



RESEARCH ARTICLE

Green synthesis of silver nanoparticles using leaf extract of *Cinnamomum tamala* and its antimicrobial activity against clinical isolates of bacteria and fungi

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Abstract

Silver nanoparticles (AgNPs) have been synthesized using the aqueous leaf extract of *Cinnamomum tamala*. The synthesized particles were characterized by UV-Visible Spectroscopy, SEM, TEM, FTIR and EDX. TEM analysis showed the size of the nanoparticles was in the range of 10-15 nm. The antimicrobial activities of green synthesized nanoparticles against different bacterial and fungal stains were assessed by broth dilution and agar well diffusion methods. The MIC values show that the bacterial strains were more susceptible to AgNPs than fungal strains. The topographical and ultrastructural changes caused by AgNPs in *Candida albicans* were characterized by SEM and TEM, respectively. The electron microscopy analysis shows the attachment of AgNPs on the surface and disruption of the fungal cell membrane and cell wall.

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Introduction

Silver nanoparticles (AgNPs) have various applications due to their antibacterial, antifungal, antibiofilm and other medicinal properties (Ansari et al., 2011; Ansari et al., 2015; Panacek et al., 2009; Kim et al., 2009). Different parts of plants extract have been used for the synthesis of silver nanoparticles. Synthesis of nanoparticles by using plant extracts is known as green synthesis method and it is easy and non-hazardous. The plant *Cinnamomum tamala* belongs to family Lauracea and it is also known as tejpat in Hindi. The leaves of *C. tamala* are used as spices throughout the world. It is medium sized tree i.e., 2.5-10 meter tall and grows on tropical and sub-tropical Himalayas, nilgiri hills and the lower part of Sikkim Himalayas.

In the present work, we have used leaf extract of *C. tamala* for the synthesis of AgNPs. *C. tamala* leaves contain various phytochemicals such as terpenoids, flavonoids, enzymes, protein, antioxidants, triglycerides, polysaccharides. The leaves of *C. tamala* are used in the treatment of anorexia, bladder disorder, diarrhea, nausea

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(Sharma & Nautiyal 2011). It has also antimicrobial (Goyal et al., 2009), hypoglycemic, hypolipidemic, antidiabetic (Chakraborty & Das 2010), antioxidant (Devi et al., 2007), gastroprotective (Eswaran et al., 2013), anti-diarrheal (Rao et al., 2008) and anti-inflammatory properties (Gambhire et al., 2009). The objective of present work was the biogenic synthesis of AgNPs using leaf extract of *C. tamala* and evaluation of their potential antimicrobial properties against various bacterial and fungal clinical isolates.

Materials and Methods

Silver nitrate (AgNO_3) was purchased from Sigma-Aldrich. The leaves of *C. tamala* was washed 3-4 times with double distilled water to remove the dust particles and other impurities and then sun dried to remove the moisture from the leaves. The dried leaves were then crushed by using mortar and pestle. 10 grams of dried powder of leaves was mixed with 100 ml double distilled water and boiled for 15-20 min. The extract was then filtered with Whatman no.1 filter paper to remove the heavy materials from the extract.

The aqueous extract of leaves was centrifuge 1000 rpm 10-15 min. A volume of 5 ml of leaf extract was mixed with 95 ml of 1mM AgNO_3 solution and after 24 h observed any color change in the solution.

Characterization of green synthesized AgNPs

UV-Vis spectrophotometer analysis

The green synthesized AgNPs were scanned by UV-Vis spectrophotometer at the wavelength of 300-600 nm on Perkin Elmer Lambda 25 spectrophotometer. This method is used for characterization of colloidal silver nanoparticles which possess strong surface plasmon resonance (SPR).

FTIR Spectroscopy

The FTIR of green synthesized AgNPs was recorded using Perkin Elmer spectrum between 400-4000/cm and the FTIR spectrum obtained from *C. tamala* leaves extract displayed a number of absorption peaks reflecting its complex nature.

Transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDX)

TEM (JEOL JEM 6510 LV) was performed to analyze the shape and size of the nanoparticles. Thin film of the sample was prepared on a carbon coated copper grid. EDX was used to prove the presence of silver nanoparticles in the liquid suspension.

Evaluation of antimicrobial activity of AgNPs

Two-fold broth dilution methods were used to assess the minimum inhibitory concentration (MIC) values of AgNPs. Further, agar well diffusion were also used to evaluate the zone of inhibition of green synthesized AgNPs against various microorganism such as *Candida albicans*, *Candida tropicalis*, *Candida dubliensis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

The pure cultures of microorganisms (bacteria and fungi) were sub-cultured on nutrient broth and sabouraud dextrose broth at 37°C on a rotatory shaker at 150 rpm for 24 hours. By using the sterile cotton swab each culture of microorganism was swabbed on nutrient agar (Bacteria) and sabouraud dextrose agar plate (Fungi), respectively. Wells was made by puncturing the agar gel plates. Each well was filled with different concentration of synthesized AgNPs and incubate 37°C for 24 hours.

Effects of AgNPs on the morphology of Candida cell as examined by SEM

The effects of AgNPs the morphology of candida cells were observed by SEM as described previously with slight modification (Jalal et al., 2016).

Ultrastructural changes in Candida albicans caused by AgNPs as examined by TEM

Interaction of AgNPs with cell wall and membrane of Candida cells and their penetration and localization inside the cells were analyzed by TEM as described previously with slight modification (Jalal et al., 2016).

Results and Discussion

The color of the solution change from light yellow to dark brown after 24 h due to the reduction of Ag^+ to Ag^0 by the reducing agents present in the leaf extract and the change in color of the solution is indicative of formation of AgNPs (Fig 1). The UV-Vis spectroscopic spectrum shows that the surface plasmon resonances of AgNPs were 450 nm (Fig 2).



Figure 1: (a) leaf extract and silver nitrate at 0 h; (b) leaf Extract + AgNPs after 24 h. Change in color is an indicative of formation of AgNPs.

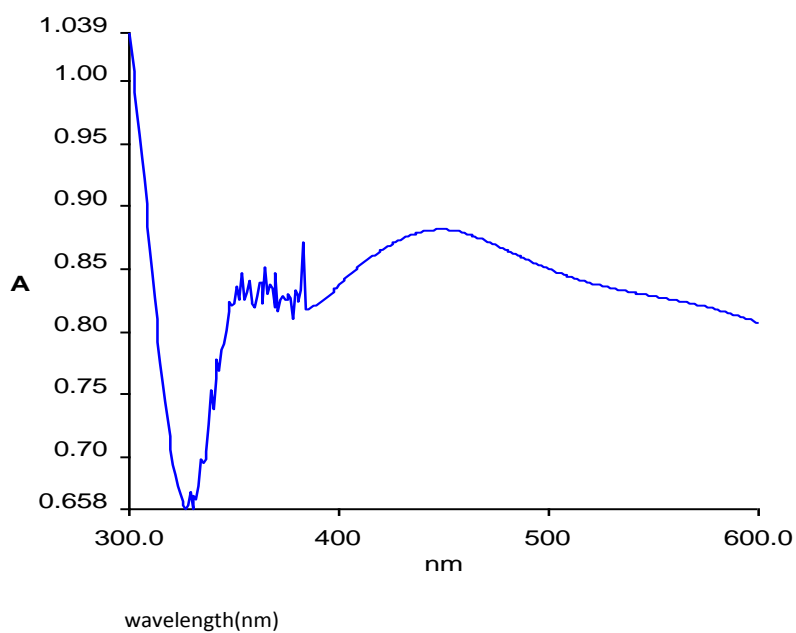


Figure 2. UV-Vis spectra of synthesized nanoparticles

Figure 3 shows the FTIR spectra of aqueous silver nanoparticles prepared from the *C. tamala* leaf extract. FTIR measurements were carried out to identify the potential biomolecules in *C. tamala* leaf responsible for reduction and capping of the bioreduced AgNPs. The absorbance peak at 3425 cm^{-1} indicates polyphenolic O-H stretching (Sathyavathi et al., 2010). Absorption peak at 2075.51 cm^{-1} corresponds to the stretching of $-\text{CH}$ band. The peak located at 1635 cm^{-1} could be assigned to the C=O (carboxyl) stretching in protein or C=N bending in the amide I group (Bankar et al., 2010). A shift in this peak indicated the possible involvement of carboxyl or amino groups of the *C. tamala* leaf extract in nanoparticle synthesis. The peak at 663.90 corresponds to C-H (Alkane) and C=H bonding (Alkene) (Fig 3).

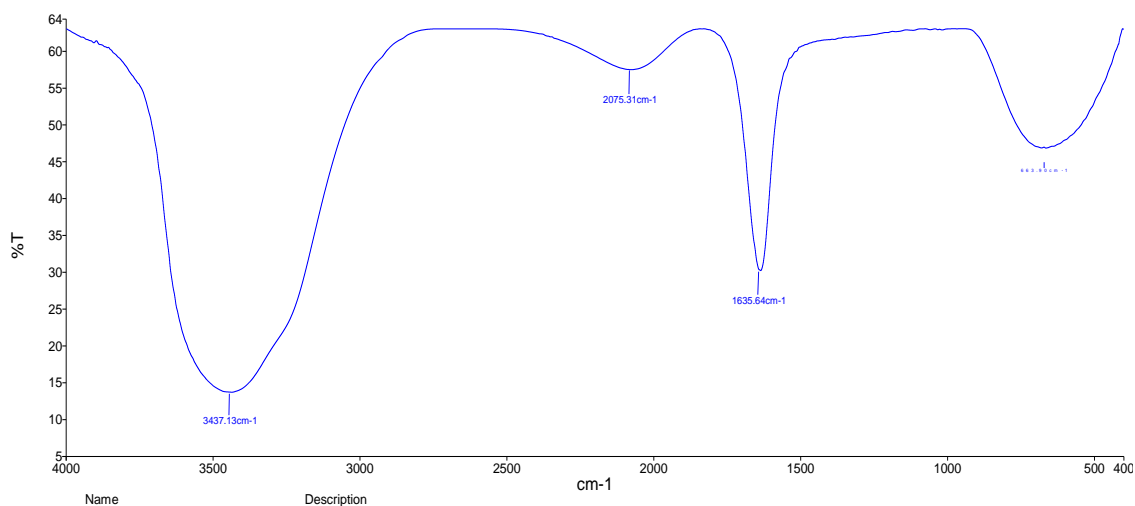


Figure 3. FTIR spectrum of silver nanoparticles synthesized by leaf extracts of *C. tamala*

The size and shape of the synthesized AgNPs by TEM were shown in figure 4. The size of the AgNPs was found in the range of 10-150 nm and nanoparticles were predominantly spherical in shape (Fig. 4). EDX analysis was performed to confirm the composition and distribution of the nanoparticles through spectrum and elemental mapping. The presence of Ag peak in figure 5 confirms the formation of AgNPs in liquid suspension.

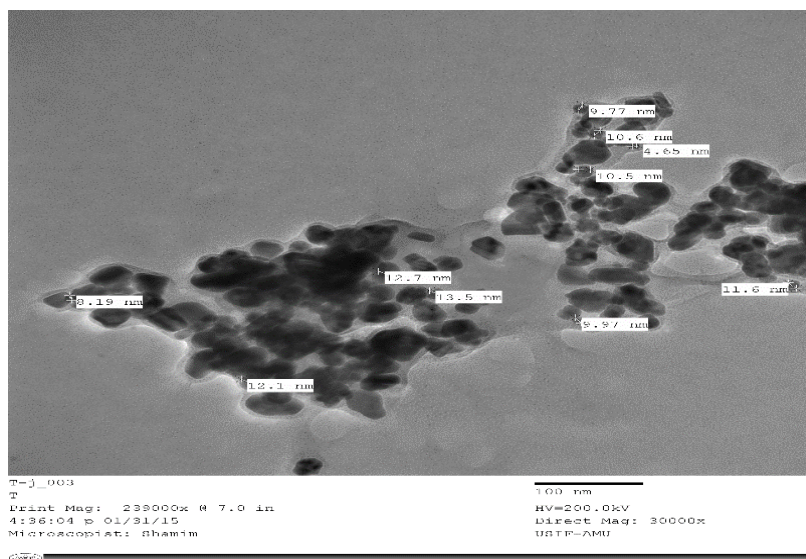
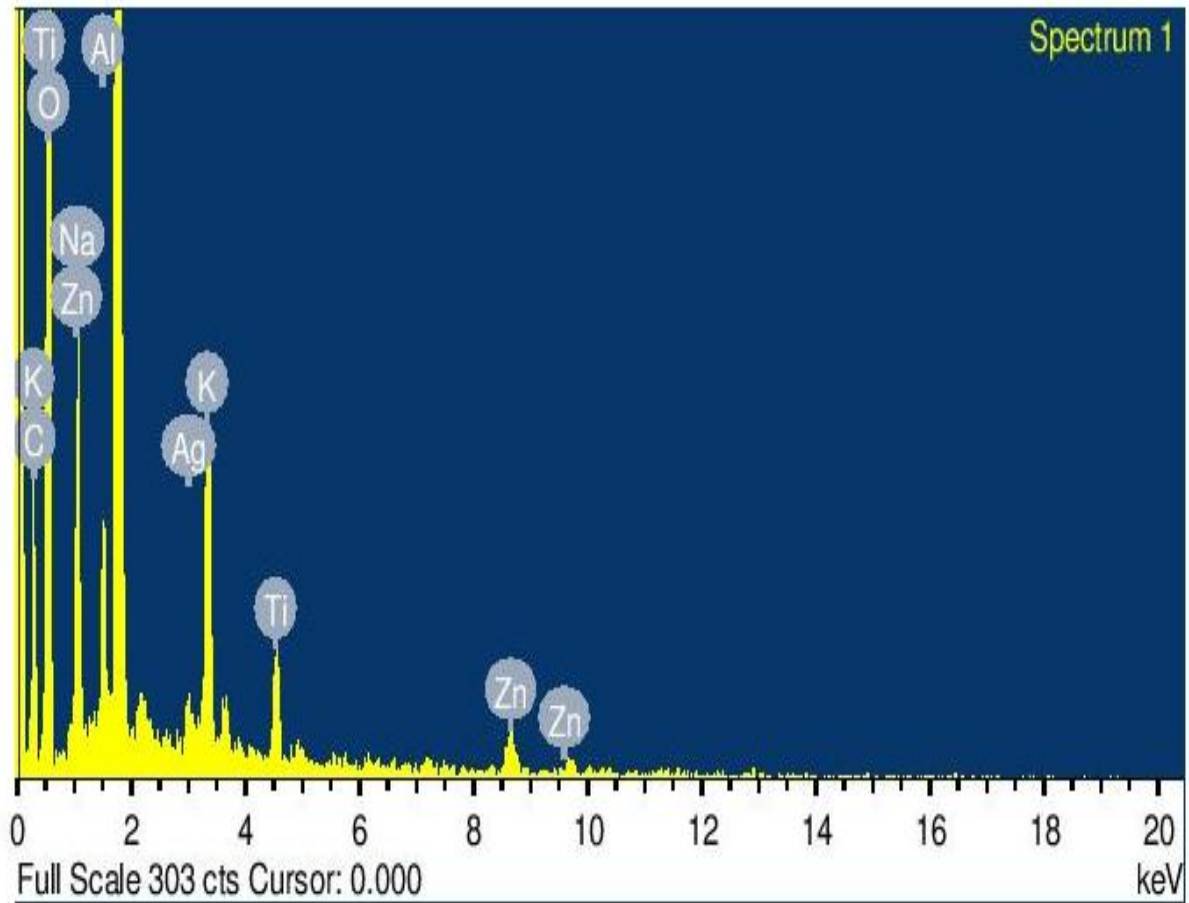


Figure 4. TEM images of green synthesized AgNPs



Element	Weight%	Atomic%
C K	17.78	25.74
O K	56.77	61.69
Na K	6.41	4.84
Al K	4.05	2.61
K K	5.64	2.51
Ti K	3.05	1.11
Zn K	4.72	1.26
Ag L	1.58	0.25
Totals	100.00	

Figure 5. Element analysisi of green synthesized AgNPs by EDX spectra

The antimicrobial properties of AgNPs against various bacterial and fungal strains were characterized by determining the minimum inhibitory concentration (MIC) using two-fold serial dilution method. MIC is defined as the lowest concentration of antimicrobial agents that prevent the visible growth of microorganism (Ansari et al., 2011, Ali et al., 2015).

The MC values for bacterial strains range from 25-50 $\mu\text{g/ml}$; whereas for fungal strain it was 125-250 $\mu\text{g/ml}$. From MIC results it is cleared that the bacterial strains were more susceptible than fungal strains (Table 1).

Table 1. Minimum inhibitory concentration of AgNPs against fungal and bacterial strains

Microorganisms	MIC ($\mu\text{g/ml}$)
<i>Candida albicans</i>	125
<i>Candida tropicalis</i>	125
<i>Candida dubliensis</i>	250
<i>Escherichia coli</i>	25
<i>Staphylococcus aureus</i>	50
<i>Pseudomonas aeruginosa</i>	25

The antifungal activity was further evaluated by measuring the zone of inhibition. Zone of inhibition test has been performed on nutrient agar and SDA plates at different concentration of AgNPs. The presence of a clear inhibition zone around the wells in which AgNPs were added clearly indicates the antimicrobial efficacy of AgNPs (Fig 6 A-C & 7 A-C). The zones of inhibition were found to be larger at highest concentrations of AgNPs, whereas zone of inhibition was smaller at lowest concentrations of AgNPs (Jalal et al., 2016).

From figure 6 & 7; it is clear that as the concentration of AgNPs increased; the size of zone of inhibition also increased. These results are in agreement with previous work carried out on green synthesis of AgNPs by using extract from different parts of the plant and their antimicrobial activity against bacterial and fungal strains (Kaviya et al., 2011; Masurkar et al., 2011; Khalil et al., 2014; Ali et al., 2015). The results have clearly indicated that AgNPs has a potential as an antifungal agent in treating fungal infectious diseases (Selvaraj et al., 2014).

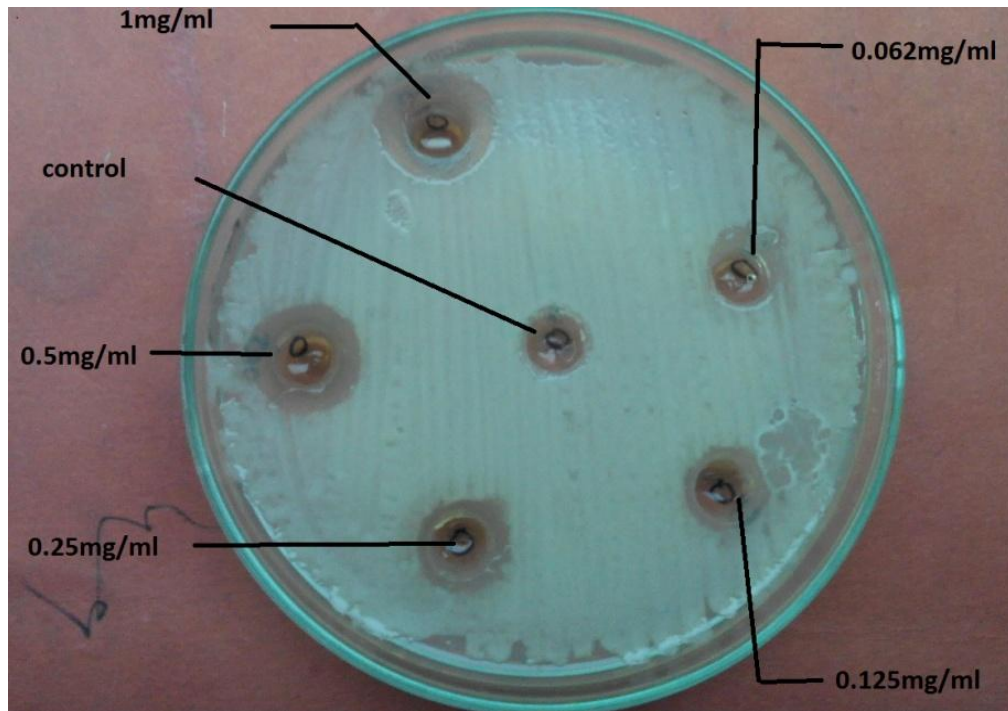


Figure 6(A): SDA plates showing zone of inhibition of AgNPs against *C. albicans*

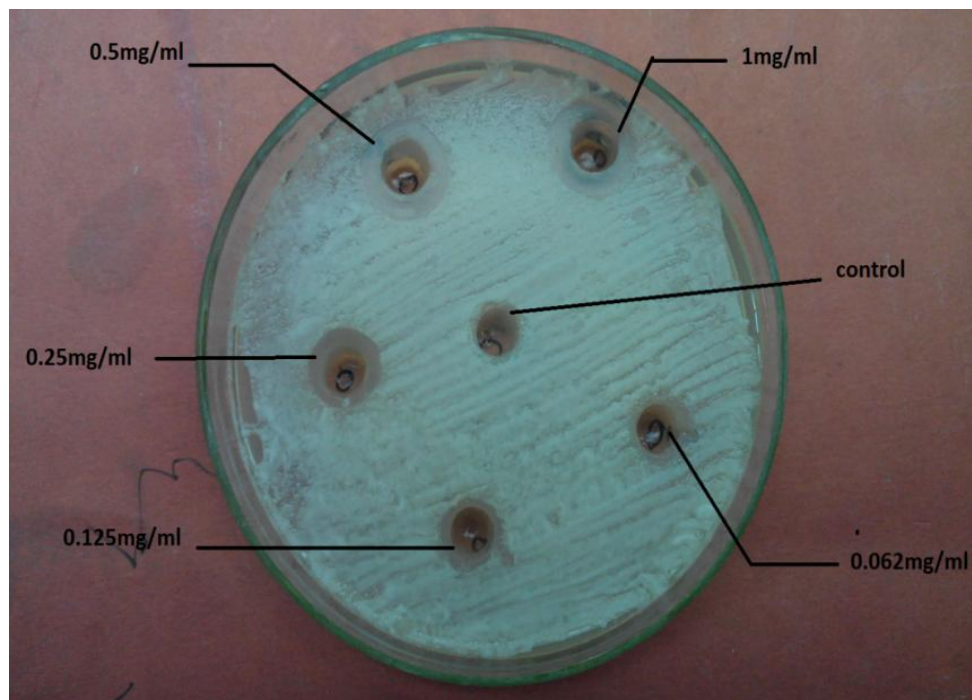


Figure 6(B): SDA plates showing zone of inhibition of AgNPs against *C. tropicalis*

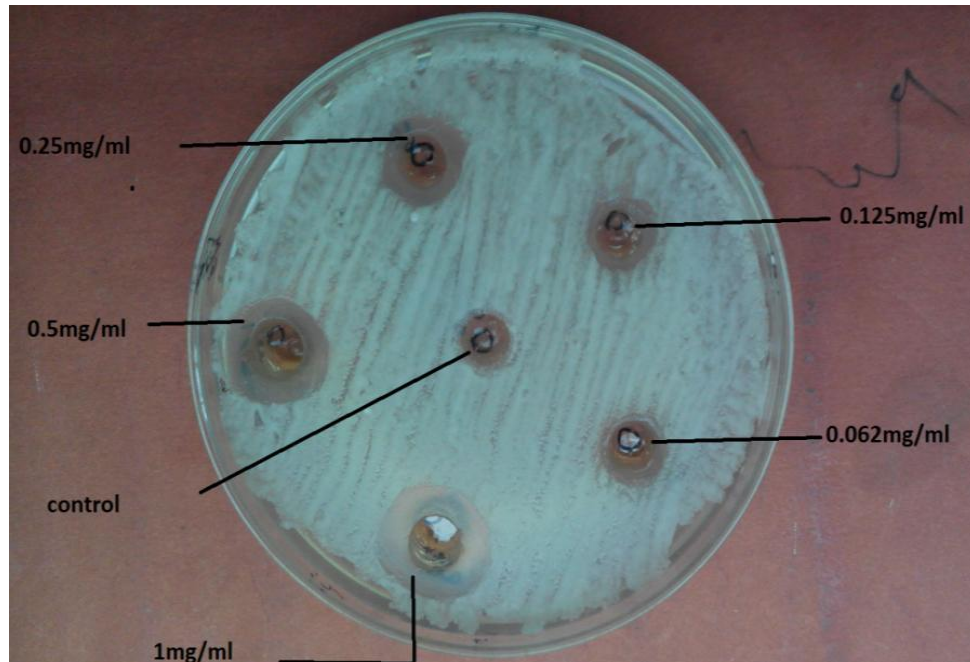


Figure 6(C): SDA plates showing zone of inhibition of AgNPs against *C. dubliniensis*

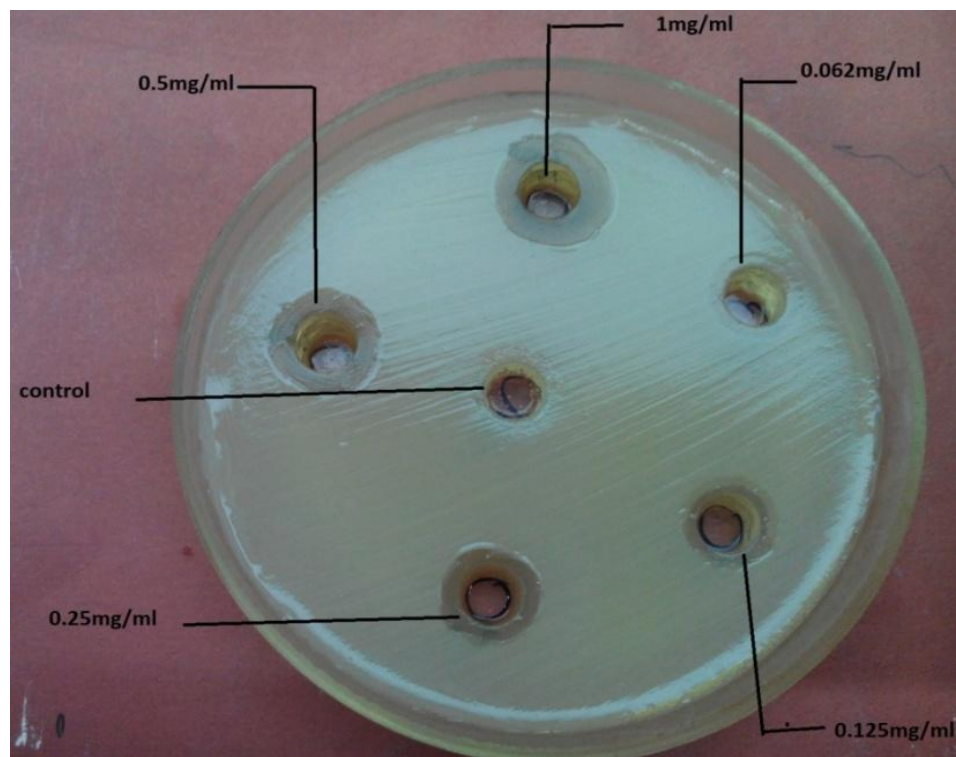


Figure 7(A): Nutrient agar plates showing zone of inhibition of AgNPs against *E. coli*

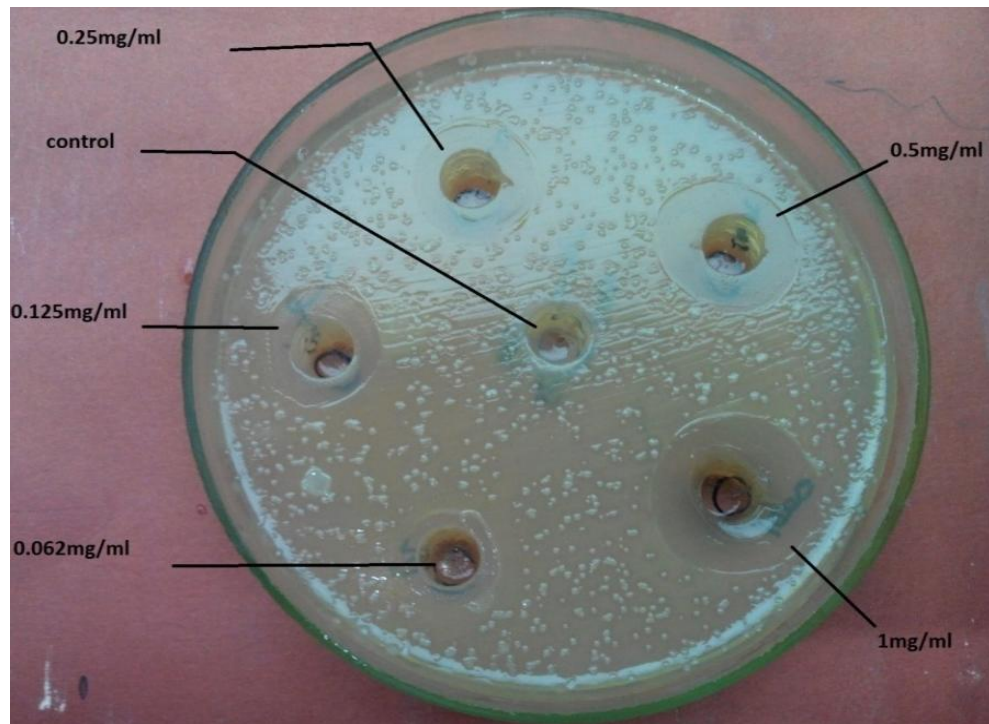


Figure 7(B): Nutrient agar plates showing zone of inhibition of AgNPs against *S. aureus*

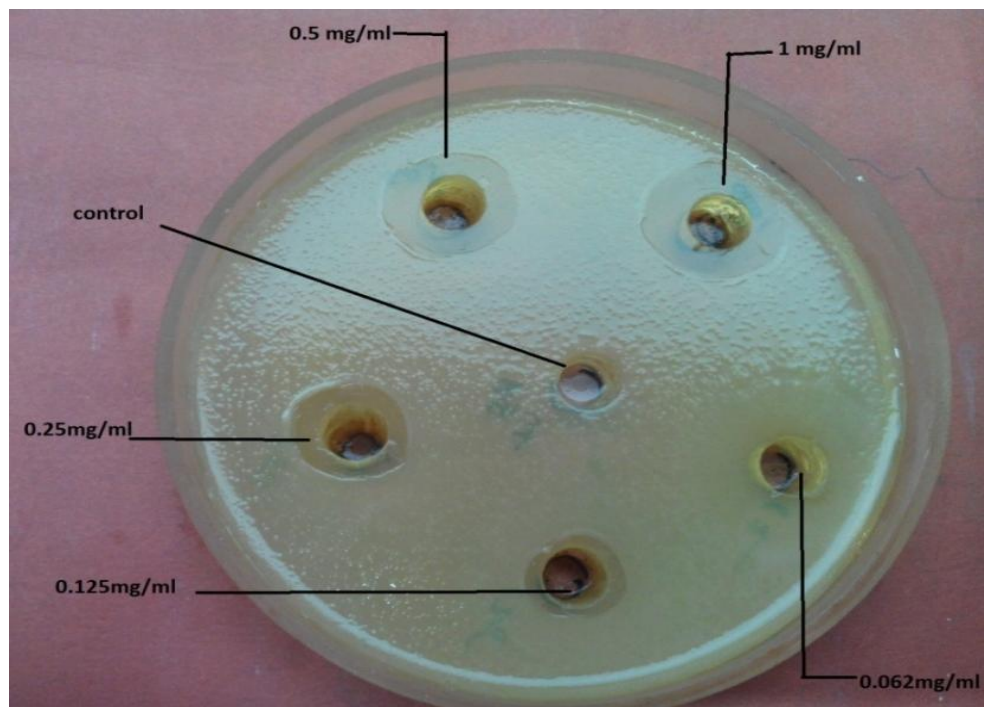


Figure 7(C): Nutrient agar plates showing zone of inhibition of AgNPs against *P. aeruginosa*

The scanning electron microscopy (SEM) was performed to assess the topographical alterations caused by AgNPs in *Candida* cells. The *Candida* cells treated with AgNPs exhibited significant morphological alterations including deformation and shrinkage (Fig 8 A-B). It was observed that AgNPs treated cells were elongated with irregular and rough fragments instead of normal structure (Fig. 8 A-B).

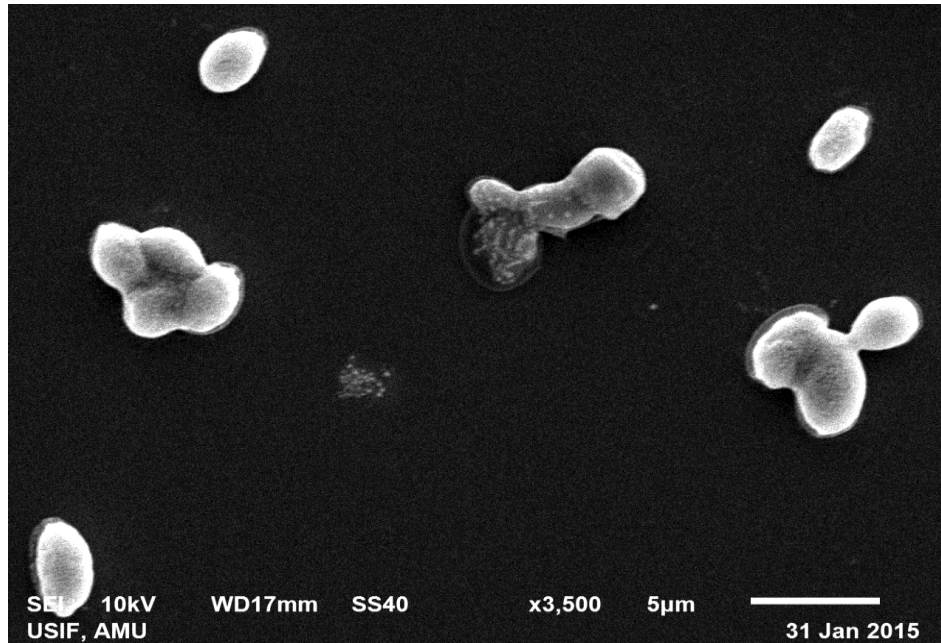


Figure 8 (A). SEM micrograph of *C. albicans* cells treated with AgNPs (100 µg/ml)

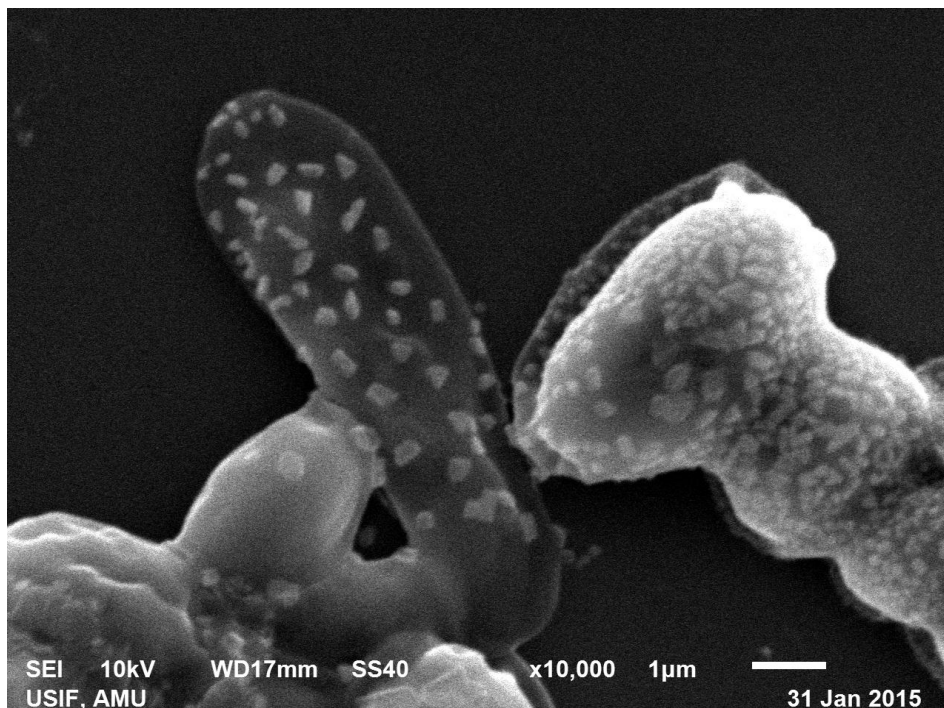


Figure 8 (B). SEM micrograph of *C. albicans* cells treated with AgNPs (200 µg/ml)

The transmission electron microscopy (TEM) analysis shows that a large number of nanoparticles were adhere on the surface of the cell. The cells treated with AgNPs were highly deformed and the cells had shrunken to a greater extent. Alteration in the cell wall and cell membrane was also observed (Fig. 9). It has been reported that when *Candida albicans* were treated with AgNPs; cell membrane and cell wall lost its structural integrity by changing the membrane potential (Selvaraj et al., 2014; Monteiro et al., 2015), creating the pores (Kim et al., 2009) and generating apoptosis (Hwang et al., 2012) and finally caused cell lysis.

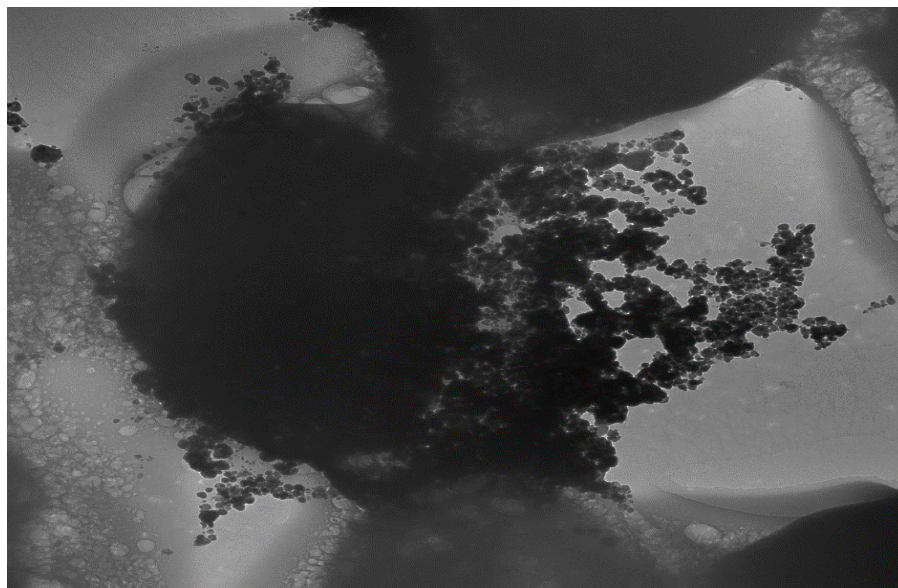


Figure 9. Ultrastructural (TEM) changes in *Candida albicans* caused by AgNPs

Conclusion

Eco-friendly, cost effective and non-hazardous biogenic method were applied for the synthesis of silver nanoparticles by reduction of Ag^+ to Ag^0 using aqueous leaf extracts of *Cinnamomum tamala*. Different techniques such as UV-Vis spectrophotometer, FTIR, SEM, TEM, and EDX were used for characterization of synthesized AgNPs. MIC and zone of inhibition data shows that AgNPs (10-15 nm) possesses excellent antibacterial and antifungal activities against various clinical isolates. Furthermore, SEM micrograph confirmed the interaction of AgNPs onto the cell wall; TEM analysis gives a picture of the loss of cellular integrity, vacuolation, and deformation of the cells. In future, the biogenic AgNPs could be used as an effective and safer topical antifungal and antibacterial agent in clinical applications.

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