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# Biosynthesized silver nanoparticles using green chemistry approach and its evaluation as antifungal agent

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Silver nanoparticles (Ag-NPs) have emerged as potential antifungal agents due to their exceptional properties. These nanoparticles can be synthesised in the laboratory by using green synthetic methods. Hence, in the present study dark brown colour Ag-NPs have been synthesized from 0.01 mM $AgNO_3$ using *Azadirachta indica* leaf extract as a reducing agent and characterised via different spectroscopic and microscopic techniques. The average particle sizes of the synthesized Ag-NPs are found to be 36 nm and all the particles are mostly spherical in shape. These biosynthesized Ag-NPs effectively have mitigated the mycelial growth of *F. oxysporum* and *F. moniliforme*. However, the inhibition has been found better for *F. oxysporum* as compared to *F. moniliforme*. At 500 ppm concentration of Ag-NPs, the reductions in mycelial growth have been found to be 100 and 80% for *F. oxysporum* and *F. moniliforme*, respectively. So, it can be concluded from the study that Ag-NPs prepared from *A. indica* leaf extracts could be explored for developing fungicidal formulations.

**Keywords:** Antifungal agent, Biosynthesis, Green chemistry, Silver nanoparticles

The pioneering innovations in the field of nanotechnology have left no field untouched. Nanotechnology has also stepped forward in the management of fungal pathogens. The anti-fungal properties of silver nanoparticles (Ag-NPs) have been explored by many researchers globally[1–7]. Ag-NPs can be synthesized through different ways including laser ablation[8], gamma irradiation[9], electron irradiation[10], chemical reduction[11], photochemical methods[12] and biological synthetic methods[7,13–16]. But generally, the chemicals used in the synthesis of Ag-NPs pose threat to the environment by being toxic in nature[13]. Moreover, the shape, size, morphology, physicochemical properties, stability and the interaction of reducing agents with metal ions vary with the experimental conditions[13,17]. To achieve the sustainability, there is a need to eliminate the use of toxic chemicals in Ag-NPs synthesis. Hence, focus has been shifted towards the synthesis of Ag-NPs through green processes[13]. Moreover, the use of biocompatible reducing and stabilizing agents used in such green processes enhance the scope of application of such Ag-NPs in the biological fields[13]. *Azadirachta indica (A. indica)* contains numerous chemical constituents which can possibly be utilized as reducing agents for green synthesis of Ag-NPs. The exploration of such compounds in *A. indica* may also be helpful in facilitating the control over size and morphology of synthesized Ag-NPs.

Plant pathogens (i.e., bacteria, fungi, virus etc.) causes various diseases in many crops of economic importance[15]. As a result, crop losses due to such phytopathogens accounts for nearly 16% of the total crop produced globally[18]. The losses occurred due to these pathogenic organisms are already huge and they may intensify further subjected to the pathogen virulence and environmental factors[4]. All the crops cultivated globally suffer substantial losses owing to phytopathogenic fungi. *Fusarium oxysporum* and *Fusarium moniliforme* are such fungi which are responsible for diseases in economically important crops like banana[19], tomato[20] and potato[21]. In the present study, we report the synthesis of Ag-NPs using aqueous leaf extracts of *A. indica* and

evaluation of their antifungal potential against *F. oxysporum* and *F. moniliforme*. The present research will surely add-on to the existing knowledge about the antifungal effects of eco-friendly Ag-NPs.

## Experimental Section

$AgNO_3$ (>99.9% assay) was purchased from CDH (P) Ltd. India. Fresh leaves of *A. indica* were collected from Rambagh area of Jaipur. Double distilled water was used in entire study wherever required. Potato dextrose agar was obtained from HiMedia Pvt. Ltd., India. Only borosilicate glasswares were used throughout the study.

### Biosynthesis of Ag-NPs

The leaves were plucked and washed with ample amount of tap water to remove any dust particles and other impurities and lastly with double distilled water. The leaves were then cut into small pieces with the help of a chopper and 10 g of finely chopped leaves were then boiled in 200 mL of double distilled water for 15 min. The extract was then allowed to cool down and filtered with Whatman filter paper no.1. The filtered extract was then stored in amber coloured bottle away from light under refrigerated conditions for further utilization in the synthesis of Ag-NPs. For *A.indica* mediated synthesis of Ag-NPs the protocol followed by Al-Otibi *et al.* was followed with slight modifications[15]. For synthesis of Ag-NPs, 5 mL of *A. indica* extract was mixed with 45 mL of 0.01 mM $AgNO_3$ and the mixture was heated at 60°C for 45 min. The colour of the solution changes from yellowish orange to dark brown which is the key indicator for the successful synthesis of Ag-NPs.

### Characterization of biosynthesized Ag-NPs

Shimadzu UV-1800 UV-visible absorption spectrophotometer was used to ascertain the synthesis of Ag-NPs. The absorbance spectra of synthesized Ag-NPs were measured between 200-700 nm. The FT-IR spectra of Ag-NPs on KBr pellets were recorded in the wavenumber range of 400–4000 $cm^{-1}$ by using Nicolet™ iS50 FTIR spectrometer. SEM analysis was done through Zeiss® EVO 18 to study the surface morphology. Ag-NPs were further characterized on the basis of their particle size performed through Zetatrac$^{TM}$ particle size analyser. The particle size analyser was based on the principle of dynamic light scattering (DLS) which analyse the fluctuation of the scattering intensity of light as a result of the Brownian motion of particles present in the suspension or colloid. Particle size determination was conducted with 200 ppm solutions of Ag-NPs in distilled water at room temperature. The morphologies of Ag-NPs were further confirmed by transmission electron microscopy (TEM) imaging using Technai (Model: F30 S-Twin).

### Evaluation of anti-fungal potential of synthesized Ag-NPs

#### *Fungal Culture*

Two fungal species *F. oxysporum* and *F. moniliforme* were obtained from ICAR-Indian Agricultural Research Institute, New Delhi. Both the fungal pathogens are economically important and causing various diseases in agricultural crops. These fungal cultures were kept on the slants of potato dextrose agar (PDA) and stored under refrigerated conditions at 4°C.

#### *In vitro anti-fungal activity*

The antifungal potential of biosynthesized Ag-NPs were evaluated on both the fungi through poisoned food technique assay. The PDA growth media was treated with different concentration of Ag-NPs (i.e. 500, 250, 125, 62.5 and 32.25 ppm). 5 mL of Ag-NPs solution of desired concentration was added to the corresponding growth media before plating the culture in a Petri dish of size (90 x 15 mm). PDA plates were then inoculated with 5 days old culture of fungi in corresponding treatments. The mycelial plug of 5 mm was placed at the centre of Ag-NPs containing Petri plates. Inoculated Petri plates were the finally incubated at 25 ± 1 °C until the fungal growth in control plate reaches to the edges of the Petri dish. Three replicates were taken for each treatment for higher accuracy of results. Finally, diameter of the mycelial growth of fungi was measured to assess the percent inhibition which was calculated using the formula:

$$\text{Mycelial growth inhibition (\%)} = \frac{Growth\ in\ control\ \ plate - Growth\ in\ treated\ \ plate}{Growth\ in\ control\ \ plate} \times 100$$

MS Excel 2016 was used for the analysis of bio-efficacy data.

## Result and Discussion

### Synthesis of Ag-NPs

Ag-NPs were synthesized by mixing 0.01 mM $AgNO_3$ and *A.indica* leaf extract. The mixture of these two solutions was heated at 60°C until the colour

changed from yellowish orange to dark brown (Fig. 1a). This change in colour has been attributed to the excitation of surface plasmon resonance by Ag-NPs[22]. The colour change was visible only after 5 min of heating and became more and darker with the passage of time. After 45 min no change in colour was visible. The reaction was carried out in dark to avoid photoxidation of silver. The change in colour is the first indication of synthesis of Ag-NPs[15]. The chemical constituents present in *A. indica* leaf extracts acted as an electron donor and facilitated the reduction of silver ions. The reduction of Ag-NPs through aqueous extracts of *A. indica* leaves was further confirmed by the UV-visible absorption spectroscopy. Absorption spectrum of these Ag-NPs exhibits a maximum absorption ($\lambda_{max}$) at 453 nm (Fig. 1b). Similar absorption between 430-455 nm was reported in the earlier studies[15,23–25].

**Characterisation of the biosynthesised Ag-NPs**

SEM imaging was used to envisage the size, shape and other surface characteristics of synthesized Ag-NPs (Fig. 2a). The preparation of SEM grids was done by placing a small amount of synthesized Ag-NPs over the surface of coated grid and drying of sample was done with the help of a lamp. The SEM images revealed that the dimensions of formed Ag-NPs are well within nano range having mostly spherical shapes. However, some irregularly shaped particles with mild agglomeration and lumps are also present but no flat surfaces were detected. However, very few nanoparticles are observed to be irregular in shape and the irregularities in the synthesised Ag-NPs are attributed to the coalescence among the agglomerated particles. Biosynthesized Ag-NPs having spherical morphology were also observed in other reports[13,14,26,27].

Surface features of the synthesized Ag-NPs, including shape, size and morphology were assessed through TEM analysis (Fig. 2b). Most of the particles were found to be spherical in shape with a diameter ranging from 12 to 20 nm. This size range is comparable with Ag-NPs synthesized by Narayanan *et al.* where spherical polydispersed Ag-NPs with average size of 16.14 nm were obtained[13]. Based on the studies conducted, it has been observed that smaller Ag-NPs have higher antifungal activities[28,29]. Mainly the shape of the biosynthesized Ag-NPs were spherical, however, some irregularly shaped particles and some agglomeration was observed.

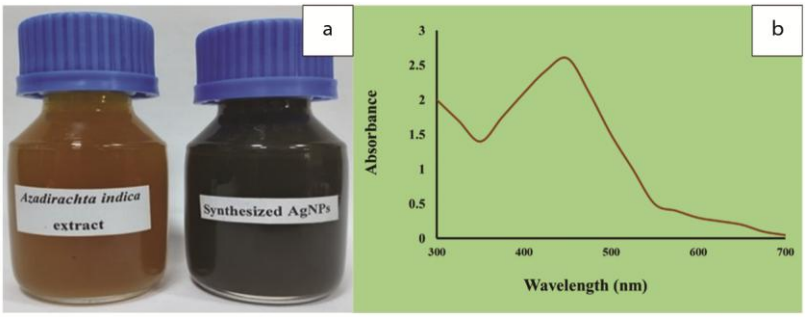


Fig. 1 — (a) Photograph showing colours of Crude extract of *A. indica* and solution containing synthesized Ag-NPs and (b) UV–visible absorbance spectrum of biosynthesized Ag-NPs

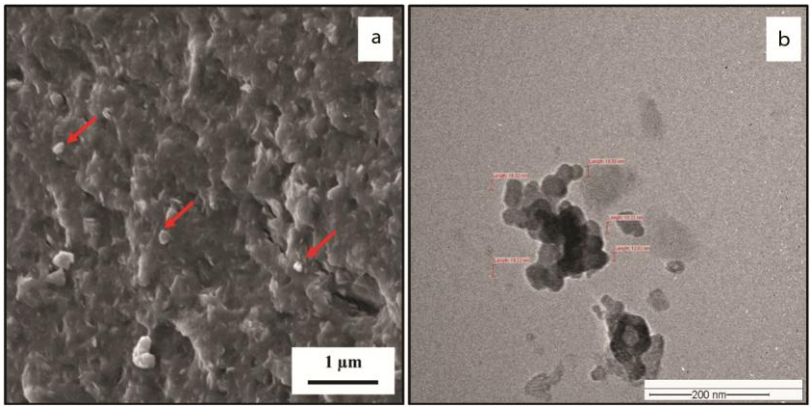


Fig. 2 — (a) SEM and (b) TEM micrographs of Ag-NPs synthesized using *A. indica* leaf extract (Ag-NPs shown by red arrow in Fig. 2a)

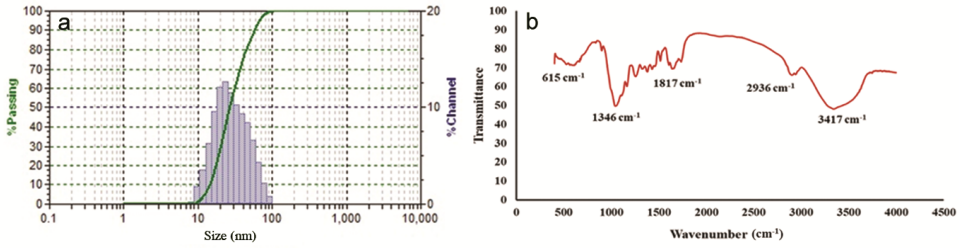


Fig. 3 — (a) DLS analysis and (b) FT-IR spectrum of biosynthesized Ag-NPs

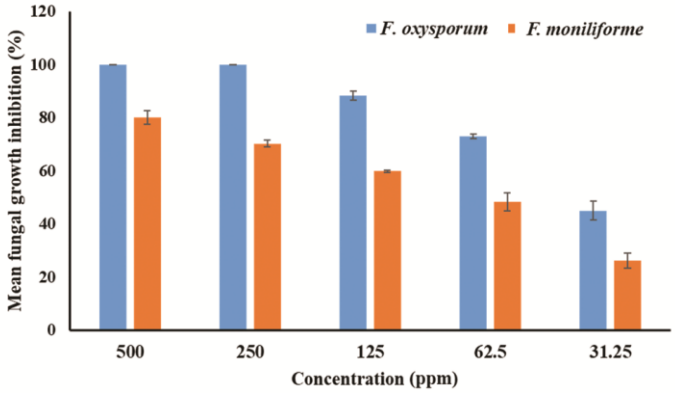


Fig. 4 — Fungal growth inhibition of *F. oxysporum* and *F. moniliforme* treated by different concentrations of Ag-NPs

DLS measurements revealed that the average hydrodynamic diameter of biosynthesized Ag-NPs was 36 nm (Fig. 3a). The zeta potential of colloidal solution of biosynthesized Ag-NPs was found to be −13.9 mV at pH 7.0 and the polydispersity index was 0.269. Negative zeta potential clearly indicates the mild electrochemical stability of the suspended Ag-NPs. Lower polydispersity index indicating that the Ag-NPs have narrow and uniform size range throughout the suspension. Comparable zeta potential for Ag-NPs is also reported by Narayanan *et al.*[13]. DLS analysis also indicates the fact that the synthesized Ag-NPs were discrete.

Various absorption bands obtained in the FT-IR spectrum indicate the presence of different chemical groups in the biosynthesised Ag-NPs (Fig. 3b). The FT-IR spectrum shows the absorption bands in regions ranging from 3417 to 615 $cm^{-1}$. The presence of broad band at 3417 $cm^{-1}$ was due to the –NH stretching of amines. The weak band at 615 $cm^{-1}$ was attributed to out-of-plane bending vibrations of C-H groups[14]. The bands at 2936 $cm^{-1}$ and 1346 $cm^{-1}$ are corresponding to C-H and C=C groups respectively[14]. Also, the absorbance at 1817 $cm^{-1}$ indicates the presence of carbonyl (C=O) group. Presence of such peaks indicate functional groups that act as capping and stabilizing agents[15].

**Antifungal activity of synthesised Ag-NPs**

The mean fungal growth inhibition as assessed via poisoned food technique revealed that Ag-NPs to be comparatively higher toxic to *F. oxysporum* rather than *F. moniliforme*. In case of *F. oxysporum* the mean fungal growth inhibition was observed to be 100%, 100%, 88.2%, 72.9% and 45.0% at the respective concentrations of 500 ppm, 250 ppm, 125 ppm, 62.5 ppm and 31.25 ppm (Fig. 4). While in case of *F. moniliforme* the same was observed to be 80.0%, 70.2%, 59.8%, 48.3% and 26.2% at the respective concentrations of 500 ppm, 250 ppm, 125 ppm, 62.5 ppm and 31.25 ppm. Fungicidal bioassay of *F. oxysporum* and *F. moniliforme* treated by different concentrations of Ag-NPs are shown in Fig. 5. The biologically synthesised Ag-NPs alone or in their formulations can be employed as powerful weapons against plant and/or human fungal infections[30]. Many available reports strongly supports the present results of absorption spectrum, SEM and FTIR analysis[16,31]. Several reports has evidenced the bioefficacy of biologically synthesized Ag-NPs against various plant pathogens[7].

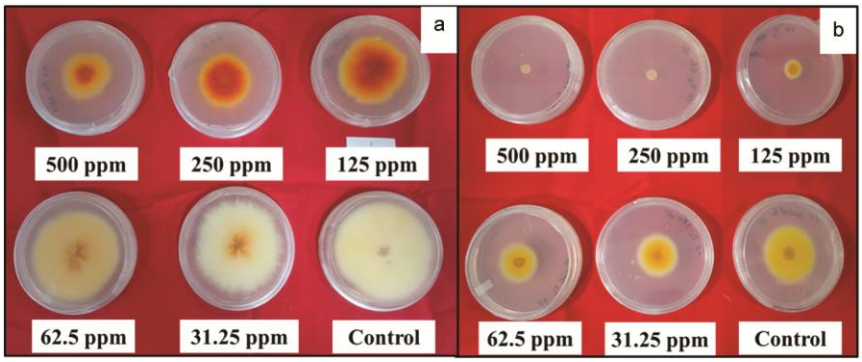


Fig. 5 — Fungicidal bioassay of *F. oxysporum* and *F. moniliforme* treated by different concentrations of Ag-NPs

## Conclusion

The *A.indica* leaf extract mediated synthesis of Ag-NPs is simple, economic and environment friendly. Moreover, *A.indica* is abundantly available and its leaves are obtained very easily. Biosynthesis of Ag-NPs by *A.indica* leaf extract was confirmed by UV-visible spectrophotoscopy, DLS, SEM, TEM and FTIR analysis. Antifungal activities of the biosynthesised Ag-NPs were then evaluated against *F.oxysporum* and *F.moniliforme*. These NPs can be explored for developing fungicidal formulations and exploited for the management of various plant fungi, pathogenic fungi during pre and post-harvest period.

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