Characterisation and antifungal activity of silver nanoparticles biologically synthesised by *Amaranthus retroflexus* leaf extract

Bahram Bahrami-Teimooria, Yaser Nikparastb, Mostafa Hojatianfarc, Mahdi Akhlaghiab and Hamid Reza Pourianfar

aIndustrial Fungi Biotechnology Research Department, Research Institute for Industrial Biotechnology, Iranian Academic Centre for Education, Culture and Research (ACECR)-Mashhad Branch, Mashhad, Iran; bDepartment of Agronomy, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran; cDepartment of Agricultural Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT
Following the emergence of resistant fungal pathogens, silver nanoparticles (AgNPs) biosynthesized by plants have been recognized as promising tools to combat parasitic fungi. This study evaluated the potency of *Amaranthus retroflexus* in producing AgNPs, followed by testing their antifungal effects. The AgNPs exhibited a maximum absorption at 430 nm through ultraviolet-visible spectroscopy, while the X-ray diffraction indicated that they were crystal in nature. Fourier transform infrared spectroscopy confirmed the conversion of Ag⁺ ions to AgNPs due to the reduction by capping material of plant extract. The transmission electron microscope analysis further revealed that the AgNPs were spherical ranging from 10 nm to 32 nm in size. The AgNPs at the concentrations of 50, 100, 200, and 400 μg/mL were applied to the growth of plant, mushroom, and human pathogenic fungi. The 50% minimum inhibitory concentrations (MIC₅₀) against *Macrophomina phaseolina*, *Alternaria alternata* and *Fusarium oxysporum* were observed to be 159.80 ± 14.49, 337.09 ± 19.72, and 328.05 ± 13.29 μg/mL, respectively. However, no considerable inhibition was observed regarding *Trichoderma harzianum* or *Geotrichum candidum*. These findings may suggest *A. retroflexus* as a green solution for biosynthesizing AgNPs with potent antifungal activities against plant pathogenic fungi.

KEYWORDS
antifungal activity; *Amaranthus retroflexus*; silver nanoparticles; characterisation; *Macrophomina phaseolina*

1. Introduction
Recent studies have suggested various biological sources for synthesising silver nanoparticles (AgNPs), including fungi, bacteria and plant extracts [1–4]. Among other suitable plant species, *Amaranthus* spp. have been reported to serve as good biological sources for AgNPs synthesis. *Amaranthus* spp. are flowering plants that belong to *Amaranthaceae* family and have long been used for food and medicinal purposes [5]. Few studies have shown that extracts obtained from *Amaranthus* spp. possess...
antimicrobial activities. According to the studies conducted so far, *A. hypochondricus* protein extract possesses potent antifungal activities towards *Alternaria alternata*, *Fusarium solani*, *Candida albicans*, *Fusarium oxysporum*, *Trichoderma* sp. and *Aspergillus ochraceus* [6].

In addition to plant extracts, the antimicrobial potential of AgNPs biosynthesised by *Amaranthus* spp. has been investigated. A recent study reported green synthesis of AgNPs using an aqueous solution of silver nitrate and *A. gangeticus* Linn (Chinese spinach) leaf extract. The biosynthesised AgNPs were demonstrated to exert antimicrobial activities towards *Bacillus subtilis*, *Shigella flexineri* and *Sclerotinia* sp [7]. Another study reported fabrication of an Ag-polyvinyl alcohol (Ag/PVA) nanocomposite using *A. tristis*-synthesised AgNPs. *Pseudomonas fluorescens* and *Klebsiella pneumoniae* were found to be inhibited by the Ag/PVA nanocomposite membrane [8].

To the best of our knowledge, no study has reported the biosynthesis of nanoparticles by *Amaranthus retroflexus*. In addition, there is limited quantified data regarding the susceptibility of parasitic fungi to AgNPs synthesised by *Amaranthus* spp. Here, we report the design and development of a green method to biosynthesise AgNPs employing dried leaves extracts of *A. retroflexus*. Further, antifungal activities of these synthesised AgNPs were quantifiably evaluated against several plant, mushroom and human parasitic fungi.

2. Materials and methods

2.1. Chemicals

Potato dextrose agar (PDA) media and silver nitrate salt (AgNO₃, 99%) were purchased from Quelab (Canada) and Merck (Darmstadt, Germany), respectively. All the reagents utilised in this study were freshly prepared before use.

2.2. Plant and fungi samples

Seeds of *A. retroflexus* were provided and authenticated by the Herbarium of Agronomy Department, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran. The seeds were cultivated and the resulting fresh leaves were then collected to be used for further experiments. The following fungal strains were kindly gifted from and authenticated by the Culture Collection of Plant Protection Department, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran: *Macrophomina phaseolina*, *A. alternata*, *F. oxysporum*, *Trichoderma harzianum* and *Geotrichum candidum*. The fungi were grown onto PDA and incubated at 25 °C to obtain pure cultures.

2.3. Preparation of leaf extract

The fresh leaves of *A. retroflexus* were washed several times in running tap water and finally in distilled water. Four grams of the air-dried leaves were mixed with 100 mL of distilled water, keeping in a water bath at 55 °C for 15 minutes followed by cooling to the room temperature. The extracts were then filtered through Whatman filter papers No. 1 and stored at 4 °C until use [4].
2.4. **Biosynthesis of silver nanoparticles by *A. retroflexus***

A total of 10 mL of the *A. retroflexus* leaf aqueous extract was added to 90 mL of silver nitrate solution (1 mM). The reaction was allowed to stand in at room temperature overnight, kept in the dark in order to minimise photo activation of silver nitrate. Then, the solution containing the formed AgNPs was centrifuged at 15,000 rpm for 10 minutes followed by drying in a vacuum oven at 60 °C for 24 hours [4]. The pellet was collected and re-dispersed in glass-distilled water, removing any interactive biological molecules.

2.5. **Characterisation of silver nanoparticles**

2.5.1. **UV-visible spectroscopy**

The formation of AgNPs in the leaf extract was confirmed through a colour change from pale yellow to dark brown. The colour change was recorded under the UV-visible (UV-vis) spectroscopy between 300 and 700 nm using an Agilent 8453 spectrophotometer (USA).

2.5.2. **Transmission electron microscopy**

Transmission electron microscopy (TEM) was performed by a Leo 912 AB instrument (Germany). In brief, a drop of appropriately diluted sample of AgNPs was poured on carbon-coated copper grids and allowed to stand for 2 minutes. The excess solution was removed using a blotting paper and allowed to be dried at room temperature.

2.5.3. **X-ray diffraction analysis**

The lyophilised AgNPs coated on X-ray diffraction (XRD) grid were subjected to XRD measurements. The analysis was carried out in an X-ray diffractometer with an operating voltage of 45 KV and current of 0.8 mA (Unisantis XMD-300, Swiss). The diffraction patterns were recorded by Cu-Kα radiation of wavelength 1.54 Å in the region of 2θ from 30° to 80°.

2.5.4. **Fourier transform infrared spectroscopy**

The aqueous leaf extract and AgNPs were subjected to Fourier transform infrared (FTIR) spectroscopy (Thermo Nicolet AVATAR 370) in order to analyse their spectra. The analysis was carried out with KBr pellets, recorded in the range of 500–4000 cm⁻¹.

2.6. **Antifungal activity of AgNPs**

The antifungal potential of the biosynthesised AgNPs was investigated according to a modified method described elsewhere [9]. Five millilitre of the AgNPs at various concentrations (50, 100, 200 and 400 μg/mL in sterile distilled water) was added into 5 mL of the autoclaved media before it solidified. The mixture was poured into the sterile 8-cm Petri dishes. Negative controls containing media only were also considered. The Petri dishes were then incubated in the dark at 25 °C for 48 hours, after which the Petri dishes were inoculated with agar plugs of the growing fungal mycelia (5 mm in diameter). The plates were incubated in the dark at 25 °C for further five days. The radial growth of fungal mycelia was calculated using the mean of two fungal colony diameters at right angles. The
inhibition potency of AgNPs towards each fungal strain was calculated using the following equation: Inhibition rate (per cent) = \( R - \frac{r}{R} \times 100 \); where \( R \) is the radial growth of fungal mycelia in the negative control (cm) and \( r \) is the radial growth of fungal mycelia challenged with the AgNPs.

2.7. Statistical analysis

All the experiments were independently repeated at least three times. In each experiment and for each fungal strain, three Petri dishes were used to calculate the radial growth rate. GraphPad Prism version 6 was utilised to conduct statistical analyses and one-way analysis of variance (ANOVA) tests. Means were compared using Tukey multiple comparisons test with a significance level of 0.05. The 50% minimum inhibitory concentration (MIC\(_{50}\)) of the AgNPs was interpolated using linear regression analysis of the relevant dose-response curve.

3. Results and discussion

3.1. Characterisation of the biosynthesised AgNPs

3.1.1. UV-vis spectroscopy

In this study, dried leaves of freshly growing \( A. \) \( \text{retroflexus} \) plants (Figure 1) were utilised for extraction. Following the addition of silver nitrate to the dried leaf extract, a colour change from pale yellow to dark brown was observed (Figure 2). This colour change has previously been attributed to the excitation of surface plasmon resonance by AgNPs [10]. The reduction of AgNPs in the aqueous \( A. \) \( \text{retroflexus} \) leaf solution was further confirmed by the UV-vis spectroscopy. After 24 hours of incubation, the sharpening of the absorption at 430 nm confirmed that the particles were mono-dispersed (Figure 3).

3.1.2. Transmission electron microscopy analysis

Microscopic features of the AgNPs, including morphology and particle size, were assessed through TEM analysis. Figure 4 illustrates the TEM image of AgNPs synthesised by the leaf extract of \( A. \) \( \text{retroflexus} \). As depicted by the TEM image, the particles were mostly spherical in shape with a diameter ranging from 10 to 32 nm (Figure 4). This size range is smaller than those of AgNPs synthesised by many other \( A. \) \( \text{amaranthus} \) species, including \( A. \) \( \text{viridis} \); 10–45 nm [11], \( A. \) \( \text{tristis} \); 20–40 nm [8] and \( A. \) \( \text{dubius} \); 10–70 nm [12]. By contrast, the size range of AgNPs biosynthesised by \( A. \) \( \text{gangeticus} \) was reported to be 11–15 nm which was smaller than that reported by us here [7]. It has been known that reducing the size of nanoparticles may enhance their antibacterial activities [13]. However, no comparison could be made here with regard to antifungal activity, as any of the aforementioned studies have not reported quantified antifungal activities of AgNPs.

3.1.3. X-ray diffraction pattern

The XRD results showed clear diffraction line at low angles, ie 30°–80° (Figure 5). The Bragg reflections at angles 2\( \theta \) of 38.18°, 44.35°, 64.4° and 77.3° corresponded to 111, 200, 220 and 311 sets of lattice planes, respectively. This band pattern confirmed the structure
Figure 1. *Amaranthus retroflexus* plant leaves grown in this study.

Figure 2. Optical photographs of the AgNPs synthesis by *Amaranthus retroflexus*. *A. retroflexus* leaf extract (a); the biosynthesised silver nanoparticles 24 hours after the addition of silver nitrate to the leaf extract (b).
of silver as a face-centred cubic structure [14]. Therefore, the XRD data clearly demonstrated the presence and crystal structure of the silver in the A. retroflexus leaf extract.

3.1.4. Fourier transform infrared spectra
Various absorption bands in the FTIR spectra showed different chemical groups in the extract containing biosynthesised AgNPs. While a broad band at 3273 cm\(^{-1}\) showed the stretching vibrations of \(-\text{N-H}\) and \(-\text{O-H}\) groups, the absorption bands at 2924, 1640, 1383 and 1033 cm\(^{-1}\) corresponded to \(-\text{C-H}\), \(\text{C=O}\), \(\text{C=C}\) and \(\text{C-O-}\) groups, respectively. Additionally, the absorption bands of 1155 and 1068 cm\(^{-1}\) confirmed the presence of \(\text{C-N}\) group. The weak bands at 726 and 612 cm\(^{-1}\) were due to the out-of-plane bending vibrations of \(-\text{O-H}\) and \(\text{C-H}\) groups, respectively. In addition, the peaks recorded

\[ \text{Figure 3. UV-vis spectrum of the AgNPs biosynthesised by A. retroflexus leaf extract.} \]

\[ \text{Figure 4. Transmission electron microscopy (TEM) image of biosynthesised AgNPs.} \]
at 1234 cm$^{-1}$ indicated that the amino groups were partially utilised for the encapsulation and stabilisation of the biosynthesised AgNPs (Figure 6).

The presence of $\text{--N--H}$, $\text{--O--H}$, $\text{C=C}$ and $\text{--C--H}$ groups in the FTIR spectra suggests that the A. retroflexus extract contained the hydroxyl and amino groups substituted flavonoids, as similarly reported elsewhere [4]. The flavonoids can act as reducing agents (which reduce Ag$^+$ to Ag$^0$), whereas the amino group serves as a stabilising agent in the green synthesis of AgNPs [15]. Thus, the FTIR spectra obtained in this study may explain the interaction of the leaf biomolecules of A. retroflexus with silver nitrate, leading to biosynthesis of AgNPs. In addition, the FTIR data reveal the multi-functionality of the aqueous extract of A. retroflexus where reduction and stabilisation occur simultaneously.

Figure 5. X-ray diffraction patterns of AgNPs synthesised by A. retroflexus.

Figure 6. Fourier transform infrared spectroscopy of biosynthesised Ag nanoparticles with its leaf extract.
3.2. Antifungal activity of Amaranthus retroflexus-synthesised AgNPs

The inhibition of the mycelia growth by the AgNPs at various concentrations were daily evaluated and photographed for all the tested fungi, in comparison to the negative control. As an example, Figure 7 illustrates the mycelial growth of *M. phaseolina* challenged with the biosynthesised AgNPs in comparison to the negative control (Figure 7). Moreover, the antifungal activities of the AgNPs were quantified and statistically analysed (Table 1).

The quantified findings showed differences in antifungal activities of the AgNPs across the tested fungi and various concentrations. The AgNPs significantly prevented the growth of *M. phaseolina*, *A. alternata* and *F. oxysporum* in a dose-dependent manner as compared to the negative control (*p* < 0.05). Among the tested fungi, the highest

![Figure 7. Quantified and observational effects of AgNPs on the growth of *Macrophomina phaseolina*. The photos have been taken five days after inoculation of the AgNPs-containing media with *M. phaseolina* negative control (a); *M. phaseolina* treated with AgNPs at 50 μg/mL (b); 100 μg/mL (c); 200 μg/mL (d); 400 μg/mL (d).](image)

**Table 1.** Quantified susceptibility of pathogenic fungi to *Amaranthus retroflexus*-synthesised AgNPs.

| Fungi                  | Concentration (μg/mL), mean ± SD (n ≥ 3) | Interpolated MIC50 |
|------------------------|----------------------------------------|-------------------|
|                        | 50          | 100          | 200          | 400          |                  |
| *Macrophomina phaseolina* | 31.75 ± 2.23a | 50.35 ± 1.13b | 54.77 ± 0.88c | 71.09 ± 1.41d | 159.80 ± 14.49A |
| *Alternaria alternata*  | 9.84 ± 2.81a | 27.35 ± 2.9b  | 31.35 ± 1.47b | 57.55 ± 2.85c | 337.09 ± 19.72B |
| *Fusarium oxysporum*    | 9.403 ± 0.05a | 24.84 ± 0.55b | 40.18 ± 0.31c | 53.83 ± 1.04d | 328.05 ± 13.29C |
| *Trichoderma harzianum* | 12.90 ± 1.08a | 19.35 ± 1.63b | 24.36 ± 2.22c | 27.40 ± 2.20c | >400              |
| *Geotrichum candidum*   | Nd          | Nd           | Nd           | Nd           | >400              |

Note: Values represent amounts of prevention (percentage) of fungal mycelial growth as compared to the negative control. Superscript lower-case letters within each row indicate statistical comparisons among the concentrations of each fungus. In the column of the interpolated MIC50, superscript upper-case letters show statistical comparisons made between the fungi. Means followed by the same letters are not significantly different (Tukey, *p* value < 0.05). Nd: not determined because no measurable growth inhibition was observed; MIC50: the 50% minimum inhibitory concentration was defined as the concentration of AgNPs at which the mycelial growth of the tested fungi was inhibited as much as 50%.
inhibition rate (71.09% ± 1.41%) was observed with *M. phaseolina* at 400 μg/mL of the AgNPs while the lowest one (9.84% ± 2.81%) was seen with *F. oxysporum* at 50 μg/mL of the AgNPs (*p* < 0.05). In addition, the least effective concentration of the biosynthesised AgNPs was found to be 50 μg/mL which caused 31% inhibition of the growth in *M. phaseolina*. The antifungal effect of the AgNPs was very limited against *T. harzianum* while no measurable antifungal effect was seen with *G. candidum* even at the highest concentration of the AgNPs (Table 1).

MIC$_{50}$ values of the AgNPs were also interpolated to be 159.80 ± 14.49, 337.09 ± 19.72 and 328.05 ± 13.29 μg/mL against *M. phaseolina*, *A. alternata* and *F. oxysporum*, respectively. According to the statistical analysis, there was no difference in MIC$_{50}$ between *F. oxysporum* and *A. alternata* (*p* ≥ 0.05) even though the AgNPs demonstrated the lowest MIC$_{50}$ against *M. phaseolina* in comparison to the other fungi (*p* < 0.05). No MIC$_{50}$ of AgNPs could be determined towards *T. harzianum*, or *G. candidum* at the range of the tested concentrations (Table 1).

According to all of the observational and quantified findings, the AgNPs were found to be much more effective against the growth of plant pathogenic fungi (*M. phaseolina*, *A. alternata* and *F. oxysporum*) than that of human (*G. candidum*) or mushrooms (*T. harzianum*) pathogenic fungi. In this regard, *M. phaseolina* and *G. candidum* were the most sensitive and resistant microorganism to the *A. retroflexus*-synthesised AgNPs, respectively.

Due to the environmental concerns regarding the use of chemical agents, bio-control agents are largely used in plant disease control programmes [16]. Among others, several species of *Trichoderma* have been suggested as promising biological control agents against a number of plant pathogenic fungi, including *F. oxysporum* [16–18]. *F. oxysporum* is known as a root-infecting fungal pathogen that causes several plant diseases on a broad range of plant species. The findings of our study may have implications in bio-control disease programmes, as no inhibition was observed against *T. harzianum* even at the highest concentration while the AgNPs considerably inhibited the growth of *F. oxysporum*. These results may propose that the use of biosynthesised AgNPs along with *T. harzianum* could serve as effective green tools in order to inhibit the growth of pathogenic fungi *F. oxysporum*.

### 4. Conclusion

In the absence of knowledge on the potency of *A. retroflexus* in biosynthesising AgNPs, the present study reports the biosynthesis of AgNPs by *A. retroflexus* leaf extract, confirmed by XRD, TEM, UV-vis spectroscopy and FTIR analyses. Antifungal activities of the biosynthesised AgNPs were then evaluated towards plant, mushroom and human pathogenic fungi. There is a body of information on antimicrobial activities of AgNPs biosynthesised by a broad range of plant species [19], but little attention has been given to their antifungal activities. In conclusion, the data presented here demonstrate that *A. retroflexus* may be considered a green tool for synthesising AgNPs with efficient antifungal activity, particularly against plant fungi: *M. phaseolina* and *F. oxysporum*. Thus, the findings of this study could be adopted for several applications in the plant protection field. Further studies are underway to investigate treatment of pathogenic fungi-damaged plants by the *A. retroflexus*-derived AgNPs. However, further research is also warranted to investigate whether the application of AgNPs into the soil might cause unwanted damages to useful bacteria, as it has been well known that nanoparticles have potent antibacterial...
activity. Other biological potentialities, namely antibacterial and anticancer activities of A. retroflexus-mediated nanoparticles could be also studied in the future.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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