Silver Decorated Myconanoparticles Control Growth and Biofilm Formation in Uropathogenic *E. coli*

S. Ranjani¹ · U. Rubiya Kathun¹ · S. Hemalatha¹

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Abstract

Nanotechnology involves the synthesis of nanoparticles that have been used in the therapeutic application for treating diseases. In this present study, we have adopted the synthesis of myconanoparticles from the extracellular extract of endophytic fungi *Penicillium sclerotiorum* (PsNps) and validated its antibacterial potential against antibiotic-resistant uropathogenic *E. coli* and ATCC (25,922) strain of *Escherichia coli*. Endophytic fungi were isolated from the healthy leaves of *Tamarindus indica*. The genomic DNA from endophytic fungi was isolated and the ITS region was amplified by polymerase chain reaction (PCR) using universal fungal primers ITS1 and ITS4 and sequenced for the identification of endophytic fungal isolates. *Penicillium sclerotiorum* extract was used for the synthesis of silver nanoparticles (PsNps) and was characterized by UV–vis spectroscopy, Fourier transform infrared spectroscopy (FTIR), zeta potential, FE-SEM, and Energy dispersive X-ray analysis (EDAX). Antibacterial activity of PsNps was tested against the antibiotic-resistant uropathogenic *E. coli* and ATCC (25,922) strain of *E. coli*. Further experiments were carried out to explore the potential of PsNps in regulating the CTX-M-15 gene. The antimicrobial activity showed that the PsNps inhibited growth, biofilm formation in both the strains of *E. coli*. The expression of the gene encoding CTX-M-15 was downregulated in a resistant strain of uropathogenic *E. coli*. Our results suggest that the PsNps could be used as an alternative source for antibiotics. Thus, further studies can be conducted to prove the in vivo potential of PsNps and can be formulated for commercialization.

Keywords *Tamarindus indica* · Silver nanoparticles · CTX-M-15 gene · Antimicrobial activity

Introduction

Endophytic fungi are the endosymbionts that are present in the healthy tissue of plants. Endophytic organism plays an important role in higher plants by inducing disease resistance. Endophytes are rich in producing a large number of active secondary
metabolites which help their host combat microbial diseases. Previous literature support that isolated endophytic fungi from medicinal plants showed antifungal, antibacterial, and antimicrobial properties against different pathogens [1]. Secondary metabolites have a broad array of applications in various fields, especially in the pharmacy industry. Endophytic microorganisms are a great source of natural and novel bioactive compounds that can be used to meet pharmaceutical, medical, and industrial demands. Under optimum conditions, endophytes deliver a large number of secondary metabolites which are extremely effective against detrimental pathogens such as MDR microbes [2, 3].

Material size ranging in nanometer is known as nanoparticles. Because of their large surface area, nanoparticles are more reactive against bacterial cells and cause cell death by one or several mechanisms. The recent interest in material stabilization is the development of the efficient biological synthesis of nanoparticles for medicinal applications [4]. AgNPs had unique thermal, optical, electrical, chemical, and physical properties due to the high proportion of high-energy surface atoms [5]. Silver is highly toxic toward the microbial cells and thus can be utilized as an antimicrobial agent [6]. In recent times, more attention has been paid to the biosynthesis of AgNPs by microorganisms due to targeted drug delivery and antimicrobial agent [7]. Microorganisms are considered as a potential source for the stabilization and reduction of AgNPs, AuNPs, CdNPs, and ZnNPs. Different microorganisms have been exploited as a reduction and stabilizing agent for the biosynthesis of silver nanoparticles. The biological method of NP synthesis is more efficient than physical and chemical methods due to less toxicity [8].

In this study, endophytic fungus *Penicillium sclerotiorum* was isolated from the leaves of *Tamarindus indica*. Tamarind is a tropical fruit which is traditionally used in food. In Ayurveda, the tamarind leaves, stems, oils, and seed extracts are used to control many health problems such as inflammation, pathophysiological disorders, and immunological disorders. In Unani and Siddha, tamarind is traditionally used in all forms due to its active substances. Tamarind plays a potential role as anti-inflammatory, antidiabetic, anticaner agents, antimicrobial, antivenom, antioxidant, antimalarial, cardioprotective, antiasthmatic, and plays a potential role in the treatment or prevention of obesity and other chronic diseases [9]. Fruits, leaves, and seeds of the tamarind tree are natural sources of antioxidants related to phenolic compounds such as ascatenin, epicatechin, glucose, mucilage, pectin, uronic acid, procyanidin B2, tartaric acid, arabinose, xylose, galactose, and triterpene [10].

This current study concentrated on the synthesis of myconanoparticles using endophytic fungal extract of *Penicillium sclerotiorum* (PsNps). The synthesized PsNps were biophysically confirmed by using various characterization methods. The antimicrobial activity of PsNps was studied by performing MIC, MBC, and biofilm assays. Upon treatment of bacterial strain with PsNps, the antibiotic resistance gene CTX-M-15 gene expression was analyzed by using polymerase chain reaction. CTX-M-15 is an ESBL (extended-spectrum beta-lactamase) enzyme produced by Gram-negative bacteria that can break penicillin and make them ineffective. ESBL group of enzymes causes various infections such as urinary tract infections and other life-threatening diseases. CTX-M-15 was first discovered in India that is mostly active against cefotaxime and then identified in Turkey, France, Romania, and the UK [11]. CTX-M-15 is produced by multidrug-resistant UTI pathogens of *E. coli* and *P. aeruginosa* from hospitals in Nigeria and is a type of ESBLs enzyme produced by MDR pathogens by a continuous mutation which led to resistance towards antibiotics [11].
Materials and Methods

The *E. coli* strains such as ATCC [12] and clinical isolate of biofilm forming, uropathogenic, and multidrug-resistant (MDR) strains of *E. coli* were isolated from the Tagore Medical College and Hospital, Chennai, after proper ethical approval from BSACIST (Ref. no. BSAU: REG-OFF: 2016/02SLS).

Isolation and Identification of Endophytic Fungi from *Tamarindus indica*

Healthy leaves of *Tamarindus indica* were collected from B.S. Abdur Rahman Crescent Institute of Science and Technology, Vandalur, Chennai. The leaves were washed several times under running tap water to avoid contamination. Leaves were cut into small pieces (1 mm) and surface sterilized with distilled water and followed by sequential rinsing in 70% ethanol for 1 min, 1% sodium hypochlorite for 1 min, 90% ethanol for 5 min, and finally rinsed with sterile distilled water for 2–3 times and then allowed to dry under sterile conditions in laminar airflow. The cut surfaces of the segments were placed in petri dishes containing PDA supplemented with Ampicillin (1 mg/lit) in a laminar airflow chamber and incubated at 28 °C for 4–7 days. Plates were periodically observed for the growth of endophytic fungi. After 7 days, endophytic fungi were growing out from the explants were subcultured in new PDA plates to obtain pure culture [13–16].

Identification of the Endophytic Fungi Isolated from *Tamarindus indica*

Entirely, 1 g of fresh fungal mycelium was carefully cleaned with sterile water and ground into a fine paste in a mortar and pestle. The fungal DNA was isolated using the CTAB method [17]. Further identification of endophytic fungi was carried out through the amplification of fungal DNA using universal ITS primers ITS1 (5′TCC GTA GGT GAA CCTG G 3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′). The polymerase chain reaction was performed as prescribed previously [18]. The amplified PCR product was used for sequencing and further utilized for the construction of the phylogenetic tree using MEGA X based on maximum likelihood algorithm, and the endophytic fungal sequence was submitted to the NCBI GenBank [13–16].

Biosynthesis of Myconanoparticles Loaded with Metabolites of Endophytic Fungus *Penicillium sclerotiorum*

Extracellular extract of endophytic fungi *Penicillium sclerotiorum*, isolated from the leaves of *Tamarindus indica*, was used for biosynthesis of myconanoparticles (PsNps) [19]. An equal volume of endophytic fungal extract and 1 mM AgNO₃ solution was mixed with gentle swirling and incubated in the dark for 48 h at room temperature. The color of the solution changed from colorless to brown confirmed the synthesis of myconanoparticles [13–17, 19].
Physio-chemical Characterization of Green Silver Nanoparticles

The synthesis of silver-mediated myconanoparticles was confirmed by measuring the absorbance using UV–visible spectroscopy (300–700 nm), with a double beam spectrophotometer (Jasco V-730 spectrophotometer). FTIR spectrum was measured between 400–4000 cm\(^{-1}\) to identify the potential chemical groups present in PsNps. This helps us to identify the possible biomolecules, which could play the role in reduction, stabilization, and capping during the synthesis of myconanoparticles (PerkinElmer Spectrum 100 FTIR) [20–23]. Colloidal nanoparticle solution was used to identify the hydrodynamic size (Z-average), polydispersity index (PDI), and surface charge (zeta potential) of the synthesized nanoparticles using particle size analyzer combined with zeta analyzer (Malvern Instruments Ltd, UK). Particle size analysis was performed at the scattering angle of 90°, medium viscosity 0.895 mPa·s, count rate of 210 kCPS, at 25 °C. FESEM combined with EDAX was used to study the morphology and elemental composition of nanoparticles (SIGMA HV–Carl Zeiss with Bruker Quantax 200–Z10 EDS Detector) [12, 24–26].

Antibacterial Assay of Myconanoparticles

The efficacy of silver nanoparticles was evaluated for their antibacterial and antibiofilm activity by assessing minimum inhibition concentration, minimum bactericidal concentration, growth, and antibiofilm effect. Further, the study was extended to validate the expression of antibiotic-resistant gene CTX-M-15 upon treatment with synthesized nanoparticles. The minimum inhibitory concentration was performed in 96-well microtiter plates (Ranjani et al. 2020a-k). The growth of the tested organism was followed by treating with synthesized silver nanoparticles and ampicillin treatment and compared with control up to 24 h of incubation [27–31].

The gene expression of CTX-M-15 was analyzed by amplifying the treated and control DNA from strains with CTX-M-15 primers [30].

Results

Isolation and Identification of Endophytic Fungi Isolated from Tamarindus indica

The leaves of Tamarindus indica were used as explant, and endophytic fungi were isolated as described in the methods. After sequencing of the selected strain, the phylogenic tree was constructed using a maximum likelihood algorithm by aligning closely related sequences and as per sequence similarity and clustering of the phylogenetic tree, the sequence was identified as Penicillium sclerotiorum and deposited in the NCBI Genbank with accession number MK942602. Penicillium is one of the widely distributed fungi in the environment (Fig. 1a).

Penicillium sclerotiorum–Mediated Synthesis of Silver Nanoparticles (PsNps) and Biophysical Characterization

Pure cell-free extract of Penicillium sclerotiorum was used for the synthesis of silver nanoparticles, and the color change was noted from pale white to brown color which indicates
the synthesis of *Penicillium sclerotiorum* mediated synthesis of PsNps (Inlet Fig. 1b, c, d). The UV–visible absorption spectrum was taken between 200–800 nm, which showed the SPR peak around 400 nm which confirmed the synthesis of *Penicillium sclerotiorum* mediated synthesis of silver nanoparticles (PsNps) (Fig. 1e).

FTIR spectrum of PsNps was taken to observe the possible biomolecules involved in the reduction and capping to achieve the synthesis of stable PsNps. The peak wavelength (cm⁻¹) of 3146, 2902, 2107, 1992, 1887, 1610, 1311, 1078, 985, and 627 correspond to functional groups of carboxylic acid (O–H stretching), N–H stretching amine

**Table 1** The major peaks and the functional group present in PsNps synthesized using *Penicillium sclerotiorum*, endophytic fungus isolated from *Tamarindus indica*

| S. No | Peak wavelength | Functional group                          |
|-------|-----------------|------------------------------------------|
| 1     | 3146            | Carboxylic acid (O–H stretching)         |
| 2     | 2902            | Alcohol (O–H stretching) N–H stretching amine salt |
| 3     | 2107            | Isothiocyanate (N=C=S stretching)         |
| 4     | 1992            | Aromatic compound (C–H bending)          |
| 5     | 1887            | Aromatic compound                        |
| 6     | 1610            | α, β-unsaturated ketone (C=C stretching)  |
| 7     | 1311            | Sulfone (S=O stretching)                  |
| 8     | 1078            | Primary alcohol (C-O stretching)          |
| 9     | 985             | Alkene (C=C bending)                      |
| 10    | 627             | Halo compound (C–Br stretching)          |
salt and alcohol (O–H stretching), isothiocyanate N=C=S stretching, aromatic compound (C–H bending), aromatic compound, β-unsaturated ketone (C=C stretching), sulfone (S=O stretching), primary alcohol (C–O stretching), alkene (C=C bending), and halo compound (C–Br stretching), respectively (Fig. 1f) (Table 1).

The DLS technique was utilized to identify the size of nanoparticles based on Brownian movement, and it was observed as 331.2 nm and the negative charge-20.6 mV confirms the stability of the nanoparticles synthesized using Penicillium sclerotiorum extract (Fig. 2a, b).
From the field emission microscopic image at 25.00 KX magnification at 1-µm scale, 50.00 KX magnification at 200 nm scale, and 100.00 KX magnification at 100 nm scale, the nanoparticles were observed as different shapes and different within nanometer in range (Fig. 3a, b, c). The elemental composition reveals the presence of Ag by showing a peak at 3 keV. Apart from silver, other elements such as C, O, Mg, Si, S, Ca, Al, and Cl were present in the nanoparticles (Fig. 3d). The percentage weight of each element was found as 35.37%, 29.49%, 18.32%, 6.95%, 6.91%, 1.58%, 0.82%, and 0.56% for Ag, O, C, Ca, Cl, Si, S, and Al, respectively. The percentage of atom was found as 7.87%, 44.23%, 36.61%, 4.16%, 4.68%, 1.35%, 0.61%, and 0.5% for Ag, O, C, Ca, Cl, Si, S, and Al, respectively (Fig. 3e). These elements may derive from the secondary metabolites of fungal extract, which act synergistically in imparting efficient antibacterial activity.

**Antibacterial Potential of PsNps**

Many pathogens are developing resistance toward routine antibiotics. Hence, there is a pressing need of the hour to find an alternative solution by synthesizing nanoparticles using *Penicillium sclerotiorum* extract. *Penicillium* is a very important genus used for the mass production of valuable products such as penicillin. *Penicillium sclerotiorum* was reported to produce antimicrobial secondary metabolites (Petit et al. 2009) which were utilized in this study to synthesize silver nanoparticles and validated against *E. coli* ATCC (25,922) and urinary tract infection causing antibiotic-resistant *E. coli*. The minimum inhibitory concentration is the minimum concentration of PsNps which inhibit the growth of the test organism. The visual turbidity of *E. coli* ATCC (25,922) and UTI-causing clinical pathogen was observed and the well with invisible growth was considered as MIC. The MIC of *E. coli* ATCC (25,922) and UTI causing MDR pathogen was calculated as 0.75 µg/
ml and 6.25 µg/ml, respectively. The decrease in the growth rate of the bacterium at their MIC concentration was represented graphically in (Fig. 4a). The minimum bactericidal concentration is the lowest concentration of PsNps which completely kills the test organism. The MBC of *E. coli ATCC* (25,922) and UTI causing MDR pathogen was calculated as 6.25 µg/ml and 12.5 µg/ml, respectively (Table 2). This study focused on an assay to validate the antibiofilm effect of PsNps on biofilm forming UTI causing *E. coli*. From our experiments, it was observed that upon treatment with 12.5 µg/ml of PsNps, the biofilm formation was reduced by 87% and 85% for *E. coli* ATCC (25,922) and UTI causing MDR *E. coli* pathogen (Fig. 4b). In addition, this research work focused on studying the amplification of the CTX-M-15 gene upon treatment with PsNps and ampicillin. From the results, it was observed that the expression of the CTX-M-15 gene was found in control and ampicillin-treated strain; however, the expression of CTX-M-15 gene in PsNp treatment was suppressed (Fig. 4c).

**Discussion**

Endophytic fungi produce a plethora of secondary metabolites, which have potent antibacterial activity. Among that, *Penicillium* species are reported to produce pharmaceutically important secondary metabolites. They are reported to have antibacterial, antifungal, lowering cholesterol levels, and immunosuppressant properties. *Penicillium* species produce important secondary metabolites such as compactins, mycophenolic acid, kojic acid, viridicatol, quinolines, diketopiperazines, alkaloids, quinazolines, etc. [32]. *Penicillium sclerotiorum* was reported to produce azophilones sclerotiorin, pencolide, isochromophline, and pipergalone. Pipergalone is reported to have potent antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. Sclerotiorin was reported to block the RAS signaling pathway, inhibits lipase, hence patent utilized for the production of acne creams and antiobesity biscuits, aldose reductase enzymes, which plays an important role in diabetes complications such as neuropathy, nephropathy. Sclerotiorin has antibacterial activities against *Escherichia coli*, *Lysteria monocytogenes*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Salmonella typhimurium* [33]. Thus, the effect of these pharmacologically important, bioactive secondary metabolites can be harnessed for the synthesis of silver nanoparticles to offer a potent antibacterial activity against UTI causing *E. coli* [34].

The UV–visible absorption spectrum showed the SPR peak around 400 nm. The SPR depicts the size, shape, and agglomeration of PsNps. When the light wave is passed into the colloidal solution, collective oscillation of electrons present in silver nanoparticles triggers

| Table 2 Minimum inhibitory concentration and minimum bactericidal concentration of PsNps against *E. coli ATCC* (25,922) and uropathogenic MDR *E. coli* |
|---------------------------------|---------------------------------|---------------------------------|
| Strains                         | Concentration of PsNps synthesized using *Penicillium sclerotiorum* | Minimum inhibitory concentration (µg/ml) | Minimum bactericidal concentration (µg/ml) |
| *E. coli ATCC* (25,922)          | 0.75                            | 6.25                            |
| Uropathogenic, MDR *E. coli*    | 6.25                            | 12.5                            |
the free electrons to form a SPR absorption spectrum. There was literature supporting the fact that 320–580 nm is characteristic \( \lambda_{\text{max}} \) for silver nanoparticles, where the absorption spectrum of nanoparticles mainly depends on particle size, environment, and dielectric constant [35]. In addition, phytoreduction and phytocapping of silver nanoparticles were also achieved by secondary metabolites such as flavonoids, terpenoids, saponins, alkaloids, and other compounds such as proteins; enzymes which were present in the *Penicillium sclerotiorum* extract [36]. It was observed that broad spectral bands at 3146 cm\(^{-1}\) are characteristic to the O–H stretching of hydroxyl group of a polyphenols and N–H stretching vibrations in primary and secondary amines of amino acids, peptides, and proteins. There were previous reports supporting that protein molecules are involved in nanoparticle synthesis by interacting with their amide bond. Thus, protein-capped silver nanoparticles will help to maintain the stability of particles without agglomeration [37].

Based on DLS, the size of PsNps was 331.2 nm, which was measured due to the Brownian movement of PsNps in colloidal solution and the zeta value was –20.6 mV, which confirms the stability of the nanoparticles synthesized using *Penicillium sclerotiorum* extract. FESEM image of PsNps helps us to observe the morphology, size, and shape of the PsNps. All the physiochemical and other instrumental analysis gives the information on morphology, size, and shape of nanoparticles, which supports in providing effective antibacterial activity through its mode of action via several mechanisms.

The MIC of *E. coli* ATCC (25,922) and UTI causing MDR pathogen was calculated as 0.75 µg/ml and 6.25 µg/ml, respectively. The minimum bactericidal concentration is the lowest concentration of PsNps which completely kills the test organism. The MBC of *E. coli* ATCC (25,922) and UTI causing MDR pathogen was calculated as 6.25 µg/ml and 12.5 µg/ml, respectively (Table 2). The growth of *E. coli* ATCC (25,922) and UTI causing MDR pathogen was observed up to 24 h of time period on treatment with 12.5 µg/ml of PsNps, and it was observed that the growth rate was decreased by 90% and 82%, respectively, when compared with the control, whereas the ampicillin treatment could not control the growth of UTI causing MDR pathogen (Fig. 4a). These results confirmed that PsNps have potent bacteriostatic and bactericidal activity against the tested organism. Silver nanoparticles showed antibacterial activity in liquid medium because of the good dispersion ability of silver ions without any aggregation. Due to the larger surface area of silver nanoparticles, a greater number of bacterial cells will interact with silver ions which ultimately become toxic to bacterial cells and cause cell death. There were several reports on the mechanism of silver nanoparticles and their action on the bacterial cell. First, the silver ions of silver nanoparticles inactivate the bacterial cell by damaging the cell membrane, protein inactivation, lipid peroxidation, creates pits on the cell membrane, followed by the disintegration of membrane integrity, disturb the transport chain, and causes leakage of the cell membrane [38]. It is reported that silver ions have a higher affinity toward sulfur and phosphorous. When the silver ion comes in contact with the cell membrane it interacts with sulfur containing protein of the cell membrane and when it enters inside the cell silver ions interact with sulfur containing protein and phosphorous containing DNA [39]. This interaction induces drastic changes in cellular metabolism, cellular respiration, and a decrease in the production of ATP, which ultimately leads to cell death [37]. Thus, silver ions along with phytochemicals enhance the bactericidal activity of silver nanoparticles by coordinating with several biochemical and molecular mechanisms.

Biofilm is the major causative agent for several deadly diseases. UTI causing uropathogenic *E. coli* accounts for 80% of UTI infections in humans. Biofilm formation causes resistance to antimicrobial agents and makes it very difficult to penetrate inside the biofilm. From our experiments, it was observed that upon treatment with 12.5 µg/ml of PsNps, the
biofilm formation was reduced by 87% and 85% for *E. coli* ATCC (25,922) and UTI causing MDR *E. coli* pathogen (Fig. 4b). This shows that PsNPs have potent antibiofilm activity against biofilm causing pathogens. Silver nanoparticles interact physically and chemically with the biofilm and effectively evade and stop the synthesis of extra polysaccharides, which is responsible for the formation of biofilm [40]. There are reports supporting the fact that silver nanoparticles inhibit the transcription of biofilm-associated genes. There were previous reports showed that the exposure of silver nanoparticles results in intemperance of proton motive force [41]. The biocidal silver ions induce DNA assortment as a defense mechanism to protect the bacterial cell from a toxic environment but simultaneously nullify its replication ability, subsequently reducing the bacterial population, which in turn reduces the biofilm formation. The ROS production inside the cell also increases the toxicity inside the cell by inhibiting the enzymatic action, which paves the way for cell death [42, 43].

CTX-M-15 is the antibiotic resistant gene which produces enzymes which cleave the beta-lactam ring of antibiotics before its action. CTX-M-15 gene is predominant in South India, which was found to be present in most of the antibiotic resistant organisms isolated from clinical samples. From the amplification study it was found that the presence of the CTX-M-15 gene was suppressed (Fig. 4c). This shows that PsNp directly targets the DNA thereby reduces the ability of transcription and translation of the cell. Ultimately replication of the cell would have stopped without multiplication of bacterial cells, thereby decreasing the whole population in nanoparticles environment [31, 39].

**Conclusion**

In this research work, the systemic methodology was adopted to synthesize myconanoparticles using *Penicillium sclerotiorum*, to explicate the superior antibacterial and antibiofilm effects against biofilm forming, multidrug-resistant UTI causing *E. coli*. PsNps showed excellent bacteriostatic, bactericidal, and antibiofilm activity against UTI causing *E. coli*. Further validation on commercial production of PsNps can be formulated as nanogels and vaginal wash, which could be used to prevent the urinary tract infection caused by uropathogenic organisms.

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**Author Contribution** SH conceived and designed the research. SR and URK conducted the experiments. All authors wrote the manuscript. All authors read and approved the manuscript.

**Data availability** Data will be available on request.

**Code Availability** Not applicable.

**Declarations**

**Ethics Approval** Not applicable.

**Consent to Participate** Not applicable.
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