans* forms a structured biofilm embedded with exopolysaccharides (Figure 4(a)). In treatment with AgNPs, there was no exopolysaccharide and only scant cells were seen to be adhering to the matrix (Figure 4(b)).

3.3. Cytotoxicity Studies Using HeLa Cells. The IC_{50} value for the HeLa cells was determined as 0.393 μg mL^{-1}. Morphological changes due to AgNPs were visualized using AO/EB under the fluorescence microscope. Figure 5(a) represents the control group of HeLa cells with green fluorescence due to the intact nuclei with clear cell boundaries. HeLa cells treated with AgNPs showed a fluorescence color of yellow-green which corresponds to the early apoptotic nucleus. The orange-red fluorescence represents the necrotic cells with uneven shapes. Thus, from AO/EB staining, the apoptotic characteristics of HeLa cells due to the treatment of AgNPs were revealed. Cytotoxic effect of AgNPs at different concentrations (0.25, 0.5, and 1 μg mL^{-1}) was determined against HeLa cells using MTT assay. In the presence of AgNPs, the survival rate of HeLa cells was 62%, 45%, and 28%, respectively (Figure 5(b)).

3.4. Toxicity Assay Using Brine Shrimp. The *in vivo* effect of AgNPs (0.5 μg mL^{-1}) on *C. albicans*-infected brine shrimp at their nauplii stage was tested. Nauplii infected with *C. albicans* showed 45% survival at 24 h and 30% survival at 48 h. In the presence of AgNPs, the survival rate of nauplii infected with *C. albicans* showed 80% survival at 24 h and 75% survival at 48 h (Figure 6). However, there were 100% survival nauplii in the presence of AgNPs (0.5 μg mL^{-1}) which is a comprehensive evidence that AgNPs are not toxic for the nauplii.

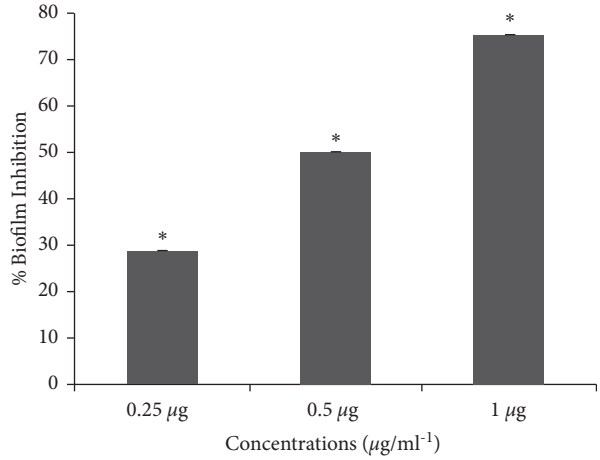


Figure 2: Antibiofilm activity of AgNPs against *C. albicans* biofilm at different concentrations. A biofilm inhibitory concentration (BIC) of AgNPs was determined as 0.5 μg mL^{-1} showing the maximum inhibition of 50%. $^*P < 0.05$ statistical significance was calculated using one-way ANOVA.

4. Discussion

Green synthesis of nanoparticles is considered to be eco-friendly [26] comparing with other materials such as microorganisms which are laborious and time-consuming. In green synthesis, any part of the plant materials is used and their extracts are used as a capping agent to fabricate the materials. In this study, the leaf extracts of *N. arbor-tristis,* a well-known medicinal plant, were used for biosynthesis of silver nanoparticles. The biosynthesis of AgNPs was observed within 15 min with a change in color from green to dark-brown color with an average size of 67 nm having a spherical shape. Most of the reports with AgNPs synthesized from plant extracts show the color change from yellow to brown with a plasma resonance peak appearing in a region of 406–453 nm which demonstrates the fabrication process [27, 28]. The leaf extract used in the study has a dual role, which can reduce as well as cap the AgNPs, which was confirmed using the FTIR technique. The AgNPs were further investigated for biofilm inhibition and sequential reduction was observed.

However, 0.5 μg/mL showing 50% biofilm inhibition was determined as BIC and further experimented. To purge mature *C. albicans* biofilm is very difficult due to host defense mechanisms and antimicrobial resistance. In addition, *C. albicans* entering the phenotypic state change from yeast to hyphal transition in a biofilm are more complicated to be treated. Therefore, it is always easier and beneficial to eradicate the initial stages of biofilm [29–31]. Microscopic images (light and SEM) substantiate that AgNPs prevent the attachment of yeast cells to a glass slide. Hence, AgNPs exclude *C. albicans* biofilm formation and therefore it is envisaged that AgNPs could facilitate the eradication of biofilm that is difficult during treatment with antibiotics. *C. albicans* undergoes morphological switching from yeast cells to hyphal forms, which is very important for adherence and biofilm formation. Indeed, this step is very essential for

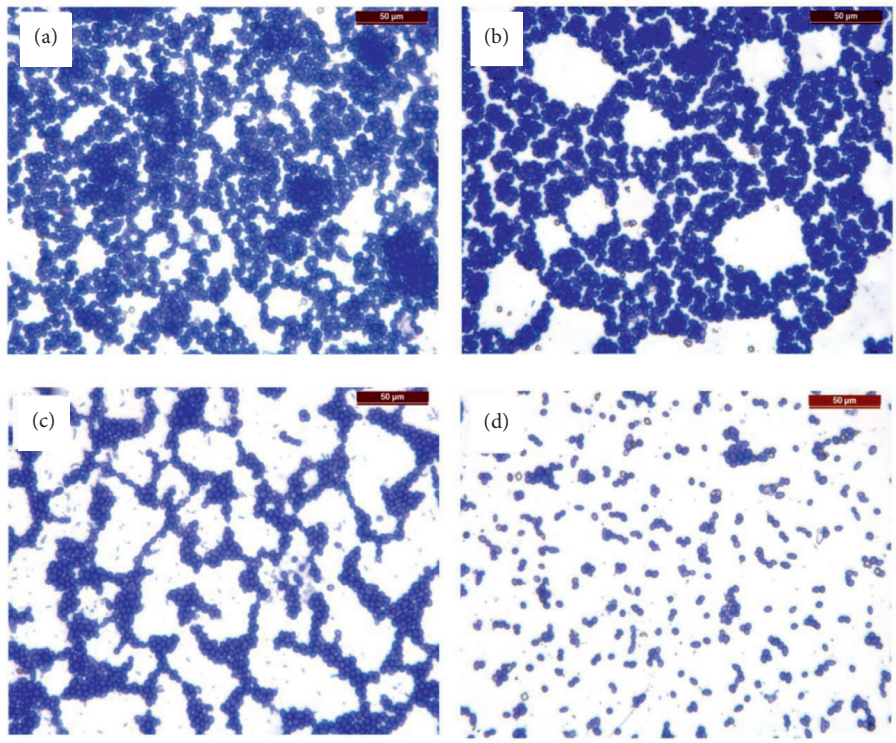
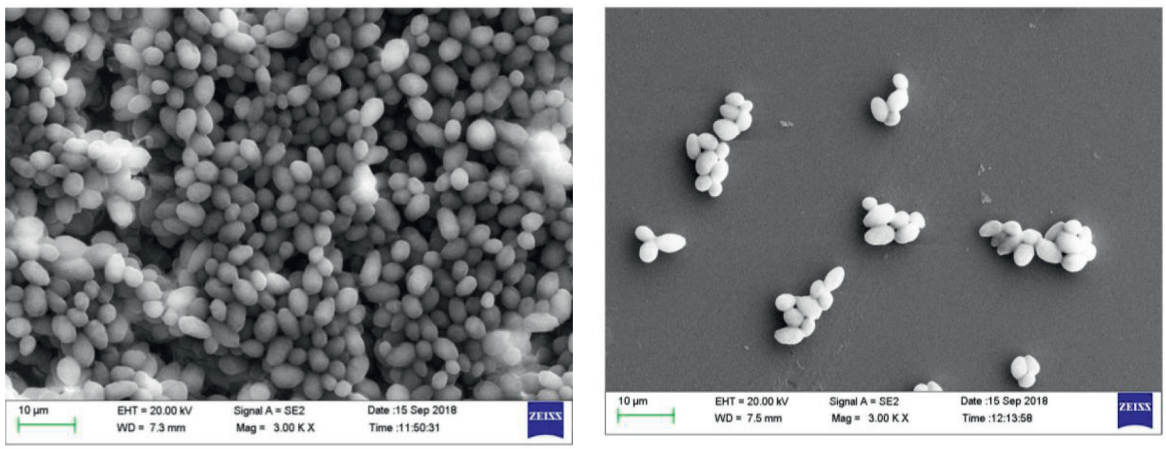


Figure 3: Light microscopic images for the antibiofilm activity of AgNPs against *C. albicans* biofilm. (a) *C. albicans* biofilm control. (b–d) Biofilm formed in the presence of 0.25 $\mu g\,mL^{-1}$, 0.5 $\mu g\,mL^{-1}$, and 1 $\mu g\,mL^{-1}$ of AgNPs showing dose-dependent reduction of biofilm with different concentrations of AgNPs.



(a) (b)

Figure 4: SEM images showing the structure of *C. albicans* biofilm in the presence and absence of silver nanoparticles. (a) Control. (b) The biofilm treated with 0.5 $\mu g\,mL^{-1}$ silver nanoparticles.

colonization and pathogenesis [32]. From the light and SEM images, it was clear that AgNPs significantly reduced the adhesion of yeast cells which has typically proven that hyphal production was also remarkably inhibited and prevented the colonization and pathogenesis of *C. albicans*. Molecular investigation of aqueous leaf extract of *Polyalthia longifolia* has shown inhibition of Ras-mediated signal transduction, which includes activation of cascade of genes such as Elongation gene (ECE1), hyphal transition genes (Tup1 and Rfg1), and hyphal inducer gene (Tec) [33]. Interestingly, chitosan nanofibrous mats used for wound dressing loaded with silver nanoparticles have shown to possess significant antimicrobial and antioxidant property [34]. Thus, from the present study, it is envisaged that AgNPs of *N. arbor-tristis* might have adapted similar mechanism for inhibition of yeast cell adhesion and yeast to hypal switching.

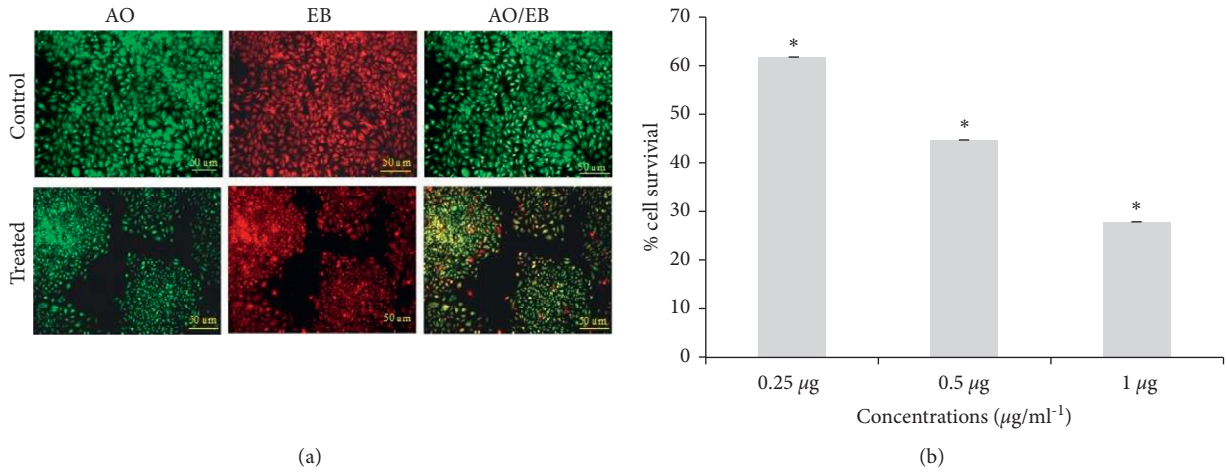


Figure 5: (a) AO/EB staining showed the morphological changes of HeLa cells visualized under a fluorescent microscope. The cytotoxic effect of AgNPs showed apoptotic cell death. The green circle represents the live cells, the red circle represents the necrotic cells, and the blue circle represents the apoptotic cells. (b) Viability of HeLa cells was examined on exposure to AgNPs after 24 h incubation. The cytotoxicity was determined by an MTT assay. Data are expressed as the percentage of inhibition compared with a negative control in which cell survival was assumed to be 100%. Statistically significant was indicated as " $*$ " with $P < 0.05$.

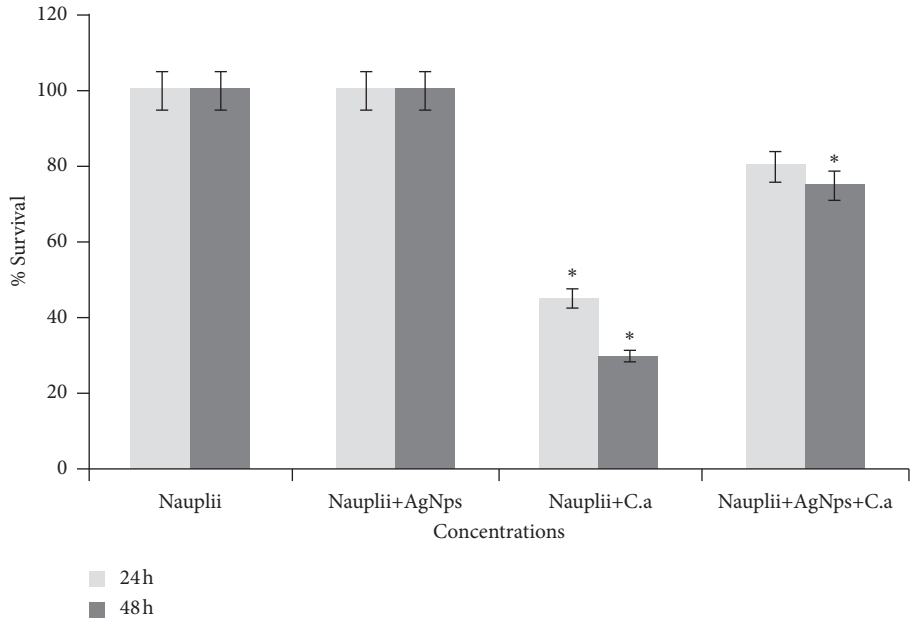


Figure 6: The *in vivo* effect of AgNPs (0.5 μg mL^{-1}) on *C. albicans*-infected brine shrimp at their nauplii stage showing 45% survival at 24 h and 30% survival at 48 h. In the presence of AgNPs, the survival rate of nauplii infected with *C.albicans* was 80% at 24 h and 75% at 48 h. $^{*}P < 0.05$ was indicated as statistically significant.

Later, AgNPs investigated against HeLa cells showed cytotoxic effect as wells morphological changes, which represented the apoptotic cell death. Apoptosis occurs with the decrease in the antioxidant level. The primary role of anticancer drugs is to trigger apoptosis in cancer cells, which was very clear from Figure 5(b), showing cytomorphological changes in the presence of AgNPs. Various reports have also shown that silver nanoparticles from different plant extracts have anticancer activity against human cells [24, 30]. AgNPs synthesized using *Ficus religiosa* leaves have shown similar effect on HeLa cells with the shrinkage cells due to rounding of the nuclei [35]. Therefore, the present study also supports the anticancer property of the synthesized AgNPs from *N. arbor-tristis* leaf extract.

The toxic effect of AgNPs on *C. albicans* infections was tested using *Artemia* at different periods. Due to various ease features like the hatching of the cyst, short life span, and high vulnerability to toxin, brine shrimp has been a suitable model organism for *in vivo* experimental studies [36–38]. AgNPs tested against this model organism are nontoxic and significantly able to inhibit the *C. albicans*. Therefore, AgNPs have been suggested as an antibiofilm agent that can be used

against *C. albicans*. However, several studies were focused on the identification of active ingredients such as plant compounds, nanoparticles, and antimicrobial peptides with multiple roles for multitargeting approach. Garlic clove extract-coated silver nanoparticles have shown several roles as antibacterial, antibiofilm, antihelminthic, anticancer, and anti-inflammatory activity [39]. Similarly, catechin overlaid graphene oxide and zinc oxide nanocomposites were reported to have anticancer and antibiofilm activity [40]. Recently, histone H2A-derived antimicrobial peptide has shown potential effect as antibacterial, anticancer, and antibiofilm agent [41]. Thus, from the present study, medicinal plant (*N. arbor-tristis*) extracts fabricated as AgNPs are reported to have antibiofilm and anticancer effect. In general, immunocompromised and cancer patients are at high incidence of bacterial and fungal infection [42]. Therefore, it is envisaged that identification of nanomaterials with multiple biological potential will surely pave a way to target multiple diseases in the field of biomedical application employed either as coating device or as therapeutic targets at controlled sites.

5. Conclusion

In this study, the leaf extract from the medicinal plant (*N. arbor-tristis*) was used for fabrication, which is cost-effective and eco-friendly. The biosynthesized AgNPs showed potential antibiofilm activity against *C. albicans*. Additionally, AgNPs have also shown cytotoxic effects against HeLa cells. *C. albicans*, a nosocomial fungal pathogen, affects immunocompromised patients and prolonged hospitalized patients. *C. albicans* being opportunistic with the disturbance in their environment can cause infection by forming biofilm. Thus, biofilm eradication is most critical since patients do not respond to gold standard antibiotics used for treatment. In recent trends, using nanomaterial-coated devices has been considered as a novel strategy to eradicate the biofilm-related infection. Thus, the present work supports the fabrication of medicinal shrub into AgNPs with dual role as antibiofilm and anticancer agent with least toxicity which can facilitate the usage of AgNPs further as coating material for medical devices and prevent fungal biofilm mediated infections.

Data Availability

Sufficient data have been included in the manuscript. Additional data can be kindly requested from the corresponding author.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Figurer S1: EDX analysis and a peak at 3 KeV represents the silver ions as the main component, indicating the fabrication of *N. arbor-tristis* leaf extract into AgNPs. (*Supplementary Materials*)

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