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RESEARCH ARTICLE

Synthesis and characterisation of *Celosia cristata* L. flower extract-based silver nanoparticles as effective antimicrobial agent for plant protection

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Abstract

Plant systems are a natural reservoir of phytochemicals that may act as agents for reducing silver ions into nanoparticles. This study reports the green synthesis and characterisation of silver nanoparticles using *Celosia cristata* L. flower extract and evaluates their antimicrobial efficacy against key plant pathogens: *Alternaria solani*, *Fusarium graminearum* and *Xanthomonas oryzae* pv. *oryzae*. The biosynthesis involved the use of phytochemicals as natural reducing/stabilising agents and silver nitrate. The formation of silver nanoparticles was indicated by a colour change and confirmed by a surface plasmon resonance peak at 426 nm in UV–visible spectroscopy. X-ray diffraction analysis revealed a face-centred cubic crystalline structure with dominant (111) orientation and transmission electron microscopy showed mostly spherical nanoparticles with an average size of 13 nm. Fourier-transform infrared spectroscopy identified functional groups responsible for capping and stabilisation. Antimicrobial activity was demonstrated through agar well diffusion with notable inhibition zones: 26 ± 1.00 mm (*A. solani*), 23 ± 2.00 mm (*F. graminearum*) and 21 ± 0.5 mm (*X. oryzae* pv. *oryzae*). Minimum inhibitory concentrations (MICs) as observed in the investigation was 78.1 mg/mL (for *A. solani*) and 156.2 mg/mL (for *F. graminearum* and *X. oryzae* pv. *oryzae*). The findings highlight the potential of *C. cristata*-derived silver nanoparticles as eco-friendly biocontrol agents in sustainable agriculture, offering a promising alternative to conventional chemical pesticides.

Keywords: antimicrobial activity; biocontrol; eco-friendly synthesis; transmission electron microscope

Introduction

Nanotechnology has become a significant field of study in contemporary science providing creative answers for various uses in fields such as environmental sciences, agriculture and medicine (1). Among the many kinds of nanomaterials, silver nanoparticles have drawn a lot of interest because of their special physicochemical characteristic antimicrobial activity (2, 3). Utilising biological entities, including plants (4), bacteria and fungi to sustainably create nanoparticles by green synthesis has been investigated as an environmentally benign substitute (5). Nanoparticles are widely utilized in water purification, food preservation, biomedicine and most recently in agriculture. Silver nanoparticles are a promising option for managing plant diseases because of their efficiency in inhibiting plant infections (6). Silver nanoparticles show broad-spectrum action with little toxicity to plants and the environment in contrast to traditional chemical treatments (7). There are several ways to create silver nanoparticles including chemical, physical and biological processes. Although, the use of physical and chemical processes frequently entails environmental risks, excessive energy consumption and toxic reagents, biological methods proved to be eco-friendly (8).

The Amaranthaceae family includes the widely common plant *Celosia cristata* L. commonly known as cockscomb which has considerable medicinal and decorative significance (9). It is widely recognised for its therapeutic potential which comprises of anti-inflammatory, anthelminthic, antifungal and antibacterial effects. The green route adopted not only provides a sustainable and eco-friendly alternative to the previously prevalent chemical methods but also provides a strong evidence for presence of rich phytochemicals in *C. cristata* flower, which acts as reducing and capping agents during nanoparticle formation (10). Globally, plant pathogens including *Xanthomonas oryzae* pv. *oryzae* (6), *Alternaria* species (11) and *Fusarium* species cause large crop losses (12). These pathogens reduce the yield and quality of a variety of commercially significant crops by causing serious illnesses. Chemical fungicides and bactericides among other traditional means of disease management have sparked worries about pollution of the environment, pathogen resistance and hazards to human health. Chemical bactericides and fungicides are used in conventional ways to manage these diseases. Though these approaches are successful but they have several disadvantages including the development of resistance, environmental contamination and negative effects (13). Green-synthesised silver

nanoparticles are a possible substitute because they are ecologically benign and biocompatible and they have strong antibacterial action (6, 14). These nanoparticles interact with genetic information, break cell membranes, produce reactive oxygen species and inhibit enzyme function among other methods to produce their antimicrobial effects (15, 16). Silver nanoparticles have a multi-targeted mode of action that lowers the possibility of pathogen resistance development as compared to traditional chemical insecticides (17).

The extensive use of chemicals leads to their accumulation in the food chain and absorption by the soil. They create disturbance in the physicochemical and biological properties of the soil, pollutes groundwater and leaches the soil ingredients, which adversely affects the healthy microflora of soil, plant and animal health, thereby raising environmental issues. Addressing these constraints, nano-technological research notably green-synthesised nanoparticles, have come up as a promising alternative offering two-fold benefits in terms of non-biodegradable durability along with enhanced performance at minimal concentrations. Their high surface to volume ratio helps them to interact with biological systems and reduce loading with improved performance. Therefore, this approach presents a biocompatible strategy of eliminating chemical-based soil-deterioration and bioaccumulation.

The purpose of this work is to study the potential applications of *C. cristata* mediated silver nanoparticles as powerful antimicrobial agents against *Xanthomonas oryzae* pv. *oryzae*, *Alternaria solani* and *Fusarium graminearum*. To provide insight into the practical use of silver nanoparticles in the control of plant diseases, the study concentrates on their production, characterization and antimicrobial assessment. The characterization techniques used to analyse the absorbance peak, crystalline structure, functional groups, shape and size of the synthesised nanoparticles are UV-Vis spectroscopy, X-ray diffraction, Fourier-transform infrared spectroscopy and Transmission Electron Microscopy respectively. The investigation confirms and conceptualize the use of green synthesized silver nanoparticles for formulation as antimicrobial agent.

Materials and Methods

Plant pathogens used in study

Plant pathogens chosen for present investigation includes *A solani* (MTCC-10690), *F. graminearum* (MTCC-2089) and *X. oryzae* (MTCC-11107) which are known to cause serious illnesses in plants. These pathogens were chosen because of their substantial negative effects on crop health which results in the economic losses in crop production.

Plant sample collection and flower extract preparation

Fresh flowers of *C. cristata* were collected from regions in vicinity of Meerut which were identified by Dr. Garima Bartariya (Botanist, IIMT University, Meerut). The specimen was also compiled in the herbarium and then authenticated by Dr. Vijai Malik (CCS University, Meerut). The flowers of *C. cristata* were properly cleaned and washed with distilled water to remove any surface contaminants like dust, dirt or microorganisms. The flowers were shade dried as direct sunlight might break down the bioactive substances which stabilize and reduce silver nanoparticles. To ensure consistent particle size for effective extraction of bioactive components, the flowers were completely dried to remove any remaining moisture (18, 19). They were then crushed into a fine powder using a high-Speed grinder (Model: SA4001, Glen Appliances Pvt. Ltd., Faridabad, India). 10 g of flower powder was weighed and added to 100 mL distilled water to prepare the extract. Following this, the extract was heated to 80 °C for 30 min and then allowed to cool to room temperature. After that, cooled extract was further filtered through Whatman filter paper No. 1 and stored in a sterile container at 4 °C (20).

Silver nanoparticle synthesis

The prepared extract of *C. cristata* flower was used as a stabilising agent in synthesis of silver nanoparticles. In a sterile container under controlled conditions, 12 mL of plant extract and 88 mL of 10 mM silver nitrate solution were mixed. Following mixing, the reaction mixture was placed in an incubator shaker and incubated for 120 min at 37 °C and was observed for colour change (21).

Characterisation

Visual observation

When the plant extract and the silver nitrate solution are mixed, then the interaction between the precursor silver ions (Ag^+) and the bioactive metabolites in the plant extract causes a visible colour change. The phytochemicals function as stabilising and reducing agents that enables the bioreduction of Ag+ ions to elemental silver (Ag) nanoparticles. This initial indication of nanoparticle formation was the detection of a noticeable colour shift from red to brown which can be easily examined through visual observation (22).

UV-Visible spectrophotometer

The reaction mixture was examined using the UV-Vis spectrophotometer (LABMAN model number UV1900), to identify the particular absorbance peak, since silver nanoparticles show a distinctive surface plasmon resonance absorption peak in the 300–500 nm region (23). The successful reduction of silver ions into nanoparticles would be indicated by a peak within the expected surface plasmon resonance range. The wavelength and intensity corresponding to the peak revealed information about particle aggregation and dispersion (24).

X-ray diffraction

For examining the crystalline structure and phase purity of the produced nanoparticles, X-ray diffractometer was used. A Siemens X-ray diffractometer (model XPert PRO PANalytical) which was furnished with a Cu–Kα radiation source (λ = 1.54 Å) was utilized to acquire diffraction patterns (23, 25). To provide thorough coverage of distinctive diffraction peaks, the diffractogram of the silver nanoparticles was recorded throughout a scanning range of the 2θ angle extending from 20° to 80°. To determine the presence of crystalline phases, the acquired diffraction peaks were contrasted with standard reference card from the Joint Committee on Powder Diffraction Standards (JCPDS) (26).

Fourier transform infrared spectroscopy

This technique was used to identify the functional groups present in *C. cristata* flower extract which stabilizes and caps the produced silver nanoparticles (27). The biomolecules which reduced Ag (I) ions to Ag (0) nanoparticles were evaluated using a SHIMADZU FT-IR spectrophotometer. The samples were prepared using the KBr pellet procedure which guaranteed ideal transmittance and precise spectrum capture. The FT-IR spectra were obtained for a wide

variety of functional groups in the range of 500 to 4000 cm^{-1} (28).

Transmission electron microscopy

The size, shape and distribution of the produced silver nanoparticles were investigated using this technique which also provided high-resolution images to confirm their structural properties. A Model: JEOL, JEM2100F, was used for the experiment allowing for fine-grained imaging at nanoscale levels. For the preparation of the samples, a drop of the nanoparticle solution was drop-coated onto a copper grid which was coated with carbon to ensure uniform dispersion of the nanoparticles (29, 30). The samples were then allowed to dry naturally before being put into the specimen holder for imaging. The images were processed and size of nanoparticles were displayed and images were captured. ImageJ software was used to examine the TEM pictures. Microsoft Excel 2016 and Origin software was used to further process the data and create a size distribution histogram which allowed for the determination of the frequency of nanoparticles of various sizes.

Antimicrobial activity

Preparation of inoculum (McFarland)

A 0.5 McFarland standard bacterial and fungal suspension was made to inoculate in the nutrient agar and potato dextrose agar respectively. To ensure consistency in microbial content the bacterial and fungal spore suspensions were standardised using a 0.5 McFarland standard. Using a UV-Vis spectrophotometer, optical density (OD) of the suspension was determined at 600 nm and settled to meet the necessary microbial concentration around 1.5×10^{6} CFU/mL (31, 32).

Antimicrobial activity for agar well methods

The antibacterial activity of the produced silver nanoparticles against bacterial and fungal pathogens was evaluated using the agar well diffusion technique. A 0.1 mL of microbial suspension was spread on sterile petri dishes containing solid agar medium using a sterile cotton swab. The fungal strains were cultivated on potato dextrose agar and the bacterial strains onto nutrient agar. Using a sterile cork borer, wells of 6 mm diameter were drilled in the agar plates making sure that there was an equal distance between them to permit the appropriate diffusion of fabricated silver nanoparticles (33). Each well was filled with a predetermined amount of the silver nanoparticle solution (100 µL). For optimal microbial development, fungal plates were incubated at 25 °C for five days while bacterial cultures were kept at 29 °C for 48 hr. The efficiency of silver nanoparticles against the examined bacteria was determined by measuring the width of the inhibition zone that developed around each well. Microbial inhibition was shown by the appearance of a distinct circular zone surrounding the well. Bigger zones were associated with better antibacterial action (34). All experiments were conducted in triplicate to reduce experimental errors and establish consistency, reproducibility and reliability of the data.

Evaluation of minimum inhibitory concentration and minimum microbicidal concentration

Minimum inhibitory concentration is the minimum dose of any agent that do not allow the growth of microorganism in its presence, whereas the minimum microbicidal concentration is the minimum amount of the same agent to kill all the microorganism present in the sample. Both the parameters were assessed using macro broth dilution technique. In a sterile 96-well microplate, the macro broth dilution technique was used to measure the minimum inhibitory concentration and minimum microbicidal (fungicidal/bactericidal) concentrations of the produced silver nanoparticles. This technique made it possible to accurately assess the antibacterial potential of these nanoparticles against bacterial and fungal infections. To ensure consistent conditions, 100 µL of the culture media was added to each well of columns 1–10 of the microplate. A gradient of nanoparticle concentrations was ensured by first adding 200 µL of the silver nanoparticle solution to column 11 and then serially diluting the solution twofold by continuing 100 µL from one well to the next until it reached column 2. A 100 µL amount of the standardised microbial inoculum was then added to each well in columns 1–11. To verify microbial growth, column 1 was used as the positive control which included simply the culture media and the bacterial or fungal pathogens devoid of nanoparticles. In column 12, 200 µL of sterile medium in an uninoculated well served as a negative control to verify sterility. To facilitate MIC determination, 10 µL of resazurin dye (5 mg/mL) was added to each well acting as an indicator of microbial viability based on colourimetric changes (35). The microplates were incubated for 48 hr at 29 °C for bacterial strains and 28 °C for fungal strains which were optimum conditions (31). The wells were observed for colour changes where a shift from purple to pink or colourless indicated microbial growth while the retention of purple signified inhibition. The MIC was recorded as the lowest concentration at which no visible colour change occurred. To determine the MBC/MFC, aliquots from wells showing no growth were plated on agar media and the plates were observed for the bacterial growth after 48 hr and 5 days for fungal growth (35).

Results and Discussion

Silver nanoparticle synthesis

Using *C. cristata* flower extract, that function as a reducing agent and stabilizer in the process, silver nanoparticles were synthesized. The observable colour changes from light yellow to dark brown on adding *C. cristata* flower extract to silver nitrate solution confirms the successful bioreduction of Ag^{+} ions, serving as a qualitative indicator of nanoparticle formation through surface plasmon resonance (Fig. 1). This phenomenon is also reported in similar studies using extracts from *Portulaca oleracea* (11). They exhibited similar colour transitions from red to brown upon mixing with silver nitrate confirming the reduction (36, 37).

Characterisation

UV-visible spectrophotometer

The absorbance spectrum of the synthesized silver nanoparticles synthesized with flower extract of *C. cristata* as reducing agents is displayed in Fig. 2. The wavelengths showed a peak at 473 nm and maximum absorption in the visible region of the electromagnetic spectrum. Our findings are similar with those of other studies, when compared to the relevant literature that frequently demonstrate surface plasmon resonance peaks around 426 nm suggesting smaller and more spherical nanoparticles (38). This convergence across different plant-mediated synthesis underscores the robustness and universality of colour change as an immediate and reliable indicator of nanoparticle formation.

X-ray diffraction

The crystalline structure of the nanoparticles produced from flower

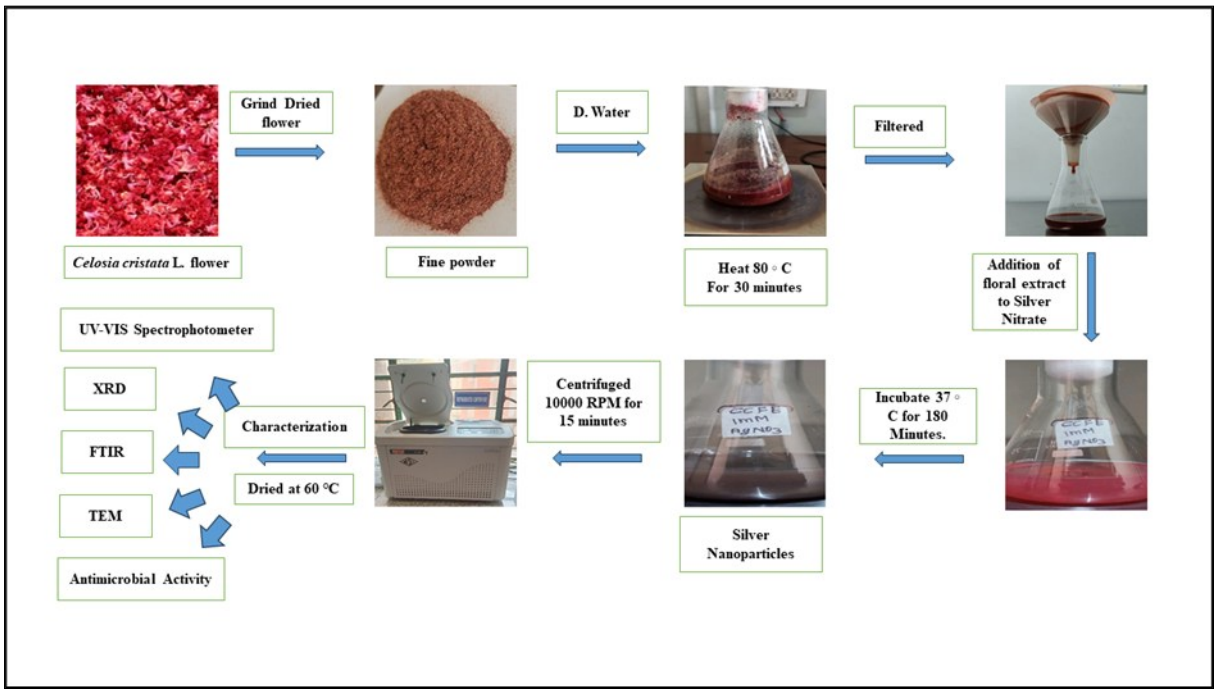

Fig. 1. Synthesis of silver nanoparticles using silver nitrate and *Celosia cristata* flower extract.

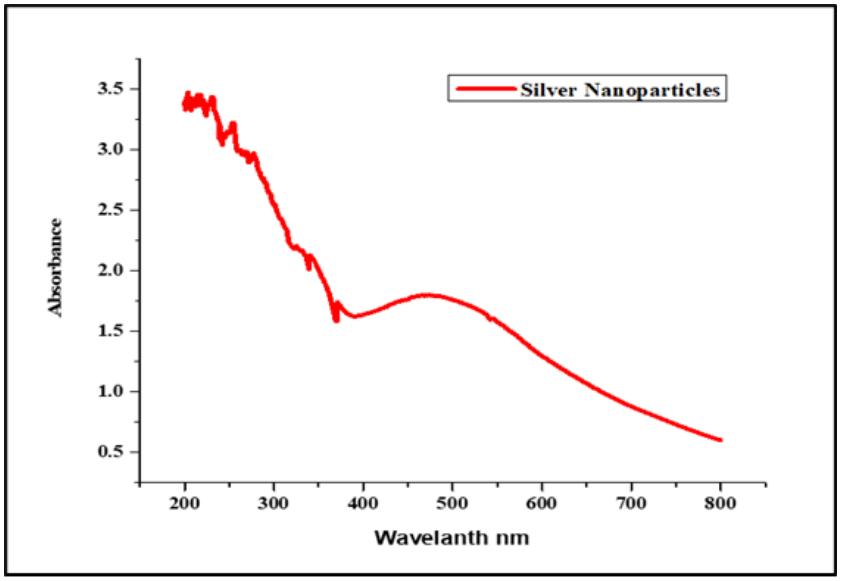

Fig. 2. UV–Vis spectra of *Celosia cristata* flower extract silver nanoparticles.

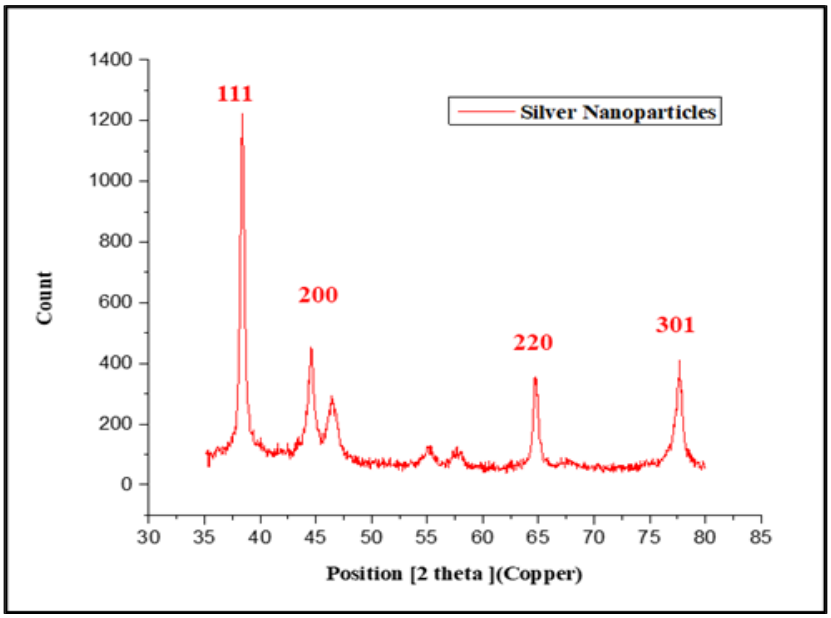

Fig. 3. X-ray diffraction pattern of the silver nanoparticles produced with flower extract from *Celosia cristata*.

extract was examined using this technique. The (111), (200), (220) and (311) hkl were represented by the occurrence of discrete diffraction peaks at 2θ values of 38.36°, 44.60°, 64.71° and 77.69° respectively in Fig. 3. This is in accordance to the Joint Committee on Powder Diffraction Standards (JCPDS) database file number 31–1238 (39). These diffraction patterns confirms that biosynthesised silver nanoparticles are having face-centred cubic (FCC) shape (40). The sharpness of the peak and intensity further supported the nanoscale regime of the nanoparticles by demonstrating their great crystallinity. As is frequently observed in metallic silver nanoparticles the (111) peak was found to be the most intense indicating that silver nanoparticles preferentially develop along this plane. The nanocrystalline size has been demonstrated by the widening of the peaks (28, 30).

Fourier transform infrared spectroscopy

The secondary metabolites present in silver nanoparticles, made using flower extract were identified using this technique. The existence of several functional groups that are in charge of stabilising the nanoparticles were shown by the appearance of multiple distinctive absorption peaks in the FTIR spectra of silver nanoparticles. The observed absorption peaks and their corresponding functional groups are 2912.31 cm^{-1} corresponding to C–H stretching of alkenes (3), 2841.91 cm^{-1} displaying C-H stretching of methyl groups (41), 1465.80 cm^{-1} representing presence of alcohol and phenol O-H in-plane bend (21), 1239.18 cm^{-1} highlighting C-H in-plane bending of alkenes, alcohols, carboxylic acids, esters and ethers (27), 1128.28 cm^{-1} showing alkyl amine group and OH stretching (6), 1058.84 cm^{-1} displaying C–N stretching of aliphatic amines (42), 1004.84 cm^{-1} confirming presence of a capping agent corresponding to C-F (43), 983.63 cm^{-1} corresponding to the ether group (C-O-C), 834.16 cm^{-1} deciphering C-O stretching in the aliphatic group (6). 774.37 cm^{-1}exhibiting N-H stretching indicating the presence of primary and secondary amines in Fig. 4. The presence of many functional groups including hydroxyl, alkene, amine and ether which are important for the bioreduction and stability of silver nanoparticles is confirmed by the FTIR analysis. The O-H and N-H stretching vibrations indicate that proteins, flavonoids and polyphenols participate in the capping process, which increases the stability of nanoparticles (42). The intricate relationship between biomolecules and nanoparticles which adds to their regulated production and functional characteristics is further demonstrated by the presence of ether groups and aliphatic amines. All the above-mentioned points highlights that FTIR spectrum offers important information about the biomolecules that stabilize and reduce the silver nanoparticles.

Transmission electron microscopy

Analysis using transmission electron microscopy is regarded as one of the most crucial methods for studying the characteristics of nanoparticles. The size, shape and morphology of silver nanoparticles were all ascertained in Fig. 5. The particle thickness is shown by the constant electron penetration suggested by the uniform contrast in TEM pictures. The polycrystalline character of the produced nanoparticles is further supported by the strong diffraction rings shown in the selected area electron diffraction (SAED). The produced silver nanoparticles are primarily spherical with a consistent size distribution and minimal aggregation seen in certain regions according to TEM images. The estimated average size was about 13 nm that comply with similar studies that shows fabrication of nanoparticles with 12 nm and even 13 nm using different plant extracts (12, 38).

Antimicrobial activity

Agar well diffusion method

The biosynthesised silver nanoparticles demonstrated significant antimicrobial efficacy as evident by the distinct zones of inhibition measured against *A. solani* (26 ± 1.00 mm), *F. graminearum* (23 ± 2.00 mm) and *X. oryzae pv. oryzae* (21 ± 0.5 mm) using this method. The clear and measurable inhibition zones around the wells indicate the potent antimicrobial efficacy of the silver nanoparticles which can be attributed to their nanoscale size and enhanced surface reactivity that facilitate strong interactions with microbial cell membranes. The effectiveness against both fungal (*A. solani* and *F. graminearum*) and bacterial (*X. oryzae* pv. *oryzae*) strains highlights the broad-spectrum activity of these nanoparticles in Fig. 6. The experiments were performed in triplicate and its average values were used to represent the data. The obtained results were consistent with the antimicrobial potency confirming the reliability of the data. The findings of this study are in accordance with the previous *in vitro* investigation which have reported remarkable antimicrobial activity of *C. cristata* extracts mediated nanoparticles against diverse bacteria (9, 44, 45). The

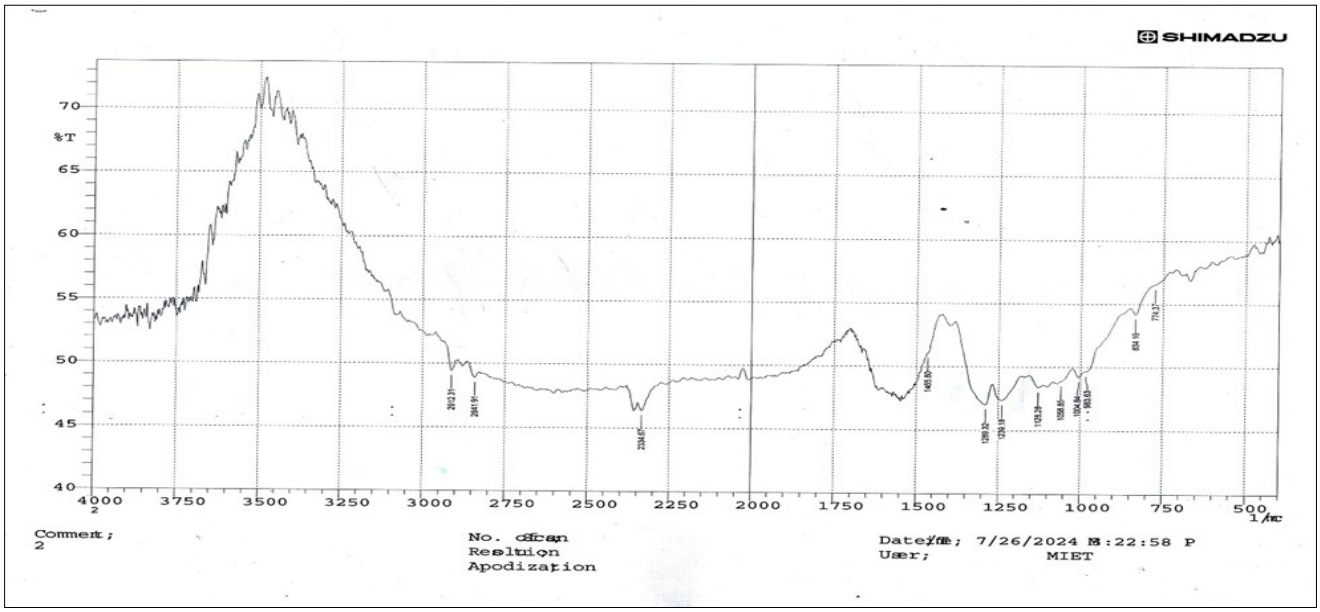

Fig. 4. Fourier-transform infrared spectroscopy of silver nanoparticles produced using flower extract from *Celosia cristata*.

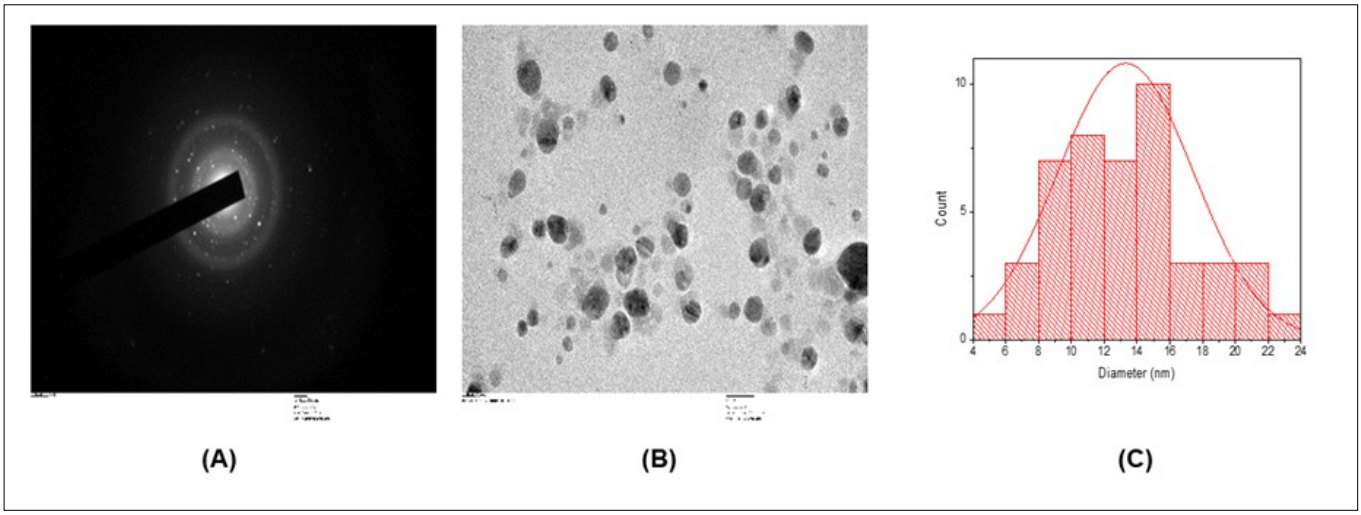

Fig. 5. Transmission electron microscopy (TEM). A- Silver nanoparticles selected area electron diffraction (SAED) image; B- TEM micrograph image of the synthesized silver nanoparticles; C- Particles size distribution.

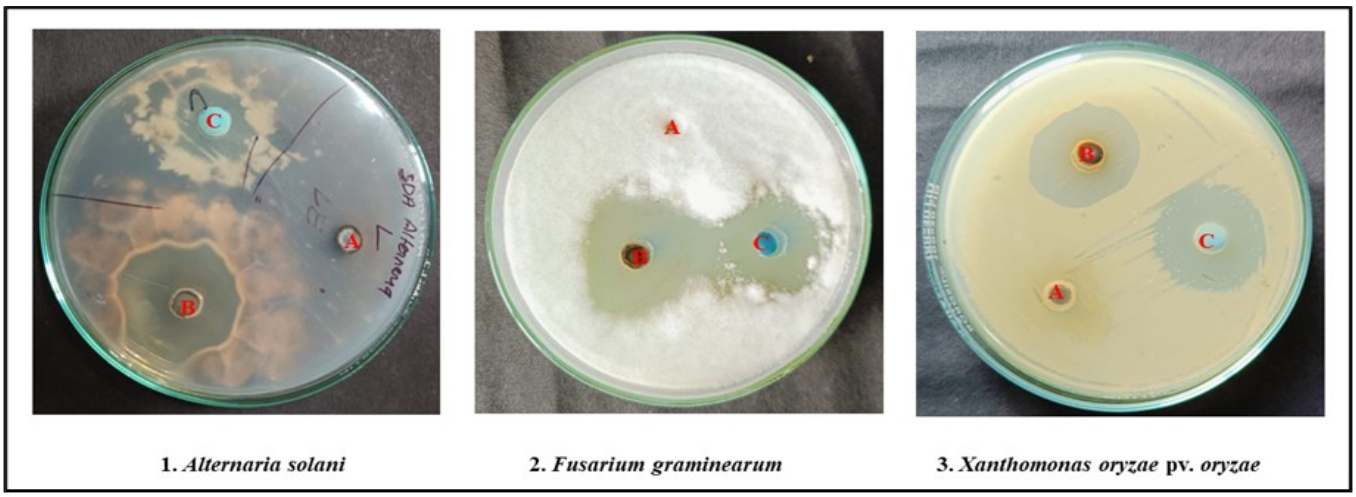

Fig. 6. Antimicrobial Susceptibility Test: A- *Celosia cristata* L. flower extract; B- Silver nanoparticles; C- Control (Carbandazim).

novelty of the present work is that although the effect of biosynthesised silver nanoparticles has been tested against a wide range of gram-positive and gram-negative bacteria but no specific work depicts their efficacy over plant pathogens. Mechanistically silver nanoparticles are known to exert antimicrobial effects through multiple pathways that includes penetration and disruption of microbial membranes leading to leakage of cellular contents and oxidative stress, caused due to the production of reactive oxygen species which lead to the structural and functional damage of DNA molecules, proteins and lipids and interaction with thiol groups in vital enzymes disrupting essential metabolic processes (15). The antimicrobial activity was assessed by calculating the inhibition zone diameter, that is reflected in Table 1. As clearly indicated by the calculated IZD, all the 3 pathogens used in the study were found to be potentially inhibited by the synthesized nanoparticles and not by the aqueous flower extract. It is evident from the study that even the flower extract is not showing any activity, but it acts as a repertoire of metabolites that helped in the formulation of silver nanoparticles having potential antimicrobial activity. The antimicrobial activity of the synthesized silver nanoparticles is aligned with the result obtained by FTIR analysis that shows presence of various plant metabolites that may have antimicrobial effect and might be used up from extract in fabrication process of nanoparticles. The result also indicates more effectiveness of synthesized nanoparticles in comparison to the control antimicrobial used. This highlights that the fabricated nanoparticles can be used effectively in formulating the preparations used for control agents.

Determination of minimum inhibitory concentration

To determine the antimicrobial efficacy of silver nanoparticles, a micro dilution assay was conducted against *A. solani*, *F. graminearum* and *X. oryzae* pv. *oryzae*. The microtiter plate method was used to assess fungal and bacterial growth based on colour change indicating metabolic activity. However, no colour change was observed in wells containing silver nanoparticle concentrations of 78.1 mg/mL for *A. solani,* 156.2 mg/mL for *F. graminearum* and 156.2 mg/mL for *X. oryzae* pv. *oryzae*, suggesting no microbial growth at these concentrations indicating

Table 1. Zone of inhibition of silver nanoparticles and plant extract against microorganisms

Concentration (mg/mL)	*Alternaria solani*	*Fusarium graminearum*	*Xanthomonas oryzae* pv. *oryzae*
CC flower extract	–	–	–
CC AgNPs 50 (mg/mL)	26 ± 1.00	23 ± 2.00	21 ± 0.5
Carbandazim	18 ± 1.00	22 ± 1.00	20 ± 0.25

– indicates no antibacterial activity. Diameter of inhibition zone, including well diameter of 6 (mm).

Table 2. Minimum inhibitory concentration and minimum microbicidal (fungicidal/bactericidal) concentration (mg/mL) of synthesised silver nanoparticles against pathogens used

Microorganism	Silver nanoparticles		Control (Carbandazim)	
	Minimum inhibitory Concentration (mg/mL)	Minimum microbicidal Concentration (mg/mL)	Minimum inhibitory Concentration (mg/mL)	Minimum microbicidal Concentration (mg/mL)
Alternaria solani	78.1	156.2	312.5	625
Fusarium graminearum	156.2	312.5	156.2	312.5
Xanthomonas oryzae pv. *oryzae*	156.2	312.5	312.5	625

No growth of organism from selected well assure that it is the concentration that not only inhibit the growth but also kill the microbes at that dose level.

complete inhibition of bacterial and fungal proliferation. This demonstrated that these concentrations were sufficient to suppress microbial metabolic activity, establishing them as potential MIC values. To confirm the results and ensure consistency of inhibitory concentrations, the same experiment was done in triplicate.

Minimum microbicidal concentration

To confirm the microbicidal concentration effects, suspensions from wells containing silver nanoparticles concentration higher than the MIC values were inoculated onto solid media. In case of fungus, suspensions were plated on potato dextrose agar and incubated for 48 hr and for bacteria, suspensions were cultured on nutrient agar and incubated for 24 hr. In all cases no microbial growth was observed at these concentrations, confirming the lethal effect of nanoparticles rather than merely inhibiting growth. Minimum bactericidal concentration and MFC for different microbial culture used are shown in Table 2.

Conclusion

Celosia cristata (flower extract) mediated silver nanoparticles exhibited strong and significant antimicrobial activity against *A. solani*, *F. graminearum* and *X. oryzae*. The investigation highlights successful synthesis of stable and predominantly spherical nanoparticles with nanoscale dimensions conducive to biological interaction. These results underscore the potential of *C. cristata* mediated silver nanoparticles as viable biocontrol agents in sustainable agriculture. By minimising the reliance on synthetic agrochemicals, this nano formulation can contribute to integrated pest management strategies which reduces environmental toxicity and mitigate the development of resistance in plant pathogens. Future research should focus on field-level validation, formulation development and safety assessment to facilitate the practical application of this promising nano biotechnological innovation.

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Authors' contributions

SM conducted the research, performed data acquisition, carried out data and statistical analysis, drafted the manuscript. AV conceptualized the study, designed the research methodology, and provided overall supervision of the project. NK handled the review and editing of the manuscript. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: The authors do not have any conflict of interest to declare.

Ethical issues: None

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