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Research Article

Eco-Friendly Synthesis of Multishaped Crystalline Silver Nanoparticles Using Hill Garlic Extract and Their Potential Application as an Antifungal Agent

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Antimicrobial resistance is a global health challenge, and the large loads of biocides dumped into the environment augment the spread of antifungal resistance and environmental contamination. Therefore, eco-friendly antifungal agents must be developed to combat antibiotic resistance and to reduce environmental contamination. In the present investigation, *Allium sativum* (Hill garlic-Malai Poondu) extract was used as a green resource to achieve silver nanoparticle (AgNP) production. AgNPs were characterized by various spectral and microscopic analyses. In vitro free radical scavenging assays were instigated to determine the antioxidant capacity of the AgNPs, and the antifungal activity was assessed using Agar well-diffusion assay again pathogenic fungal strains. The mean particle size of the AgNPs was calculated as 35.1 nm with face-centered cubic (FCC) structure. AgNPs exhibited free radical scavenging activity against 2,2-diphenyl-1-picrylhydrzyl (DPPH), 2,2-azino-bis (3-etilbenzotiazolin)-6-sulfonic acid (ABTS), and hydrogen peroxide (H_2O_2) radicals. Scavenging of DPPH radicals by AgNPs was impressive, and an IC_{50} value of $6.3 \pm 0.4\,\mu g/ml$ was obtained in this assay. Among the tested *Candida* strains, the order of the least susceptibility on exposure to AgNPs synthesized using Hill garlic extract was as follows: *C.glabrata* ≤ *C.tropicalis* ≤ *C.parapsilosis* ≤ *C.krusei* ≤ *C.albicans*. The study highlights the synthesis of environment-friendly nanoparticles using Hill garlic extract with enhanced antifungal properties.

1. Introduction

The discovery of antibiotics has been a milestone in contemporary medicine. However, the extensive consumption of antibiotics has provoked the emergence of antimicrobial resistance (AMR) which in turn has become a global health challenge [1]. Antibiotic pollution has a crucial impact on the environment apart from being a health care crisis. The overuse and misuse of antibiotics have increased the load of biocides that are being discarded into the surroundings as environmental contaminants. At a daily basis, antimicrobial agents are being disseminated into land and water

resources by pharmaceutical industries, hospitals, poultry, and livestock [2, 3]. The presence of antibiotic residues in the environment elevates the pressure on the bacteria to develop AMR through horizontal gene transfer of antibiotic resistance genes [4]. Antibiotic pollution also has a negative effect on developmental and behavioural characteristics of human and animal population. Similarly, aquatic animals are exposed to a higher dose of antibiotic residues that leads to bioaccumulation and chronic toxicity. Malformations including defects in head and body ratio and pericardial edema were identified in *Xenopus tropicalis* on tetracycline exposure [5]. The removal of pathogens from biomedical wastes has also become a meticulous process since biomedical wastes do not degrade easily in the environment. This can have a significant impact on wildlife and human beings and enhance antimicrobial resistance [6]. However, eco-friendly antimicrobial agents can be developed by the amalgamation of eco-friendly antifungal agents and modern technologies to combat multidrug-resistant microbes.

The current advancements in nanotechnology proffer new dimensions to develop unique nanoparticles (NPs) with enhanced antimicrobial properties. NPs not only act as antimicrobial agents but can also act as efficient carriers of herbal formulations and drugs [7]. Different scopes of nanomaterials and NPs have been explored to reveal their antimicrobial property; however, metallic nanoparticles such as silver and gold NPs have gained wide-spread attention due to their distinctive properties [8]. NPs are considered favourable agents in combating microorganisms due to their stability, solubility, feasibility, biocompatibility, and targeted drug release. The chief advantage of NPs over other conventional antibiotics is their small size and large surface area proportion [9]. AgNPs are considered the most effective antimicrobial agents in spite of the reasonable bactericidal effects exhibited by other NPs such as iron oxide, copper oxide, gold, and titanium oxide NPs [10–12]. The mechanism of action of NPs against microorganisms varies from that of the standard antibiotics. NPs interact directly with the cell of the microbes and inhibit the biofilm formation. They can also trigger the innate and adaptive immunity of the hosts. They can kill the microbes by initiating the generation of reactive oxygen species and effectively interact with the DNA and proteins of the pathogen [13]. Nevertheless, NPs can be promising candidates to target multidrug-resistant microbes.

Green resource employed synthesis of nanoparticles has several benefits that are not offered by other routes NP synthesis. It is highly feasible, robust, and biocompatible and can be used to produce stable and nontoxic NPs [8]. Diverse plant parts have been engaged in the production of green NPs and evaluated for their antimicrobial properties. For example, NPs synthesized from whole plant extract of *Leonurus japonicus* showed antimicrobial potential against *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Escherichia coli* [14]. AgNPs synthesized using below the ground portion of the plant *Polygala tenuifolia* possessed antibacterial activity [15], whereas *Artemisia capillaris*-mediated AgNPs inhibited the growth of gram positive (+) and gram negative (-) bacteria [16]. Results of Jalal et al. [17] revealed that AgNPs synthesized using *Syzygium cumini* seed extract conferred antifungal potential against *C. albicans* and four other *Candida* species by suppressing the biofilm and germ tube formation. NPs synthesized using *Allium sativum* extract possessed biocidal property against *P. aeruginosa* and antifungal activity against *C. albicans* [18]. This showed that plant extract-mediated NPs synthesized in an eco-friendly approach can combat microbes efficiently. Further, the use of garlic extracts in NP synthesis and evaluation of their antifungal property against multiple *Candida* strains is limited. Therefore, the current study was carried out to green synthesize AgNPs using Kodaikanal Hill garlic (Malai Poondu) extract and evaluate their antifungal property against pathogenic *Candida* strains.

2. Experimental Details

2.1. Preparation of Plant Extract. Hill garlic was purchased from a Hill garlic cultivating farm at high altitudes of Kodaikanal Hills (Poombarai village). Garlic sample was processed by removing the skin and cleaned using distilled water. The garlic pods were chopped into small pieces and crushed using a mortar and pestle. Double distilled water was added in measured amounts to obtain the extract with 1 mg/1 ml concentration.

2.2. Biosynthesis of AgNPs. 0.025 l of Hill garlic extract was added to 0.495 l of the substrate ($AgNO_3$) prepared at a concentration of 1 mM. Later, the solution was centrifuged at 12,000 rpm and the resultant pellet was thoroughly washed using deionized water to remove unreacted substances. The pellets were air-dried and used for experiments [19].

2.3. Characterization of AgNPs. Reduction of silver ions was monitored at regular intervals with by an Ultra-Violet visible (UV-Vis) spectrophotometer (Shimadzu). To identify the functional groups, FTIR analysis was executed using a Perkin Elmer spectrum 100 N Fourier Transform Infrared (FTIR) (4000 to 500 cm^{-1}) spectrometer. XRD (X-ray diffractometer) analysis was performed to identify the particle nature of the AgNPs (X' Pert Pro PXRD). Identification of charge and assessment of stability was determined using a Malvern instruments Ltd. Zeta potential analyzer, and the AgNP shapes were recorded using a Joel/Jem 2100 High Resolution-Transmission Electron Microscopy instrument.

2.4. In Vitro Free Radical Scavenging Activity of AgNPs. DPPH free radical inhibiting assay was done by adding 0.5 ml of 0.1 mM DPPH dissolved in methanol to various concentrations of AgNPs. The solution was mixed well and kept in the dark for 30 min at room temperature. Measurements were recorded at 517 nm and DPPH in methanol served as blank. For ABTS radical scavenging assay, ABTS was prepared and stored for 16 hours in the dark. To 1 ml of AgNPs, preprepared ABTS was added, and the values were observed at 734 nm. In order to evaluate the H_2O_2 radical scavenging activity, 43 mM H_2O_2 was dissolved using phosphate buffer and 4 ml of this solution was mixed with calculated amounts of AgNPs. The optical density values

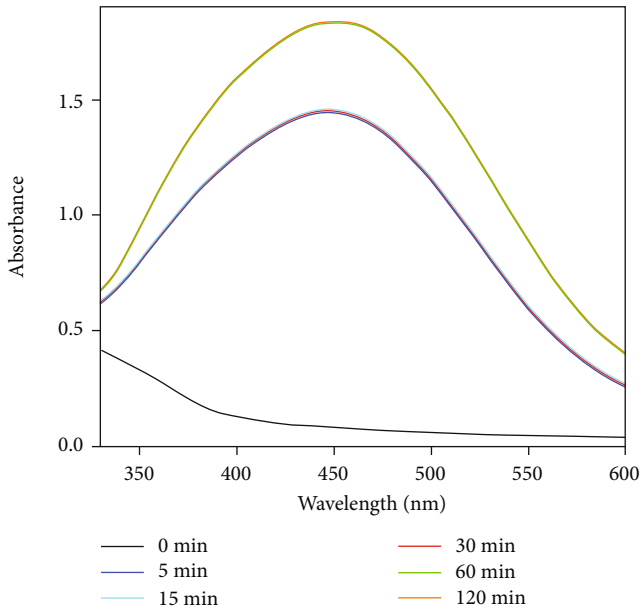


Figure 1: UV-visible absorption spectrum of AgNPs.

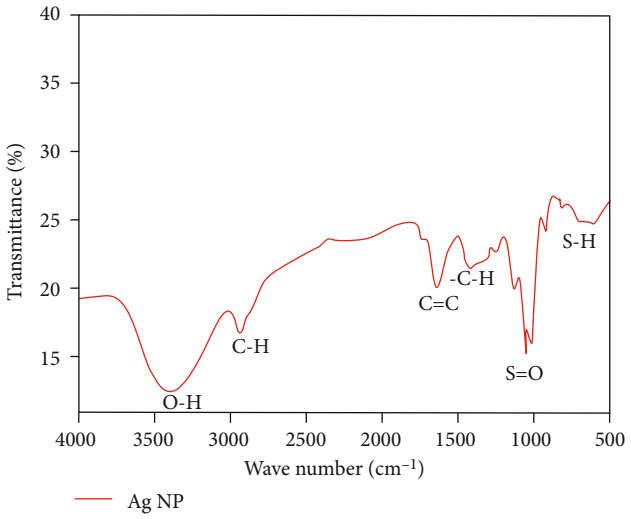


Figure 2: FT-IR spectrum of AgNPs.

were noted at 230 nm and compared to the values of standard (ascorbic acid) [20].

2.5. In Vitro Antifungal Activity of AgNPs. The potency of the AgNPs to inhibit pathogenic fungal strains was evaluated by agar well-diffusion assay. *C. tropicalis*, *C.albicans*, *C.glabrata*, *C. krusei* and *C.parapsilosis* were acquired from "Microbial Type Culture Collection and Gene Bank." The organisms were swabbed across the fungal medium, and 6 mm wells were cut. To the wells, various concentrations of AgNPs were added. The antifungal agent amphotericin B which belongs to the polyene class of antifungals was employed as the standard, and distilled water served as negative control. The plates were kept at 37°C, and the inhibition was measured post 24 h [21].

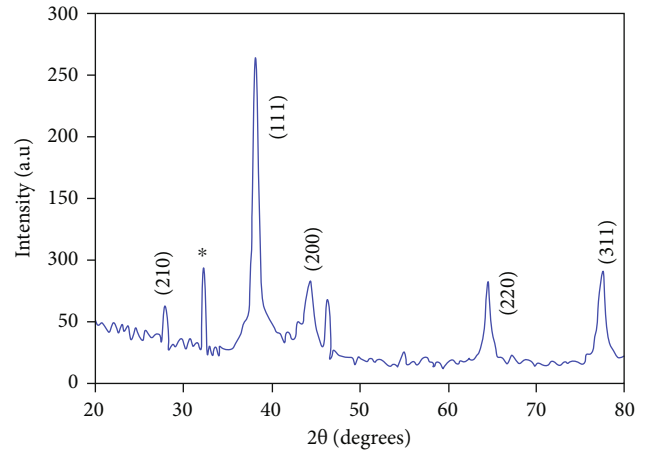


Figure 3: XRD pattern of AgNPs.

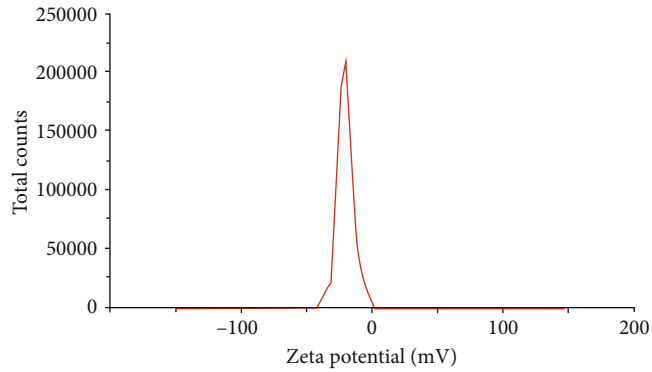


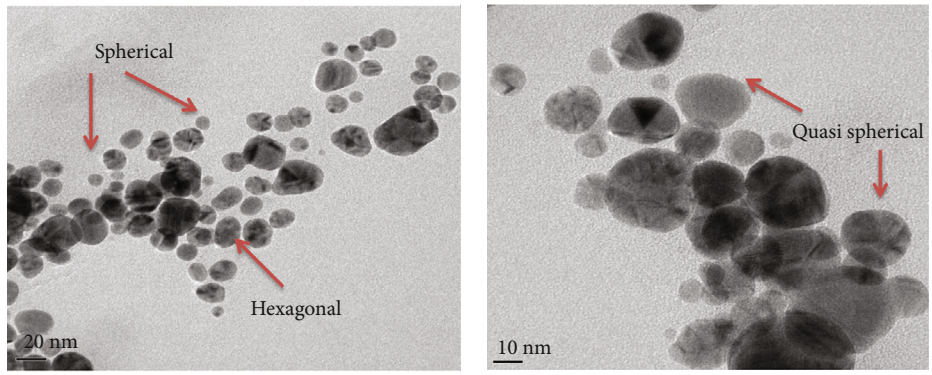
Figure 4: Zeta potential spectrum of AgNPs.

2.6. Statistical Analysis. In vitro assays were performed in triplicates and expressed as mean ± standard deviation. Origin lab software was used for data analysis.

3. Results and Discussion

3.1. Synthesis. AgNPs were obtained in a biodirected and facile approach using Hill garlic extract. AgNPs were synthesized previously by Vijayakumar et al. [22] using garlic cloves procured from Karaikudi Market; AgNPs were successfully synthesized by using 100 mg/ml concentration of garlic extract. Here, synthesis of AgNPs was feasible at a concentration of 1 mg/ml by using silver nitrate as the precursor material. The data revealed that Hill garlic extract had multiple functions in the synthesis of AgNPs which includes reduction, capping, and stabilization through organic compounds. Silver ions are greatly reduced into AgNPs based on the bioactive plant metabolites and enzymes [23].

3.2. Characterization. The transformation of silver ions into AgNPs on exposure to Hill garlic extract could be monitored by the change of color in the solution from pallid yellow to brown on the completion of the reduction reaction. The absorption spectra of the AgNPs recorded using a UV-visible spectrophotometer are shown in Figure 1. The absorbance was monitored immediately after the exposure of the



(a) (b)

Figure 5: TEM micrograph of AgNPs at (a) 20 nm and (b) 10 nm.

extract up to 120 min. The absorbance spectra gradually increased with the increase in time. AgNPs showed a strong absorbance peak at 446 nm, and there was no variance in the absorption spectra after 48 hours. The FTIR spectrum (Figure 2) represents the moieties of the AgNPs that are responsible for its characteristic chemical reactions. Peaks were recorded at 3438, 1632, 1416, 1075, and 624 cm^{-1} wave number. The broad peak at 3438 cm^{-1} paralleled to O-H vibrations and peak at 1632 cm^{-1} designated the occurrence of phenols. Peaks at 1416 cm^{-1} and 1075 cm^{-1} represented the amine and alkane groups in the AgNPs. The presence of sulfur groups was correlated to 624 cm^{-1} wave number. Spectral studies using UV-visible spectrophotometer and FTIR spectrophotometer exposed the optical properties and functional groups present in the AgNPs. The identification of sulfur groups in the FTIR spectrum supports the role of organosulfur compounds present in the Hill garlic extract in the reduction process. The synergistic activity of Allicin (Garlic Organosulfur compound) and AgNPs has been proved to be effective against Methicillin-resistant *Staphylococcus aureus* [24]. Similarly, another organosulfur compound, diallyl disulfide, was loaded into spherical AuNPs (Gold nanoparticles), and their toxic effect on colorectal adenocarcinoma cells (HT-29) cells was evaluated. Diallyl disulfide loaded gold nanoparticles exhibited potential cytotoxic effects against HT-29 and are assumed to have future possibilities as nanocatalysts and nanocarriers [25].

The XRD pattern with diffraction peaks for the synthesized AgNPs is presented in Figure 3. The crystalline features of the synthesized AgNPs were witnessed in the PXRD analysis. The diffraction peaks (Bragg's reflection) at 38.42°, 44.36°, 64.40°, and 77.49° corresponded to the typical silver lattice planes (111), (200), (220), and (311). The diffraction peaks suggest that the biodirected AgNPs were face-centered cubic (FCC) crystals, indicating the presence of one full atom on the six centers of the cubic crystal and a fraction of an atom in each corner. The XRD data was parallel to the JCPDS No: 04–0783. The XRD pattern depicted the FCC nature of the AgNP crystals; according to Shetty et al. [26], in a FCC crystal, the higher intensity of (111) plane can enhance the antimicrobial activity of the AgNPs. In Figure 3, it was witnessed that the AgNPs synthesized using Hill garlic extract showed higher intensity of (111) plane and could plausibly have enhanced antimicrobial potential.

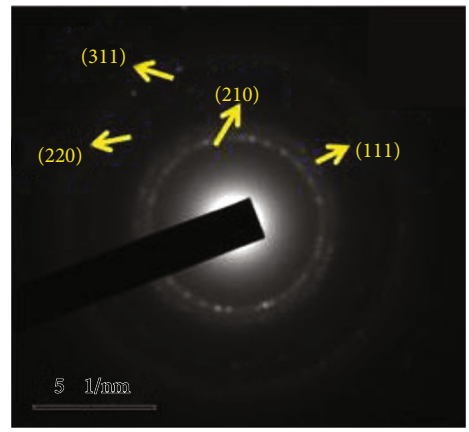
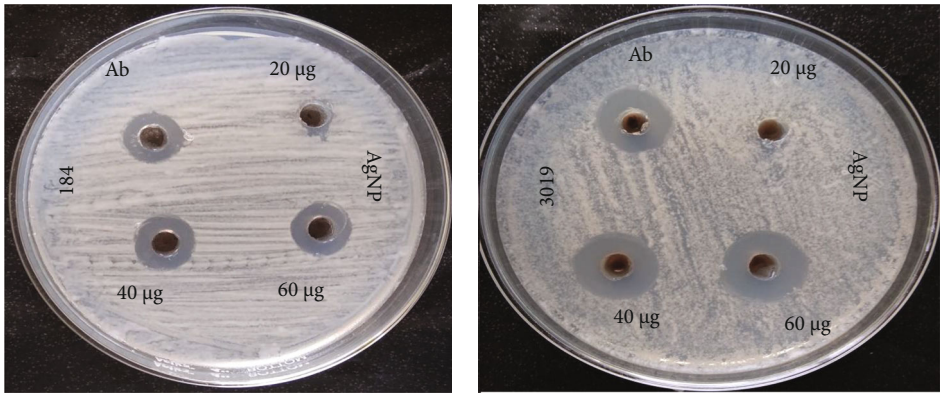


Figure 6: SAED pattern of AgNPs.

Zeta potential analysis (Figure 4) reflected the charge and stability of the AgNPs; it was observed that the nanoparticles possessed negative charge (-23.3 mV) and were moderately stable. The zeta potential measurements were helpful in identifying the stability and charge based on electrophoretic mobility of the nanoparticles. Our results (-23.3 mV) were in comparable to the results obtained on using garlic extract to synthesize AgNPs in a previous study that showed a zeta potential value of -26.1 mV [22].

The size and shape of the nanoparticles were examined using HR-TEM technique and are shown in Figure 5. HR-TEM micrographs showed that the particles were predominantly spherical shaped, whereas quasispherical and hexagonal particles were also observed. The size of the particles was within the range of 20.3 nm to 60.5 nm. The mean size was measured as 35.1 nm. Ring patterns were observed in SAED analysis (Figure 6); the crystalline nature as predicted in the XRD analysis matched with the results of the SAED pattern obtained for the AgNPs. Spherical nanoparticles are frequently obtained in the synthesis of AgNPs using plant extracts; here, multishaped nanoparticles were observed in HR-TEM micrographs. The antimicrobial potential of multishaped AgNPs was tested against three microbes including two gram positive and one negative in a study, which



(a) (b)

Figure 7: Anticandida activity of AgNPs. (a) *C. albicans* was highly susceptible to AgNP treatment, and (b) *C. glabrata* was the least susceptible species.

Table 1: Inhibition zone diameter of synthesized AgNPs.

S. no.	Pathogens	Inhibition zone (mm)		
		Distilled water	Amphotericin B	AgNPs
1	*C. albicans*	Nil	17 ± 0.3	20 ± 0.7
2	*C. krusei*	Nil	17 ± 0.2	18 ± 0.04
3	*C. parapsilosis*	Nil	15 ± 1.2	18 ± 0.1
4	*C. tropicalis*	Nil	13 ± 1.3	12 ± 0.3
5	*C. glabrata*	Nil	13 ± 2.1	9 ± 0.3

concluded that the antimicrobial properties of AgNPs were shape-dependent and Ag ion release was entirely based on the shape of the nanoparticles [27].

3.3. Free Radical Scavenging Activity of AgNPs. The free radical scavenging potential of the synthesized NPs was determined using three different *in-vitro* free radical scavenging assays. For all the assays, ascorbic acid (vitamin C) was taken as the antioxidant standard. Among the different tested concentrations, maximum inhibition of molecules containing unpaired electrons in their valence shell was evidenced at 25 μg/ml concentration. The ability of AgNPs to scavenge DPPH and H_2O_2 was excellent when compared to ABTS radicals. The IC_{50} of DPPH radicals exposed to AgNPs was $8.3 \pm 0.4\,\mu$g/ml, whereas $12.4 \pm 1.2\,\mu$g/ml and $9.6 \pm 0.2\,\mu$g/ml were recorded as the IC_{50} for ABTS and H_2O_2 radicals, respectively. The inhibition ability augmented with increase in AgNPs concentration, indicating that the reaction was concentration/dilution-dependent. Free radical scavenging percentage recorded for ascorbic acid was 89%, 62.34%, and 81.12% for DPPH radicals, ABTS radicals, and H_2O_2 radicals, respectively. The scavenging percentage of AgNPs was 83.4%, 47.3%, and 79.1% for the above-mentioned radicals in the same order; this was almost similar to the ability of the standard. The results of the present study were similar to a study where AgNPs synthesized from *A. cepa* showed impressive antioxidant activity when compared to the activity of ascorbic acid. The free radical capacity of the AgNPs can be related to the ability of the AgNPs to transfer electrons thus neutralizing the free radical production [28].

3.4. Antifungal Potency of AgNPs. The antifungal activity of the biosynthesized AgNPs examined by agar well-plate method is shown in Figure 7. *C. albicans* was observed as the least resistant species among the tested pathogens. AgNPs showed similar inhibitory effect when compared to the standard antibiotic amphotericin B (Table 1). Interestingly, all the tested pathogens were susceptible to the treatment of AgNPs. The inhibition area obtained for AgNPs against *C. albicans* was 20 ± 0.7 mm, and the lowest activity was observed for *C. glabrata* (9 ± 0.3 mm). Advancements in the development of antifungal drugs have been limited due to the eukaryotic nature of the fungi which is similar to higher organisms. Ergosterol synthesis pathway has always been a main target in the identification of antifungal drugs owing to its deviating nature from the mammalian pathways [29]. 30 different *Candida* species are estimated to affect humans; among these, 80% of the candidiasis cases are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*. It has been identified that strains of *C. glabrata* and *C. krusei* are resistant to azole antifungal agents, whereas *C. parapsilosis* is less susceptible to the treatment of echinocandins [30]. Mutation in Candida strains has a major role to play in their resistance towards azole drugs. Point mutation in ERG11 gene can confer increased resistance of C. albicans towards azoles. This can also vary in

other *Candida* organisms; for example, *C. glabrata* does not show azole resistance based or ERG mutation, but the resistance in developed due to the mutation in CgPDR1 transcription factor [31].

Over a 10-year period, *C. glabrata* has showed the maximum resistance (more than 13%) towards antifungal agents. This was evidenced in the present study, also, *C. glabrata* was the least susceptible strain when exposed to standard antifungal agent, and similarly, the inhibitory effect of AgNPs was less on *C. glabrata* when compared to the other tested organisms. AgNPs synthesized using Hill garlic extract have been rarely tested against *Candida* strains. In a previous study, AgNPs synthesized using garlic extract were found to have mild effect on *C. albicans* but exhibited excellent activity against *S. aureus* and *E. coli* [32]. Similarly, in another study, the antibacterial activity against vaginal bacterial strains exerted by eco-friendly AgNPs synthesized using garlic extract was commendable [33]. Amphotericin B, the standard antifungal agent used in the study, also exhibited inhibitory effect on the tested *Candida* strains. Amphotericin B belongs to the class of polyene antifungal agents and acts by inducing pore formation in the outer membrane of the microorganism. Ergosterol acts as the main target for amphotericin B to which it binds strongly and leads to formation of pores. It has also been identified that this antifungal agent modulates the immune system and provides a protective effect against pathogens. However, the toxicity in the host on continuous use of amphotericin B must be taken into consideration [34]. AgNPs are presumed to aggravate membrane permeability and cellular damage that can plausibly lead to the discharge of cell content including nucleic acids and proteins resulting in the death of the organism [35]. Our results showed that AgNPs synthesized using Hill garlic extract can be a potential antifungal agent against five different *Candida* strains.

4. Conclusion

In this study, AgNPs were synthesized using Hill garlic ("Malai Poondu") extract by a feasible, robust, and environment-friendly approach in a single-reduction step without the use of chemical reducing agents. Multishaped AgNPs with an average size of 35.1 nm were obtained. The nanoparticles exhibited high free radical scavenging efficacy against DPPH, ABTS, and H_2O_2 free radicals. Strong inhibitory potential against five *Candida* strains was observed indicating the possibility of AgNPs to act as promising antifungal agents that have the possibility to reduce AMR and antibiotic pollution.

Data Availability

All data generated or analyzed during this study are included in the published article.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

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