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Research Article

Biosynthesis of Silver Nanoparticles Using Pine Pollen and Evaluation of the Antifungal Efficiency

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Background: Nanoparticles have been applied to medicine, hygiene, pharmacy and dentistry, and will bring significant advances in the prevention, diagnosis, drug delivery and treatment of disease. Green synthesis of metal nanoparticles has a very important role in nanobiotechnology, allowing production of non-toxic and eco-friendly particles.
Objectives: Green synthesis of silver nanoparticles (AgNPs) was studied using pine pollen as a novel, cost-effective, simple and non-hazardous bioresource. The antifungal activity of the synthesized AgNPs was investigated *in vitro*.
Materials and Methods: Biosynthesis of AgNPs was conducted using pollen of pine (as a novel bioresource) acting as both reducing and capping agents. AgNPs were characterized using UV-visible spectroscopy, X-ray diffraction and transmission electron microscopy. In evaluation for antifungal properties, the synthesized AgNPs represented significant *in vitro* inhibitory effects on *Neofusicoccum parvum* cultures.
Results: Pine pollen can mediate biosynthesis of colloidal AgNPs with an average size of 12 nm. AgNPs were formed at 22°C and observed to be highly stable up to three months without precipitation or decreased antifungal property. AgNPs showed significant inhibitory effects against *Neofusicoccum parvum*.
Conclusion: The first report for a low-cost, simple, well feasible and eco-friendly procedure for biosynthesis of AgNPs was presented. The synthesized AgNPs by pine pollen were nontoxic and eco-friendly, and can be employed for large-scale production. The nanoparticles showed strong effect on quantitative inhibition and disruption of antifungal growth.

Keywords: Colloidal silver, Green synthesis, Low-cost synthesis, Pollen.

1. Background

Nanotechnology has attracted attentions in many researchers worldwide (1-4). Nanoparticles have gained increased use due to their specific performance in pharmacy, medicine and engineering (5-8). Silver nanoparticles (AgNPs) with strong antibacterial activity have been commercialized to control wide spectrum of bacteria (9-11). Some AgNPs-based products are being used in shampoos, soaps, detergents, toothpastes, cosmetics-hygienic and medicinal goods (12); even though not all such products have been proven safe and gained approval by United States Environmental Protection Agency (EPA) yet. AgNPs are being synthesized by various physical and chemical techniques (13, 14) including electrochemical (15) and photochemical (13). These techniques are generally expensive, time consuming, difficult to manage, and leave toxic chemicals. To avoid such hazardous effects, biosynthesized nanoparticles by microorganisms, plant extracts or enzymes have gained attentions (16-19). For instance, *Cassia auriculata* L. (20), fennel (10) or other plant extracts (21-24), fungi (25, 26), and bacteria (27, 28) were used to synthesize AgNPs and some other metal nanoparticles. These nanoparticles have

Figure 1. Male cones of pine tree used to collect pollens.

demonstrated antibacterial activities against a wide array of gram positive and negative bacteria (10).

Here, pine pollen (Fig. 1) for the first time was used to biosynthesis AgNPs.

2. Objectives

The main goal of the present study was to evaluate biosynthesis of antifungal metalic nanoparticles using green route. Green synthesis of AgNPs were studied and characterized using pine pollen as a novel, cost-effective, simple and non-hazardous bioresource. The antifungal effect of the resulting green synthesized AgNPs was investigated *in vitro* on *Neofusicoccum parvum.*

3. Materials and Methods

3.1. Materials

The male cones were collected from pine tree in spring of 2015, in Kerman University of Medical Sciences. $AgNO_3$ and potato dextrose agar (PDA) medium were purchased from Merck, Germany. The fungal isolate *N. parvum* was obtained from Shahid Bahonar University of Kerman bio-bank, Kerman, Iran.

3.2. Preparation of Pollen Water-Extract (PWE)

The male cones of pine were collected and surface sterilized using 2% (v/v) sodium hypochlorite for 3 min, followed by 3 min rinsing with deionized water (DW). Disinfested male cones macerated in DW (1 g.20 mL^{-1} DW), and incubated at 28 °C for 48 h in dark (21). Male cones were removed from the maceration mixture and the aqueous phase was collected and centrifuged at 1.4×103 ×*g* for 10 min to separate the liquid fraction from the pollen particles. The pellets were discarded and the supernatant was filtered through Whatman filter paper No.42. The cell free extract was stored at 4 °C.

3.3. Biosynthesis of AgNPs

To evaluate the reduction of silver ions to Ag, cell-free PWE was two-fold diluted by sterile DW and its aliquots were mixed with equal volumes of several concentrations of $AgNO_3$ (1, 2.5, 4.0 and 5.0 mM).A control sample was received water with no $AgNO_3$. All the procedures were repeated in triplicates. The samples were stored at 28 °C in dark for 16 h.

3.4. UV-visible Spectroscopy Analysis

The formation of AgNPs was monitored by UV-visible spectroscopy in Scan drop-type spectrometer (Analytik Jena, Germany). Absorption values were recorded in a wavelength of 300-650 nm.

3.5. Stability of AgNPs

The synthesised AgNPs were kept stationary over 3 months in dark for all concentrations and evaluated visually every week for probable colloid, aggregate or precipitate formation.

3.6. X-ray Diffraction (XRD) Analysis

To prepare a powder sample, 60 mL PWE containing synthesized AgNPs was centrifuged for 8 min at 1.19×104 ×*g*, the supernatant was discarded, and 60 mL DW was added to the pellets. This procedure was repeated in two additional cycles and the remaining pellets were dried at 60 °C for 48 h. The resultant powder was used for XRD analysis. An X-ray of 1.54 Å wavelength over a angel range of 10 to 80° was employed (29). The most widely used method for estimating the average crystallite size of AgNPs is based on the full width at half maximum (FWHM) of a diffraction peak using the Scherrer equation:

$$D = k\lambda/\beta_{1/2}\cos\theta \qquad (1)$$

where D, λ, k, $\beta_{1/2}$, θ, are the crystal size, X-ray wavelength, the Scherer coefficient, FWHM, and Bragg angle, respectively.

3.7. Transmission Electron Microscopy (TEM)

TEM images of the synthesized AgNPs were recorded using a CARL ZEISS transmission electron microscope working at 80 kV.

3.8. Bioassays of Antifungal Activity of AgNPs

To determine the antifungal activity of the synthesized AgNPs, agar diffusion method was employed, and the zones of inhibition were measured (5). AgNPs of different concentrations were mixed with PDA growth medium (5, 10, 15 and 30 µg.mL^{-1}) after autoclaving

and prior to pouring in Petri plates (75×15 mm). After solidification, each Petri plate was inoculated with a 6 mm agar plug of 7 days old culture of *N. parvum* at the center and incubated at 28 ±1 °C. Controls included PDA medium with no AgNPs and inoculated similarly. When the fungus on control plates relatively covered the entire surface of the medium (4 days), the mean radius of fungal growth in all treatments were recorded (30). The experiments were performed in triplicates and mean values were recorded. To evaluate the antifungal activity, the following formula was used to assess the rate of fungal inhibition:

$$\%\text{Inhibition rate} = (R-r)/R \qquad (2)$$

where R is the radius of mycelia growth of control and r indicates radius of fungal growth mycelia in treated samples containing AgNPs. Data was subjected to analysis of variance (ANOVA) with SAS Software (SAS Institute, version 9, Cary, NC). Statistical significance was determined at the $p \leq 0.01$. Duncan's Multiple-Range Test was used to compare the means.

4. Results

4.1. Visual Observation

Upon addition of PWE to $AgNO_3$ solution, reduction of the silver ions to Ag occurred, and the reduction reaction was noticeable in mixtures through color change from colorless to reddish brown (Fig. 2). This reddish brown color is attributed to the Surface Plasmon Resonance (SPR) arising due to the collective oscillation of induced free conduction electrons in AgNPs (31).

4.2. UV-Visible Spectrophotometry

Figure 3 shows UV-visible spectra for PWE, and after mixing with 1, 2.5, 4.0 and 5.0 mM $AgNO_3$. The AgNPs solutions represented a maximum optical absorption at

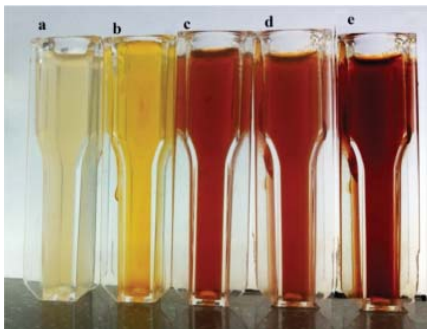

Figure 2. Color changes of PWE upon mixing with $AgNO_3$. a) PWE, b) PWE plus 1.0, c) plus 2.5, d) plus 4.0, and e) plus 5.0 mM $AgNO_3$.

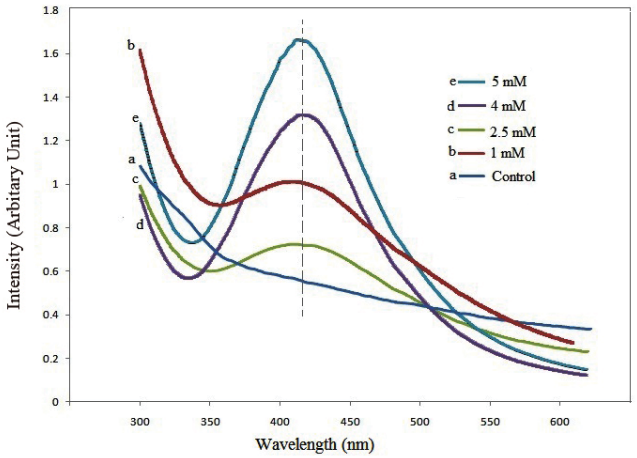

Figure 3. UV-visible absorption spectra of PWE, and after mixing with 1, 2.5, 4.0 and 5.0 mM $AgNO_3$

≈ 414 nm, and the related absorbance was increased upon AgNPs concentration increment.

4.3. Stability of AgNPs

The synthesised AgNPs represented a colloidal stability in long time at ambient temperature. It was observerd that AgNPs synthesized with 1 mM $AgNO_3$ were stable for 5 months, and those with 2.5, 4.0 and 5.0 mM were stable for 2, 1 and 1 months, respectively.

4.4. XRD Analysis

An XRD pattern recorded for the synthesized AgNPs is presented in Figure 4. The pattern contained prominent peaks at 2θ values of 38, 44, 64 and 77°, corresponding to (1 1 1), (2 0 0), (2 2 0) and (3 1 1) planes, respectively. The pattern corresponds to crystalline phase of FCC structure. It should also be added that the other peaks (at 2θ values of 32, 54, 57 and 67°) in the pattern are due to the crystallization of bioorganic phase on the surface of the nanoparticles (32). Similar results were reported elsewhere (33). The average crystalline size of AgNPs was obtained as 20 nm.

4.5. TEM Analysis

TEM images of the synthesized AgNPs at two magnifications are presented in Figure 5, and a particle size distribution histogram is shown in Figure 6. AgNPs had a spherical shape with ultra small size with an average size of 12 nm.

4.6. Antifungal Activity of AgNPs

In vitro bioassays of fungi-static properties of AgNPs were revealed by inhibition of mycelia growth of *Neofusicoccum parvum* on PDA growth medium amended with AgNPs. The antifungal activity of

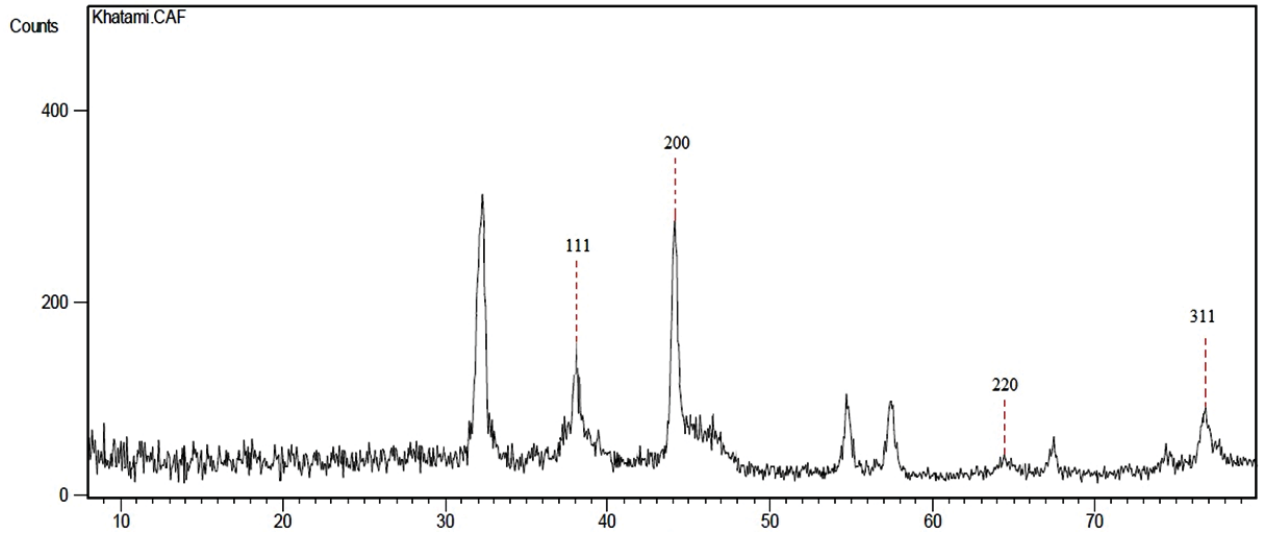

Figure 4. An XRD pattern of the synthesized AgNPs.

AgNPs for the concentrations of 5.0, 10, 15, and 30 ppm was found to be concentration dependent. Images form he bioassay plates are shown in Figure 7. The inhibition percents were obtained as 41, 58, 68 and 81% for the concentrations of 5.0, 10, 15 and 30 ppm, respectively. The data was subjected to the analysis of variance (ANOVA) and Duncan's Multiple-Range test was employed to compare the means of triplicate treatments. The results confirmed a significant inhibitory effect ($P<0.01$) of AgNPs on fungal growth at all tested concentrations.

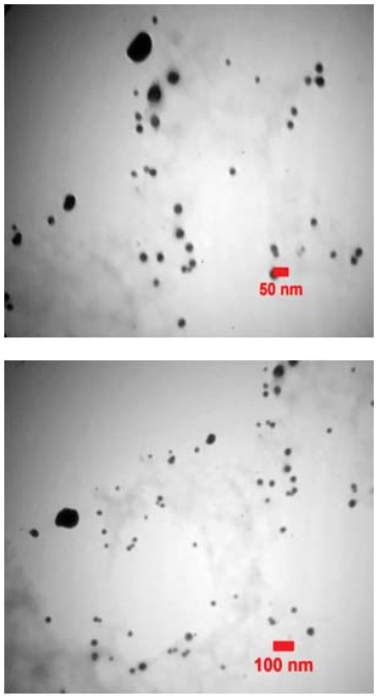

Figure 5.TEM images of the synthesized AgNPs.

5. Discussion

Kumarasamyraja and Swamivelmanickam biosynthesized spherical AgNPs with a size of 15-20 nm (20), Bonde *et al*. synthesized AgNPs in a diameter range of 18-83 nm (10), and Soltani Nejad *et al*. reported the biosynthesis of triangular AgNPs with a size range of 1-100 nm (34). Amaladhas and his coworkers showed that *Cassia angustifolia* nanoparticles were poly-dispersed with a particle size of 9-31 nm (an average size of 21.6 nm) (35). Using banana, neem and tulsi for the synthesis of AgNPs resulted in spherical, triangular and cuboidal AgNPs, respectively (33). Her, this is the first report on rapid, simple and non-hazardous method for the synthesis of very small-sized AgNPs from PWE and their strong effect on quantitative inhibition of fungal mycelium growth. PWE was efficiently and successfully used for one-step biosynthesis of

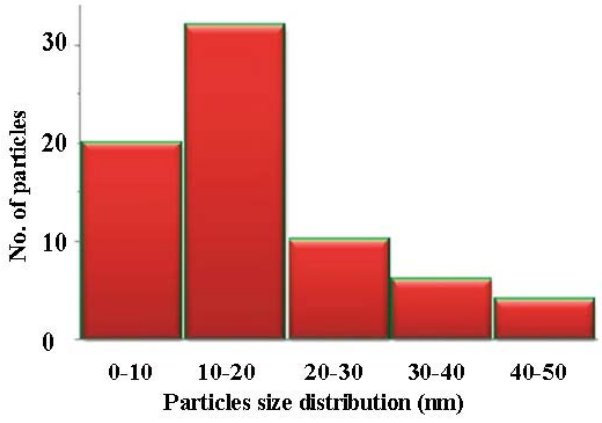

Figure 6. A particle size distribution of the synthesized AgNPs.

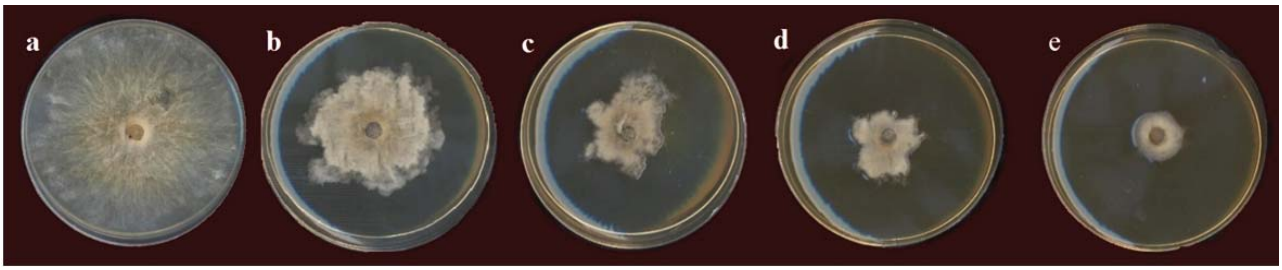

Figure 7: Fungi-static properties of AgNPs at concentrations of a) 0 (control), b) 5.0, c) 10, d) 15, and e) 30 ppm.

spherical AgNPs in ambient conditions in 10 min. Stability of AgNPs was significantly dependent on the concentration; they were more stable at lower concentrations. PWE simultaneously played both the reducing and stabilizing actions, and prevented the nanoparticles aggregation. The synthesis reaction did not require any chemical, solvent or high energy consumption. The methodology promises a rather easy scale-up procedure for production of AgNPs.

The differences in size, charge, stability, shape, and morphology of AgNPs can be characterized by UV-visible spectra. The peak in UV-visible spectra of the synthesized AgNPs had a maximum at 414 nm similar to that reported for AgNPs synthesized by *Butea monosperma* (36), while, had a blue shift compared to that measured for AgNPs synthesized by Cycas leaf (37). The difference is due to the size and shape of the AgNPs.

The antifungal and antibacterial activities of silver nanostructures have already been known (7, 16, 35, 38-41). In the present study, the inhibitory effect of AgNPs was assayed against *Neofusicoccum parvum*. AgNPs can represent this inhibitory effect through different mechanisms including i) binding to cytoplasmic membrane causing cell membrane damage, forming pits on the cell surface, and/or modifying cell wall permeability, ii) inhibition of major cellular functions such as respiration, DNA replication, and cell division, resulting in loss of cell viability (38, 42). It should be also added that this inhibitory effect is size-dependent enhancing with decrement in the size (42-44).

6. Conclusion

It was shown that pine pollen extract is a suitable reducing and stabilizing agent. The extract can be applied for the synthesis of other noble metal nanostructures. The proposed procedure for the synthesis of AgNPs can be applied for the large-scale production for antimicrobial sprays, cosmetic creams etc, although more investigations are needed to understand the toxicity and mechanism of action at cellular level.

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Authors' Contribution

All authors have participated in the manuscript preparation.

Financial Disclosure

There is no conflict of interest.

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