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Green Silver Nanoparticles for Phytopathogen Control

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Abstract

Plant diseases bring radical problem in the agriculture sector. Phytopathogens mediate diseases that pose considerable loss of yield and quality deterioration which eventually bring down the crop yield and the rural economy. The present study is, thus, focused on developing the optimized protocol for the synthesis of silver nanoparticles (AgNPs) by green chemistry approach and revealing their antimicrobial potential against phytopathogens. The synthesis of AgNPs was carried out by using aqueous plant extracts of three medicinal and aromatic plants, namely *Allium cepa* (onion), *Allium sativum* (garlic) and *Zingiber officinale* (ginger). AgNPs were characterized by various analytical techniques including UV–visible spectra, PSA, FTIR, TEM and XRD analysis. The AgNPs were spherical with size ranging from 1 to 10 nm, crystalline in nature and relatively stable up to 3 months after synthesis. The AgNPs conferred strong antimicrobial activity against selective bacterial and fungal phytopathogens. The antimicrobial activity of the AgNPs was observed against *Erwinia* sp., *Pseudomonas syringe*, *Bacillus megaterium*, *Fusarium graminearum*, *F. avenaceum* and *F. culmorum*. The effective concentration against bacterial pathogens was found to be between 50 µg/ml (garlic/*Erwinia* sp.) and 130 µg/ml (onion/*B. megaterium*). In the case of fungal pathogens, the range was 90 µg/ml (garlic/*F. avenaceum*) to 110 µg/ml (onion/*F. graminearum*) for an effective dose.

Keywords

Nanoparticles
Green synthesis
Antimicrobial
Plant pathogens

Abbreviations

AgNPs	Silver nanoparticles
FTIR	Fourier transform infrared
MAS	Marker-assisted selection
TEM	Transmission electron microscope
XRD	X-ray diffraction

Significance Statement

Higher crop losses due to phytopathogens have been a major problem in agriculture. Due to the limitations of conventional methods for disease control, there is a need for alternate approaches. The urge for an effective novel approach has shifted the focus toward nanobiotechnology. Metal nanoparticles have been reported for their efficacy in controlling various pathogens. The chemical synthesis of nanoparticles is considered as non-eco-friendly and expensive. Therefore, the biosynthesis of nanoparticles through plants has appeared as a solution of the above-mentioned problem. So, the present study was focused on the synthesis of metal nanoparticles using plants. The developed nanoparticles were found to be effective against phytopathogens, which could play a promising role in crop protection and disease management.

Introduction

Plant disease control is the need of the hour for modern agriculture because phytopathogen-based loss of the crop, unfortunately, reverses the efforts of plant breeders for increasing crop productivity and total crop production [1, 2]. The estimated crop losses due to plant pathogens in the USA, Europe, Africa and Asia were reported to be 11.5%, 13.1%, 12.9% and 11.3%, respectively [3]. Microbial infection-routed plant diseases may be extremely harmful due to the production of poisonous toxins [4]. Among top ten fungal and bacterial pathogens, *Fusarium* sp., *Pseudomonas* sp. and *Erwinia* sp. are found to be most destructive as they attack major crops viz. wheat, barley, tomato, apple, pear, quince, blackberry, raspberry carrots, potatoes, cucumbers, onions, lettuce and lower the yield at large scale [5, 6]. The loss of yield by *Fusarium* sp., *Pseudomonas* sp. and *Erwinia* sp. has been reported to be over 70%, 50% and 100%, respectively, under favorable conditions for microbial growth [7].

The alternative approaches such as antagonistic microorganisms [8], marker-assisted selection (MAS) [9], crop rotation [10] and use of chemicals (http://extension.udel.edu/factsheet/gray-leaf-spot-on-corn/) have been major past practices to control phytopathogens [11]. These methods are having several limitations like soil toxicity, higher cost as well as incomplete eradication of pathogens which means that there is considerable motivation for finding efficient antifungal agents via the eco-friendly approach. So there is a need to design some novel techniques in modern agriculture to minimize losses caused by these phytopathogens. Nanotechnology is emerging as a promising interdisciplinary field of biotechnology and nanoscience, which offers significant applications in different scientific fields such as agriculture, medicine, biotechnology, chemistry, physics and material sciences [12]. Due to the small size of

nanoparticles (1–100 nm) and large surface area to mass ratio, they exhibit tremendously unique physical, chemical and biological properties in comparison with the bulk material of the similar composition. Silver has been reported among various metallic nanoparticles to be the most effective against a wide range of pathogens [13]. Various chemical and physical methods have been employed for synthesizing AgNPs using toxic chemicals and complex processes, which require high amounts of energy and may spoil the environment. All these drawbacks thus necessitate employing some novel environmental friendly, economical as well as industrially viable approach. The current nonconventional methods employed for AgNPs syntheses such as using microbes and plant extracts have emerged as a suitable and viable approach [14, 15, 16, 17, 18, 19]. The methods, however, are still underdeveloped because problems like particle stability, aggregation and control of crystal growth are encountered which needs a lot of optimization efforts. Further, plant-mediated synthesis of AgNPs is preferred over microbe-mediated synthesis as it employs eco-friendly solvents and does not involve any special isolation and culture preparation techniques [20]. Green synthesis of nanoparticles has been reported for several plant species such as mangosteen [14], *A. cepa* [21, 22, 23] and *A. sativum* [24] which further can be used against phytopathogens. AgNPs have been used against phytopathogens, antifungal activity of *A. cepa* against *F. oxy*sp*orum* [23], and antimicrobial activity of *Z. officinale*-synthesized nanoparticles was studied against food pathogens by Velmurugan et al. [13].

Predominantly, studies were focused on the use of biosynthesized AgNPs against human pathogens. However, their antimicrobial potential against phytopathogens is not much explored for plant disease management and crop protection. Therefore, the present study was proposed to undertake the biosynthesis of AgNPs by using plant extracts (*A. cepa*, *Z. officinale* and *A. sativum*), optimizing the conditions required for maximum yield of AgNPs and their antimicrobial potential against phytopathogens such as *Fusarium* sp., *Erwinia* sp., *Pseudomonas* sp. and *Bacillus* sp.

Materials and Methods

Materials

Analytical grade silver nitrate ($AgNO_3$) was procured from HiMedia chemicals Ltd. *A. cepa*, *A. sativum* and *Z. officinale* were bought from the local market of Phagwara, Punjab. The pure cultures of *P. syringe* (MTCC 1604), *Erwinia* sp. (MTCC 2760), *B. megaterium* (MTCC 6544), *F. graminearum* (MTCC 1893), *F. avenaceum* (MTCC 10572) and *F. culmorum* (MTCC 349) for antimicrobial

studies were procured from Microbial Type Culture Collection (MTCC) facility, Institute of Microbial Technology (IMTECH), Chandigarh, India.

Preparation of Plant Extracts

The plant extracts were prepared using bulbs of *A. cepa* and *A. sativum* and rhizomes of *Z. officinale*. In a typical reaction set, respective plant component (20 g) was finely chopped, crushed using mortar and pestle, followed by boiling for 5–10 min in 100 ml of distilled water in case of *A. cepa* and *Z. officinale* [21] and without boiling in the case of *A. sativum* [24]. The extract was filtered using Whatman filter paper for further using in the synthesis of AgNPs.

Green Synthesis of Silver Nanoparticles (AgNPs)

A final volume of 100 ml was attained by adding 50 ml (5 mM) of $AgNO_3$ and 50 ml of extract. The reaction was performed in darkness. The experiments were carried out in different conditions by the varying ratio of extract, the concentration of $AgNO_3$, pH and temperature for optimization. The ratio of extract was varied from 5:5 to 9:1, concentration of $AgNO_3$ from 1 mM to 5 mM, pH varied from 2 to 12 and temperature was varied from 40 to 60 °C. The effect of these parameters on the synthesis of AgNPs was monitored by UV–Vis spectrophotometer [23].

Characterization of Synthesized AgNPs

The formation of AgNPs was indicated by the appearance of the brown color of silver nanocolloid. The optical, structural, morphological, elemental and functional characterization of the AgNPs was carried out by using the UV–visible spectrophotometer, particle size analyzer (PSA), Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and transmission electron microscope (TEM), respectively. UV–visible spectra of the AgNPs were obtained by using a Shimadzu UV-1800 spectrophotometer under wavelength range of 200–800 nm. The infrared spectrum was obtained by using Shimadzu FTIR-8400S spectrophotometer. TECNAI TEM (Fei, Electron Optics) and Hitachi (H-7500) TEM operating at 200 kV were used to study the morphology and size of the silver nanoparticles. The optical density (OD) measurements were performed at 600 nm using an Elico SL 159 UV–visible spectrophotometer.

Antimicrobial Activity of Silver Nanoparticles

For antimicrobial studies, AgNPs prepared using *A. cepa* (8:2 extract, 5 mM $AgNO_3$ at pH 12 and 40 °C), *Z. officinale* (7:3 extract, 5 mM $AgNO_3$ at pH 12, 60 °C) and *A. sativum* (9:1 extract, 5 mM $AgNO_3$ at pH 12 and 60 °C) were

chosen. The antimicrobial activity of AgNPs was studied by the minimum inhibitory concentration (MIC) test [23]. The quantitative analysis was performed by culturing *P. syringe*, *Erwinia* sp., *B. megaterium* in Luria–Bertani (LB) broth medium and *F. graminearum*, *F. culmorum* and *F. avenaceum* in potato dextrose broth medium (PDB) supplemented with variable concentrations of AgNPs (10, 30, 50, 70, 90, 110, 130 and 150 µg/ml, respectively). *P. syringe*, *Erwinia* sp., *B. megaterium* and *F. graminearum*, *F. culmorum* and *F. avenaceum* cells treated with variable concentrations of the AgNPs suspensions were incubated at 37 °C for 18 h and 28 °C for 48 h, respectively. The cells grown in the medium devoid of AgNPs served as the microbial control, and the cells grown only in the extract served as the extract control. To examine the antimicrobial activity, the absorbance of the samples was recorded at 600 nm.

Results and Discussion

Optimization and Characterization

The preliminary synthesis of AgNPs from *A. cepa*, *Z. officinale* and *A. sativum* extract was observed by a change in color from original to dark brown upon addition of plant extract along with a silver ion (Ag^+) solution (Fig. 1). The synthesis process was rapid as AgNPs were formed within 5–10 min after coming in contact with silver ion (Ag^+) solution. UV–Vis absorption spectra of AgNPs conferred absorbance maxima at 407 nm, 400 nm and 427 nm for *A. cepa*, *Z. officinale* and *A. sativum*, respectively, as shown in Fig. 2. The ratio of plant extracts to the metal precursor ($AgNO_3$) was varied from 5:5 to 9:1 for all the three plant extracts (*A. cepa*, *Z. officinale* and *A. sativum*). The maximum absorbance was observed at 9:1 for *A. sativum*, followed by 8: 2 for *A. cepa* and 7: 3 for *Z. officinale*. The effect of the concentration of $AgNO_3$ solution (1 mM to 5 mM) was also determined. The higher concentrations showed an increase in band intensity. The biosynthesis of AgNPs was confirmed by the occurrence of surface plasmon resonance (SPR) near 400 nm at 5 mM $AgNO_3$ concentration for all aqueous plant extracts.

Fig. 1

An illustrative picture of change in color from **a** original to **b** dark brown by redox reaction of plant extract along with a silver ion (Ag^+) solution

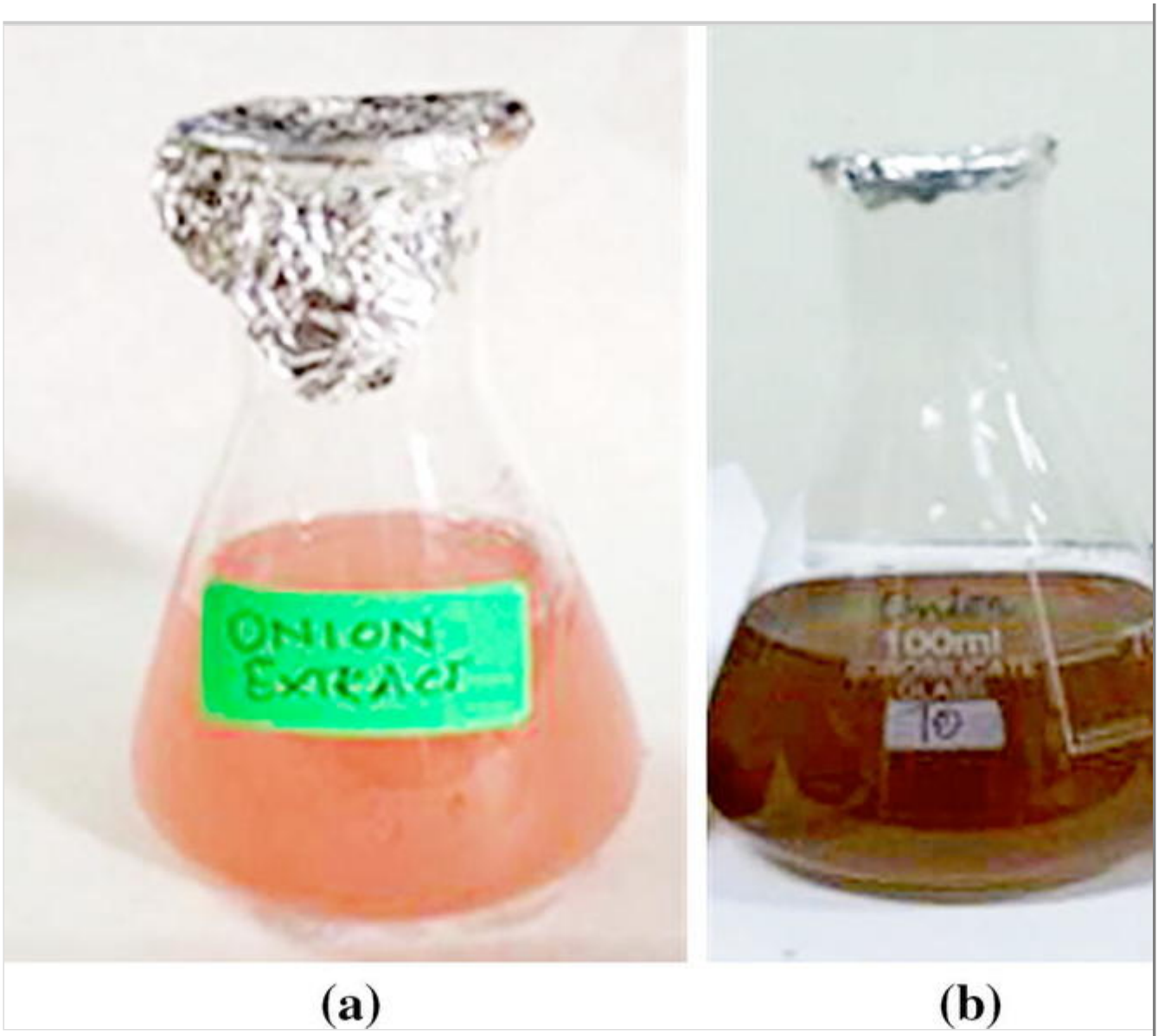
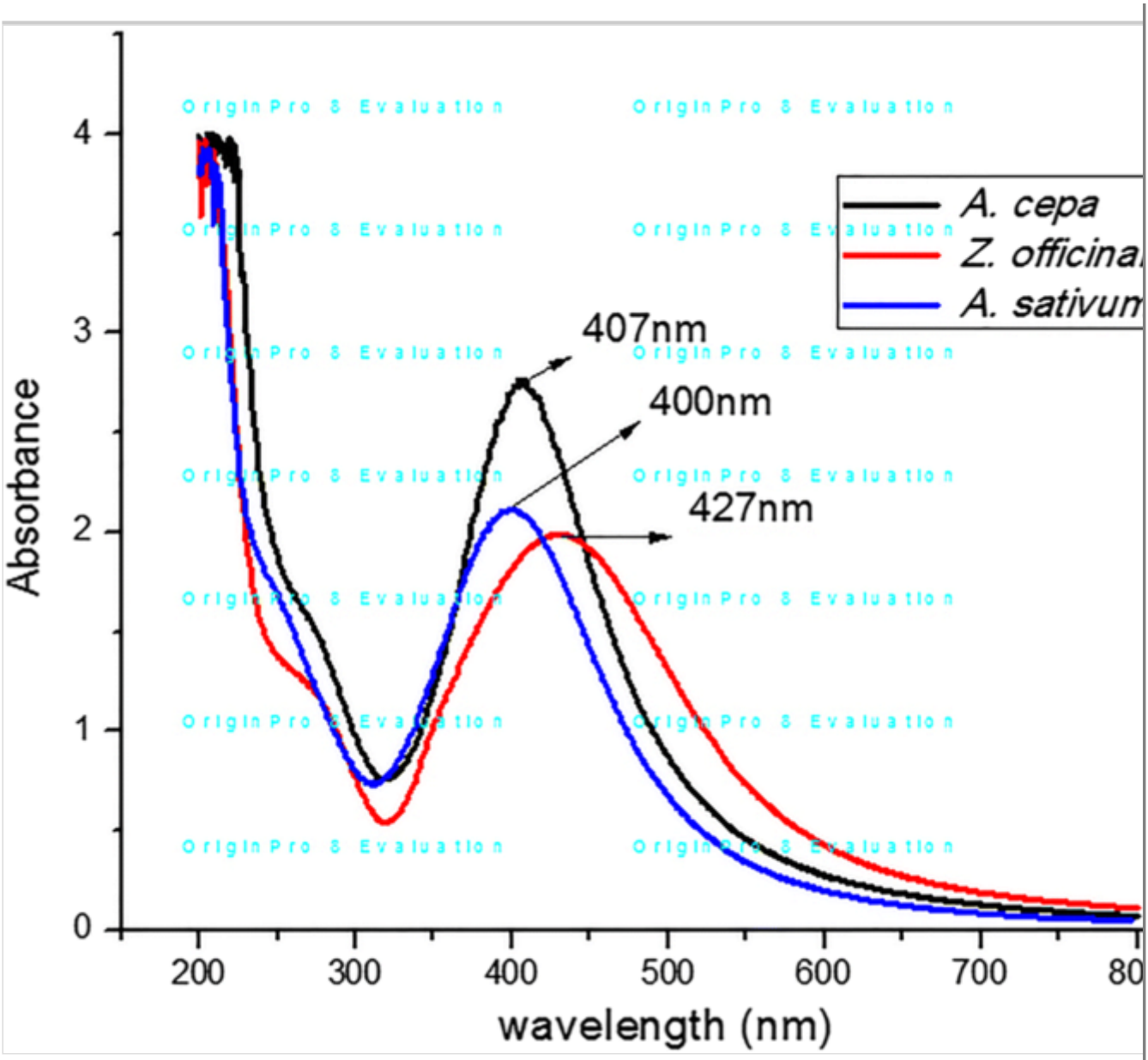


Fig. 2

Absorption spectrum of various plant extracts based on green-synthesized AgNPs



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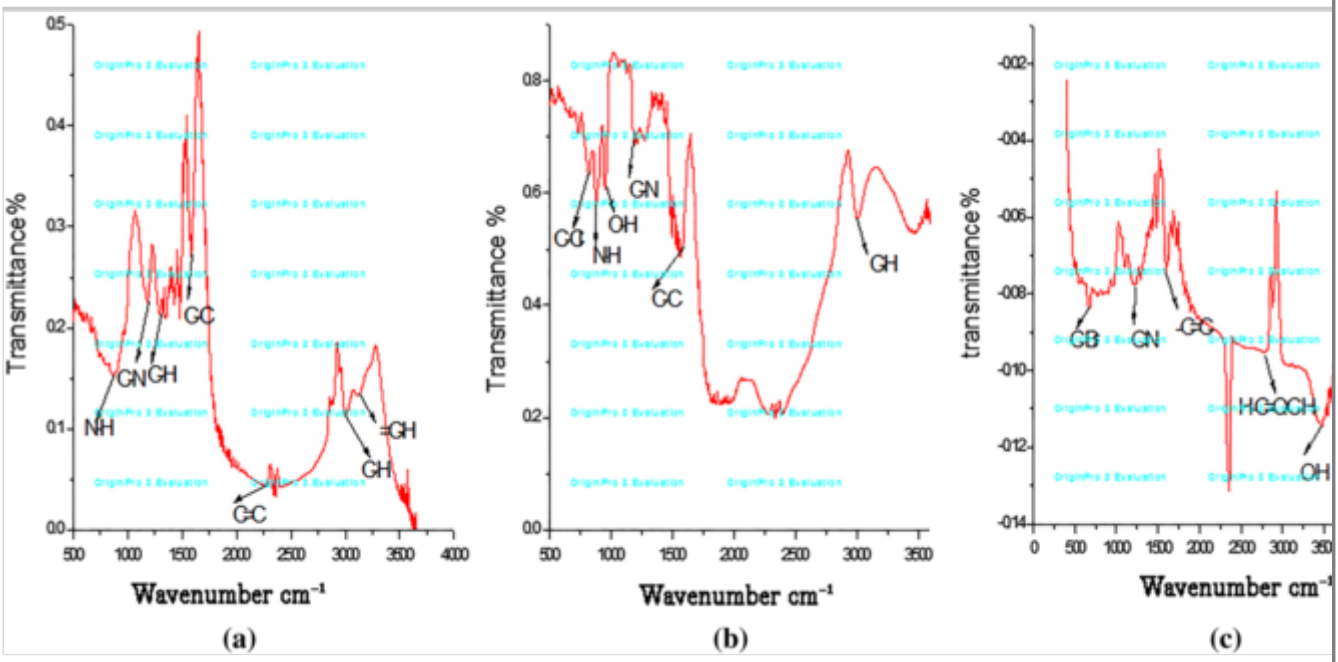
A marked variation in plasmon band intensity was observed when different temperature conditions (40, 50 and 60 °C) were applied. The optimum temperature for *Z. officinale* and *A. sativum* was found to be 60 °C, followed by 40 °C in the case of *A. cepa* leading to enhanced synthesis of AgNPs. On varying pH, the maximum yield of AgNPs was observed in alkaline conditions for all the extracts. In all the plant extracts, pH 12 was found to be the most suitable for AgNPs synthesis. It was found that higher pH showed sharp peaks than lower pH values (2, 4 and 6). The functional groups present in the nanoparticles were identified by FTIR. The bands between the wave numbers of 800–3000 cm^{-1} in the IR spectrum revealed the biochemical compositions, especially the moieties of carbohydrate, lipid, protein and polyphenols. FTIR spectrum of *A. cepa*-mediated synthesized AgNPs (Fig. 3a) showed $AgNO_3$ peaks at 878.609, 1198.13, 1298.422, 1588.62, 2256.21 and 3004.88 cm^{-1}. The IR bands in *Z. officinale*-mediated AgNPs (Fig. 3b) were observed at 808.48,

880.09, 950.33, 1188.58, 1578.32 and 3010.57 cm^{-1}. In the case of *A. sativum* (mediated synthesized AgNPs Fig. 3c), the first band appeared at 669.70 cm^{-1} followed by 1207, 1593, 2798 and 3454 cm^{-1}.

Fig. 3

FTIR analysis of biosynthesized AgNPs **a** *A. cepa*, **b** *Z. officinale* and **c** *A. sativum*



TEM images revealed spherical shape and monodisperse nature of synthesized AgNPs for all the three plant extracts. The particle size (nm) ranges from 1–9, 1–6 to 2–10 with maximum particle size (nm) distribution at 6, 2 and 3 for *A. cepa*, *Z. officinale* and *A. sativum*, respectively (Fig. 4a–c). However, particle size observed via PSA was of a higher range than what was obtained through TEM as shown in a representative graph of particle size for *A. cepa* by dynamic light scattering (Fig. 5a). The polydispersity index (PDI) of the optimized batch was found to be 0.530, and the particle diameter (*z*-average) was approximately 143.5 nm for *A. cepa* (Fig. 5b). The size obtained through PSA is usually larger than TEM, which may be due to the effect of Brownian motion of dispersed nanoparticles. The XRD pattern of AgNPs was observed at 2θ values for *A. cepa* (29.43°, 38.12°, 44.23°, 64.41° and 77.32°), for *Z. officinale* (29.42°, 38.08°, 44.35°, 64.40° and 77.38°) and for *A. sativum* (29.36°, 38.24°, 44.36°, 64.66° and 77.47°) as given in Fig. 6.

Fig. 4

TEM images of biosynthesized AgNPs and their particle size distribution. **a** *A. cepa*, **b** *Z. officinale* and **c** *A. sativum*

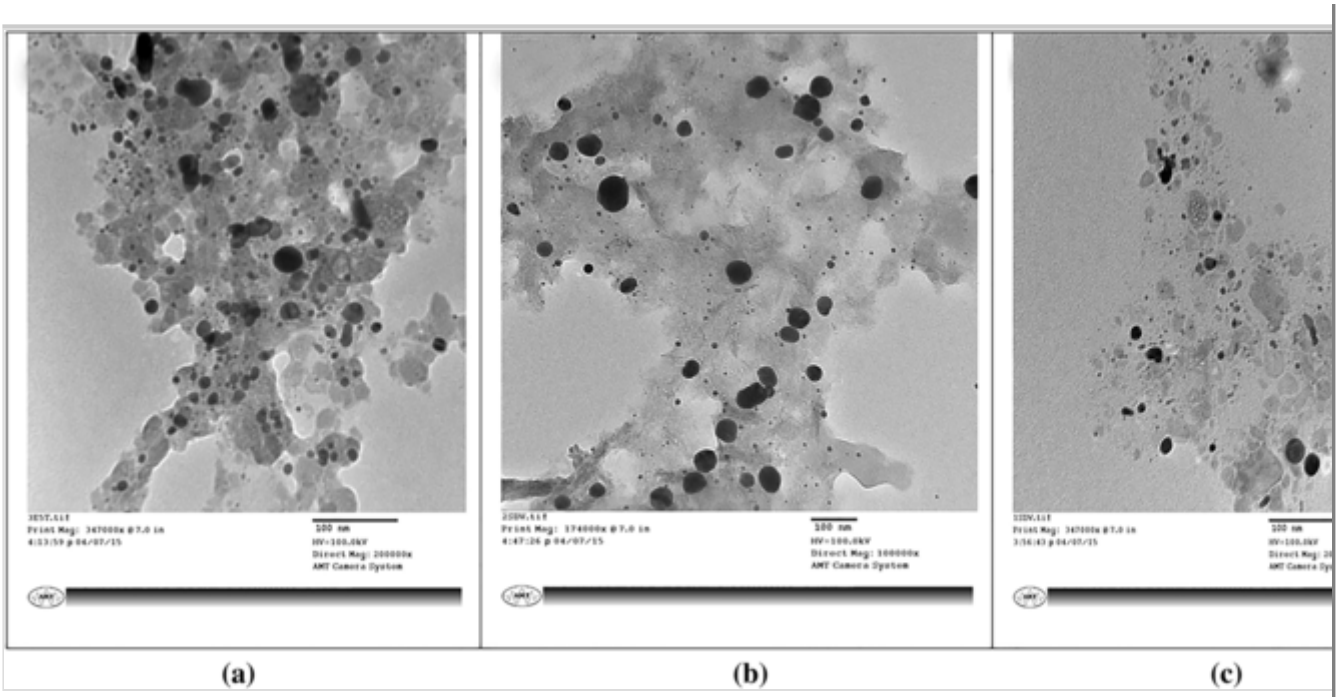


Fig. 5

Representative picture of **a** zeta potential value and **b** PSA of biosynthesized silver nanoparticles from *A. cepa*

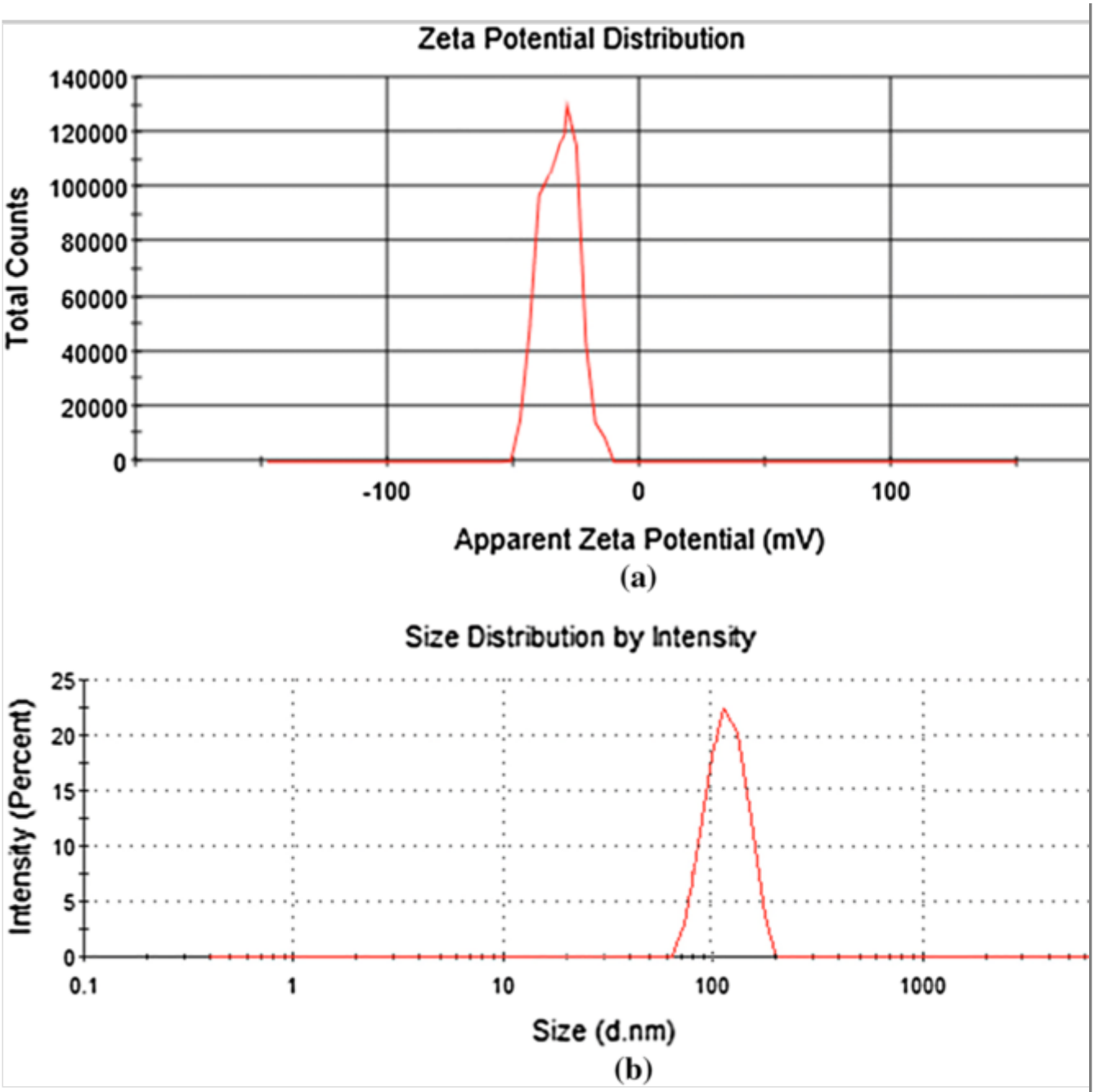
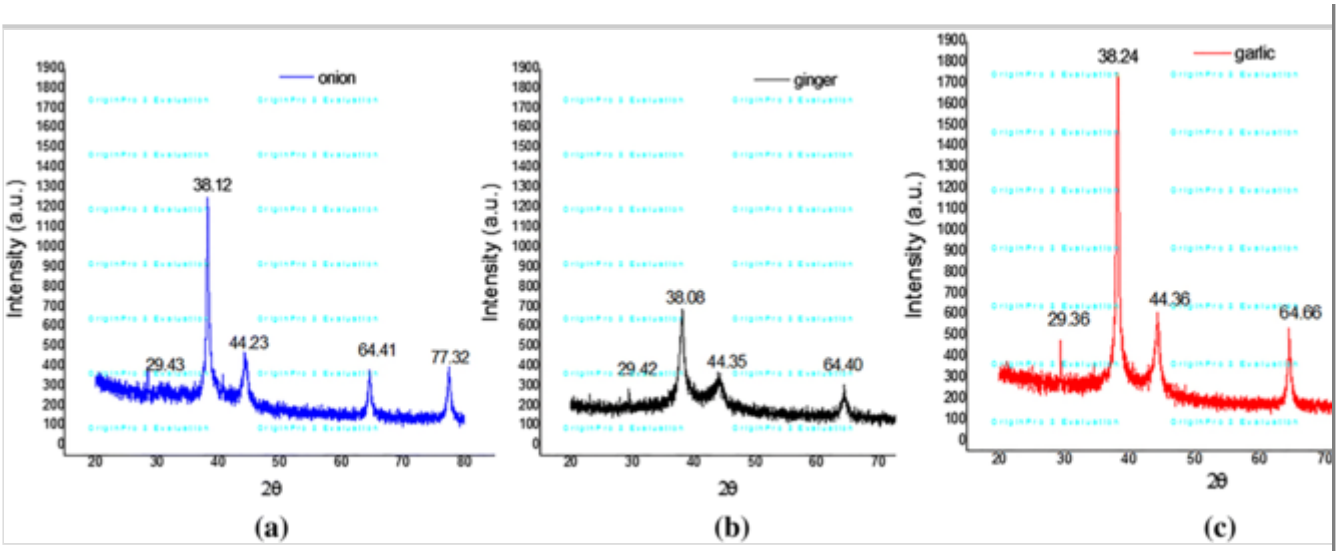


Fig. 6

XRD analyses of AgNPs biosynthesized by plant extracts **a** *A. cepa*, **b** *Z. officinale* and **c** *A. sativum*



The Ag^{+} gets reduced to silver atoms by plant extracts which nucleate to form nanocrystallites for growth. In previous studies, the color change was used as an initial parameter for AgNPs synthesis using extracts of *A. cepa*, *Musa acuminata* [23], *Helianthus tuberosus* [25], *Azadirachta indica* [18] and *Citrullus lanatus* [19]. The occurrence of SPR in the absorbance spectra confirmed the synthesis of AgNPs using UV–Vis spectrophotometer. Similar studies on *A. cepa*-synthesized nanoparticles produced absorption band at 401 nm [23], whereas, in the case of *Z. officinale*, it was found to be at 450 nm [26] and 436 nm [23]. White II et al. [24] observed maximum absorbance at 404 nm using *A. sativum* extract.

Various reaction conditions such as the ratio of plant extract, the concentration of $AgNO_3$, pH and temperature have an effect on the production of AgNPs. The ratio of plant extract to the metal precursor ($AgNO_3$) affects the shape, size and stability of AgNPs [27]. The possible effect of ratio is due to the amount of reducing and capping agents in extract for $AgNO_3$ reduction. AgNPs synthesis using mangosteen leaf extract was reported by Veerasamy et al. [14] by the varying ratio of plant extract: $AgNO_3$, and 19:1 ratio was found to be the best for the nanoparticles synthesis. The quantity of precursor plays a major role in AgNPs synthesis, as the higher amount of precursor lowers the synthesis due to the unavailability of biomolecules for reduction and capping [23]. A higher concentration of $AgNO_3$ enhances the biosynthesis of AgNPs. The present results are in agreement to the study conducted by Chandran et al. [28], and they reported 5 mM as the most effective concentration of $AgNO_3$ solution for the synthesis of nanoparticles using *Aloe vera*. The temperature elevates the activation energy of the enzymes to carry out a reaction which may provide the physicochemical environment which in turn enhances nucleation of AgNPs and also can control aggregation leading to stable nanoparticles [29]. AgNPs using mangosteen leaf extract were synthesized at different temperature conditions,

and 75 °C was reported to be optimum for the synthesis of nanoparticles [14]. The pH of the reaction mixture can significantly alter the reducing nature of the environment, playing a major role in the synthesis procedure [30]. The synthesis of AgNPs using an aqueous extract of *A. indica* (Neem) leaves was done in alkaline conditions as acidic conditions failed to produce nanoparticles [31]. Veerasamy et al. [14] optimized AgNPs synthesis using mangosteen leaf extract at various pH and reported neutral pH to be best for the synthesis of AgNPs.

The chemical nature of functional groups conferred by the NPs can be identified by FTIR technique. It is a promising technique for the identification of biomolecules that cap the biosynthesized AgNPs. IR spectrum bands of *A. cepa*-synthesized Ag NPs are observed at 878.609 (N–H waging), 1198.13 (N–O stretch and C-H waging), 1298.422 (C–O symmetric stretching and C–N stretching), 1588.62 (C=O stretching), 2256.21(C≡N stretching) and 3004.88 cm^{-1} (N–H stretching, C–H stretching and O–H stretching). The absorbance peaks could be attributed to the phytochemicals present in extract such as polyphenols, proteins, carbohydrates and water [32]. For *Z. officinale*, IR bands at 808.48 (C–H bending), 880.09 (N–H wagging), 950.33 (C=C bending), 1188.58 (C–N stretching and C–C stretching), 1578.32 (C=C stretching, C=O stretching and O–H bending) and 3010.57 cm^{-1} (C–H stretching, N–H stretching) are corresponding to phytochemicals present in extracts such as alkaloids and flavonoids, the active components of *Z. officinale*, which act as capping agents [13]. In case of *A. sativum*, IR bands are at 669.70 (C–Br stretching), 1207 (C–N stretching), 1593 (C=C stretching, C–H bending and H–C=O stretching), 2798 (C–H stretching) and 3454 cm^{-1} (O–H stretching). The observed peaks are characteristic of sugars present in the *A. sativum* extract [24]. Thus, polyphenols, proteins, carbohydrates and water in the *A. Cepa* extract, sugars present in the *A. sativum* extract and active compounds (alkaloids and flavonoids) of *Z. officinale* act as capping agents. Further, the reduction and stabilization of silver ions by a combination of several biomolecules present in the plant extracts is more efficient than using single or a few biomolecules for AgNPs synthesis. Even the AgNPs were stable longer up to 3 months after synthesis, beyond that it was observed not to be stable.

Antimicrobial Activity

AgNPs from *A.cepa*, *Z. officinale* and *A. sativum* were found to have diverse effects and distinct effective MIC against plant pathogens (Table 1). The MIC of AgNPs from *A. cepa* and *Z. officinale* against *Erwinia* sp. was 70 µg/ml, whereas it was 50 µg/ml for *A. sativum*. The strong antibacterial activity of AgNPs from *A. cepa*, *Z. officinale* and *A. sativum* against *P. syringe* was observed with MIC at 90, 110 and 90 µg/ml, respectively. Similarly, in the case

of *B. megaterium*, the MIC values were found to be 130, 110 and 110 µg/ml using AgNPs from *A. cepa*, *Z. officinale* and *A. sativum*, respectively. The strong antifungal activity of different extracts synthesized that AgNPs have been found to show MIC at 110 µg/ml, 110 µg/ml and 90 µg/ml against *F. avenaceum*. The MIC of AgNPs synthesized by *A. cepa* and *A. sativum* against *F. culmorum* was 110 µg/ml, whereas, in the case of *Z. officinale*-synthesized AgNPs, it was 90 µg/ml. In the case of *F. graminearum*, MIC values were found to be 90, 110 and 110 µg/ml for *A. cepa*, *Z. officinale* and *A. sativum*, respectively.

Table 1

MIC of AgNPs against different phytopathogens

Plant extract	***Fusarium graminearum* (µg/ml)**	***Fusarium avenaceum* (µg/ml)**	***Fusarium culmorum* (µg/ml)**	***Pseudomonas syringe* (µg/ml)**	***Erwinia* sp. (µg/ml)**	***Bacillu megate* (µg/ml)**
Onion	90	110	110	90	70	130
Ginger	110	110	90	110	70	110
Garlic	110	90	110	90	90	110

TEM images elucidated the morphology and size distribution of the synthesized AgNPs. Size and shape of nanoparticles are vital toward the efficacy of its use, and smaller silver particles are more target-specific and highly desirable as a potent antimicrobial agent [13]. XRD pattern revealed the crystal structure of the prepared AgNPs. Lattice planes (111), (200), (220) and (311) correspond to face-centered cubic (FCC) structure of silver. The obtained Bragg peaks were compared with available data on pure crystalline silver reported by the Joint Committee on Powder Diffraction Standards [33]. Past investigations advocate for AgNPs as effective antimicrobial agents. This study also supports the antibacterial and antifungal activities harbored by biosynthesized AgNPs. Similar findings were reported by Sahni et al. [23] who synthesized AgNPs using *A. cepa* and *M. accuminata* for studying the antimicrobial effect against *E. coli*, *B. subtilis*, *P. aeruginosa* and *F. oxy*sp*orum*. More recently, such inhibitory effects of AgNPs on plant pathogenic fungus viz. *Fusarium verticillioides*, *Fusarium moniliforme*, *Penicillium brevicompactum*, *Helminthosporium oryzae* and *Pyricularia grisea* further validate the agro-nanotechnological use of green-synthesized AgNPs [34]. The DNA-directed silver-based bactericides have been reported for plant disease management. The stable composites show excellent antibacterial activity against *Xanthomonas perforans* at 100 ppm dose on tomato, with no phytotoxicity [35]. In recent years, colloidal metallic

nanoparticles have gained popularity due to their potential use and wide applications [36, 37].

Conclusion

The present investigation reported a simple, rapid and eco-friendly method to synthesize AgNPs by using plant extracts from three medicinal and aromatic plant species, namely *A. cepa*, *A. sativum* and *Z. officinale*. The results clearly supported the fact that the optimization process played a crucial role in the silver precursor reduction. Crystalline AgNPs harbored potent antibacterial and antifungal activity. Hence, it is concluded that green synthesis of nanoparticles is an economic method and has immense potential for the industrial bio-fabrication of AgNPs. Due to the antimicrobial potential, it can be used in different formulations as nanofungicides, nanoantimicrobials and nanofertilizers in agriculture which protect the crop plants from different pathogens. Further, after testing of the detrimental impact of AgNPs on human health, useful insects, soil and water, the effective dose can be formulated for crop application.

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Compliance with Ethical Standards

Conflict of interest The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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