Research Article

Evaluation of the antibacterial efficacy of the polyphenol-conjugated copper nanoparticles against *Ralstonia solanacearum*

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ABSTRACT

A well-known soil-borne bacterium called *Ralstonia solanacearum* can cause catastrophic bacterial wilt in hundreds of plant species, including vital crops like pepper, tomato, potato, banana, and eggplant. Owing to the increase in antibacterial resistance, nanotechnologists around the globe are looking for novel and eco-friendly methods of synthesis, wherein they can control these plant pathogens. Keeping in view of this statement, we aimed to green-synthesise and study the efficacy of the copper nanoparticles against the *R. solanacearum*.

We extracted the polyphenols from fermented millets and synthesized copper nanoparticles using these extracted polyphenols. These NPs were characterized by FTIR, UV spectra and scanning electron microscopy tests. The synthesized NPs were evaluated for their antibacterial nature by well diffusion, MIC method and biofilm inhibition assay. From the broth dilution assay, we found that the green-synthesized NPs showed a greater reduction in the optical density value compared to the control. OD was found to reduce to 0.16 ±0.09 from 1.56 ±0.12 at 400µg/ml. We found the percentage of biofilm inhibition for NPs and E to be 84 and 41% respectively, at 15hr of incubation. This confirms that NPs are highly significant when compared to the positive control. These findings confirm the potential role of the NPs as antibacterial agents against plant pathogens.

Keywords: *Ralstonia solanacearum*, FTIR, Polyphenols, Fox tail millets, Biofilm.

1. INTRODUCTION

A soil-borne bacterial pathogen called *Ralstonia solanacearum* causes bacterial wilt, a catastrophic plant disease that damages a variety of plants, including crops like bananas, tomatoes, and potatoes and results in large financial losses. Known as the *R. solanacearum* species complex (RSSC) *R. solanacearum* is thought to exist as a complex made up of numerous genetically different strains [1]. Throughout the infection phase, *R. solanacearum* can create a variety of virulence factors that cause the host plants to wilt. Several investigations have demonstrated that the primary virulence factors of *R. solanacearum* that cause wilt symptoms are extracellular polysaccharides (EPS) and cell wall-degrading enzymes (CWDE) [2]. Reduced virulence results from decreased extracellular enzyme activity and EPS generation [3]. Furthermore, colonisation and infection behaviours of *R. solanacearum* are similarly influenced by motility and biofilm formation [4]. One of the sciences that has advanced the fastest in the twenty-first century is nanotechnology. The challenges associated with identifying and managing soil pathogens, including *R. solanacearum*, can be effectively addressed by nanotechnology [5]. Recent studies suggest that nanoparticles may be used as an antibacterial and biosensor to identify plant diseases, especially those caused by soil-borne pathogens. The possibility of a number of metallic oxide nanoparticles serving as defences against *R. solanacearum* has been studied [6]. Recently, the effects of ZnO, FeO, and CuO nanoparticles on

the tomato bacterial wilt pathogen *Ralstonia solanacearum* were studied. The results showed that NPs significantly reduced the incidence of tomato disease, especially CuO NPs. Additionally, the morpho-physiological characteristics of plants showed a significant improvement [7].

A soil-borne pathogen that creates biofilms *in-vitro* is *Ralstonia solanacearum*. This plant pathogen causes potato brown rot, banana Moko disease, and bacterial wilt. In order to move over solid surfaces and aid in the production of biofilms, *R. solanacearum* uses twitching motility. It moves from an early stage of fast growth to a later stage of slower growth using quorum sensing (QS). Extracellular DNA, a part of the biofilm matrix, is released by *R. solanacearum*. Aero taxis is necessary for *R. solanacearum* to interact with its host plant and create biofilms. Extracellular nucleases, or exDNases, are used by *R. solanacearum* to promote bacterial dispersal and biofilm development [8].

An important new tactic in the fight against harmful microbes is the use of plant extracts. Since ancient times, when they were widely utilized to treat a variety of illnesses, people have been aware of plants' therapeutic qualities. Many plant extracts still offer a wide range of advantages to people today. In this sense, polyphenols are substances derived from various plant components that contain one or more phenolic groups. Based on their structure, they are separated into multiple categories [9]. Antibacterial, antitoxin, antiviral, and antifungal properties have also been reported, in addition to their many other health benefits for humans (such as antioxidant, anti-inflammatory, antidiabetic, antiallergic, antihypertensive,antithrombotic,anticancer,cardioprotective,osteoprotective, neuroprotective, anti-ageing, and hepatoprotective benefits [10].

The main goal of this study was the environmentally friendly green synthesis of copper nanoparticles using polyphenols extracted from fermented millets. The synthesized nanoparticles were also described, and the CuNPs' antibacterial efficacy against biofilm-forming *Ralstonia solanacearum* was evaluated.

2. METHODS

Bacterial isolation: In the Tumkur district of Karnataka, soil samples were collected from the rhizospheres of *Solanum melongena* plants. Ten samples in all were gathered in the field and brought into the lab. The samples were collected in sterile sealed bags and brought to the laboratory. The bacterial strains were isolated from each serially diluted soil sample with sterile distilled water on TZC (Tetrazolium chloride) agar. Antibacterial works were performed using the following chemicals: Yeast extract: 5g; Tryptone: 10g; NaCl: 10g; Agar:15g. The dilutions of 10^{-8} and 10^{-9} were plated onto the Lysogeny agar plates, and the strains were confirmed by molecular characterization.

Molecular characterization of the strains: The strains were cultured on LB at 30°C for 20–24 hrs and characterized as described by Zhang *et al.,* [11]. A pair of universal species-specific primer pairs was used to amplify the strain DNA [AU759f: 5'-GTC GCC GTC AAC TCA CTT TCC-3'; AU760r: 5'-GTC GCC GTC AGC AAT GCG GAA TCG-3']. In brief, overnight culture was centrifuged and resuspended in 500µl of TE, 30µl of 10% SDS and 3µl of proteinase K (20 mg/ml). After lysis, the contents were added with 100µl of 5M NaCl and 80µl of CTAB/NaCl. The contents were mixed properly and added with phenol/chloroform: iso-amyl alcohol (25/24:1). The upper aqueous layer was collected to a fresh tube and added with 100% ice-cold ethanol to precipitate the DNA. The DNA obtained was stored at -20°C until further use.

PCR amplification: The DNA obtained was used for PCR amplification. PCR was performed with initial denaturation of 95˚C for 5 min followed by 35 cycles of denaturation at 95˚C for 30sec, annealing at 57˚C for 45sec and extension at 72˚C for 45sec. The PCR products were expected at 270bp and were sequenced using the same set of primers. The sequence that was produced was edited using Bioedit 7.19, BLAST was run to find the most similar sequences, and phylogenetic trees were created using the

neighbour-joining method in the MEGA 7.0 program.

Fermentation of millets: Foxtail millets were purchased from the local store, Bangalore. The millets were washed thoroughly and air-dried. Following, the millets were half-cooked at about 70°C and cooled before being added to their respective pilot vessels. About 100 g of cooked meal was added to a fermentation flask and added with 1ml of yeast culture. The contents were mixed thoroughly, added with sucrose and incubated in the dark for one week. Following incubation, the contents were filtered using a muslin cloth and then with Whatman filter paper. The supernatant obtained was used for green synthesis. Fermentation was done for 3 days, 7 days and 14 days.

Extraction of phytochemical compounds: Following fermentation, the extract was collected and used for the extraction of the polyphenols. The fermented contents were filtered with muslin cloth, and then through filter paper to further filter the contents. To 10ml of filtrate, 2 volumes of (~20 ml) chloroform was added into a separating funnel and kept on a shaker incubator for 12hrs. The bottom layer (chloroform phase) was discarded, and the upper phase was collected from the separating funnel and the aqueous layer into a clean beaker [12]. To the upper aqueous layer, two volumes of ethyl acetate was added and vortexed thoroughly for 5min at RT. The contents were filtered by filter paper, and concentrated using a rotary evaporator (62°C and 50rpm for 30min) and the concentrated extract was further freeze dried at -50°C for approximately 10hrs.

Total polyphenols determination: The Folin-Ciocalteau method, as outlined by Singleton *et al.*, [13], was used to determine the extracts' total polyphenol concentration. In short, 2.5mL of 10% Folin-Ciocalteu's reagent dissolved in distilled water, 2.5mL of 7.5% sodium carbonate, and 0.5mL of extract solution were combined. After that, the samples were incubated for 30min at 25°C. With a Shimadzu 1800 UV-vis Spectrophotometer, the absorbance was measured at 765nm. The calibration curve was created using a range of gallic acid concentrations (0 to 400µg/ml), using gallic acid as the reference solution. With $R^2 = 0.9948$, the regression equation was computed as $Y = 0.0092X + 0.1459$. The amount of total polyphenol was measured in milligrams of gallic acid equivalent (GAE) per gram of extract.

Green synthesis of copper nanoparticles: About 50ml of polyphenol extract (25%) obtained in the previous section was mixed with 50ml of 5mM copper sulphate ($CuSO_4.5H_2O$). The contents were mixed and stirred constantly for 24 to 48 hrs. For control, copper sulphate solution (5mM) was added with sodium borhydride (0.2%) for the reduction process to happen. The stirring was done 37°C, 300rpm. Colour change indicates the formation of NPs. The solutions were filtered once with filter paper. The filtrate obtained was centrifuged at maximum speed for 30min. The pellet (NPs) obtained were then washed thrice with de-ionized water to clear any unattached debris. The particles were then collected and dried in a hot air oven at 70^0C. Following drying, the particles were stored and used for the characterization and application studies.

Dynamic light scattering (DLS) and characterization: To verify the generation of CuNPs, a UV-VIS spectrophotometer (Shimadzu, 1800) with a resolution of 1nm between 200 and 800nm was used to perform UV-visible spectrum analysis in an aqueous solution. Using a Perkin Elmer Spectrum One FTIR spectrophotometer (Bomem MB100) with a 3600-400cm−1 range, Fourier transform infrared (FTIR) spectra were obtained using the KBr pellet method. Using FESEM-EDX at a 15KV accelerating voltage (SEM; PhilipsXL30ESEM), the surface morphology and atomic ratio of the CuNPs were ascertained [14]. The DLS method was used to screen the hydrodynamic size (Z average), surface charge (zeta potential), and polydispersity index (PDI) of the ecologically produced CuNPs using a Horiba SZ-100 analyzer. The particle size was measured at 210kCPS medium count rate and 90° scattering angle [15].

Antibacterial activity

Antibacterial activity of the green synthesized copper NPs was carried on *Ralstonia* isolates purified from the first section. The initial

preliminary screening was done via agar well diffusion method and MIC study. The antimicrobial activity of the plant extracts along with positive control (Ampicillin 20mg/ml).

Agar well diffusion method: The antimicrobial testing was done on LB agar plates using the agar diffusion method [16]. In brief, about 100μL of the bacterial culture was spread on the agar plates and the extracts and the NPs obtained were added to their respective samples (50μL). Polyphenol extract was used as control and CuNPs as treatment. The plates were then incubated at 37°C for about 24-48hrs. Following incubation, the mean diameter of the growth inhibition zone (in mm) was measured and recorded.

Minimal Inhibitory Concentration (MIC): The MIC of the green synthesized NPs were determined by the microdilution method using 96 multi-well microtiter plates, as described by Panda *et al.*, [17]. In brief, the green synthesized CuNPs were used at varying concentrations (5, 10, 20, 40, 80, 160µg/ml). Positive control was also used at the same concentrations. About 10μL of the bacterial suspension was added to all the wells except for the negative control, and the plates were incubated at 37°C for about 18-24 hrs. The least concentration at which turbidity occurred was taken as the MIC value. Following incubation, the plates were read for optical density under a PLATE reader (Genetrix Ltd) at 600nm and also screened for CFU/ml.

Biofilm inhibition assay: Bacterial biofilm inhibition assay was done according to Sudhakar *et al.*, [18]. In brief, 200µL of LB broth was added to all the wells of a 96-well microtiter plate. About 20µL of bacterial culture was inoculated into all the wells except the last column, which serves as a sterile control (containing only 200µL of LB broth). Ampicillin (1/4 MIC, 1/2MIC) was used as a positive control. All of the fractions were tested for the biofilm inhibition assay. About 10µL of CuNPs (1/4 MIC, 1/2MIC) were added to their labelled wells. The microtiter plates were incubated at 37°C for 24-48 hours and the absorbance of the eluted solution was read at 595nm using Plate reader (Genetrix Ltd).

3. RESULTS

The sequences obtained in FASTA were used to identify the Bacterial strains. The isolate was almost similar to *Ralstonia solanacearum* strain (Accession number: CP021766.1) with a similarity of 96.30%.

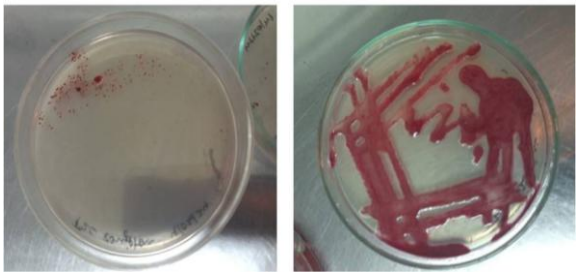

Fig. 1: Images of the plates showing *Ralstonia solnacearum* isolated from Rhizosphere samples.

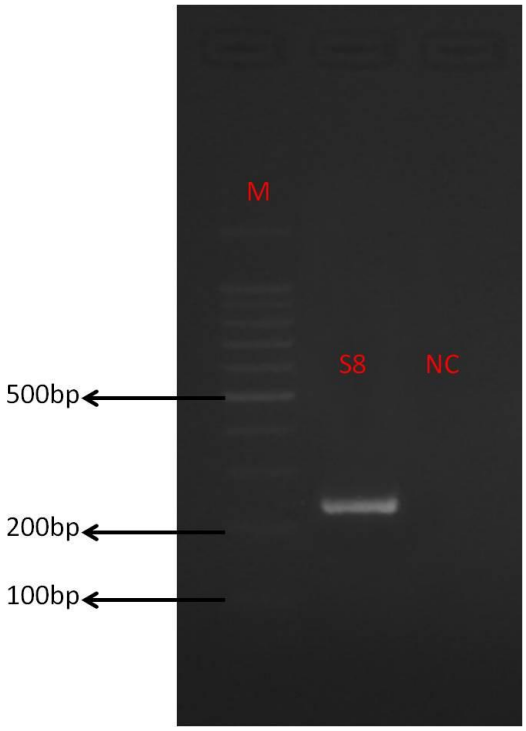

Fig. 2: Agarose gel showing the amplified product at 270bp approximately. M: Molecular marker (1Kb); NC: Negative control (sterile distilled water as template).

Total polyphenols determination: Total polyphenol content was estimated using the standard curve with gallic acid as the reference standard. With R^2 = 0.9948, the regression equation was computed as Y = 0.0092X + 0.1459. The amount of total polyphenol was measured in milligrams of gallic acid equivalent (GAE) per gram of extract. From our data, we found the total polyphenol content was found to be more after 7 days incubation and thereafter stayed steady. Polyphenol content was found to

be 47.08102 ± 3.61, 278.7178 ± 7.11 and 223.2112 ± 10.78 GAE equivalents respectively at 3, 7 and 14 days.

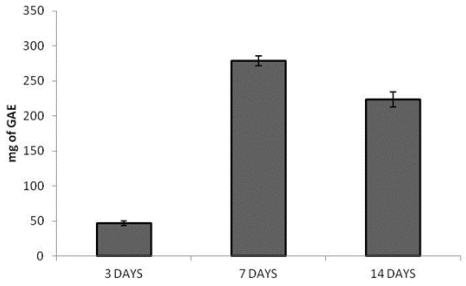

Fig. 3: Histogram showing the total polyphenol content of the samples obtained from fermented fox tail millets at different incubation times.

Characterization of NPs: From our data, the various absorption ranges for the nanoparticles were found to be 345nm, 320nm, and 482 nm for AgNPs, CHNPs, and GNPs showed absorption peaks at respectively. The change in the solution's blue colour to brown colour is the main sign that the biogenesis of metal nanoparticles took place. The characteristic absorbance peak in the UV-vis spectrum is found to be 240nm. From the SEM analysis we found the average size of the synthesized NPs to be 48 to 74nm. From the SEM we could find the particle shape to be spherical in shape. Because of the intramolecular hydrogen bonds, the relatively broad vibrational bands seen in the 3345–2969cm^{-1} range correspond to N–H and O–H stretching. Around 2969cm^{-1}, the aliphatic C–H symmetric stretching absorption bands are visible. The presence of the carbonyl group (C=O) may be verified at 1645cm^{-1}. The presence of the amide of peptide in floral extract is confirmed by a medium signal of N–H bending vibrations centred at 1367cm^{-1}. The sharp bands at 1041.70 and 872.76cm^{-1} correlate to stretching of C–O.

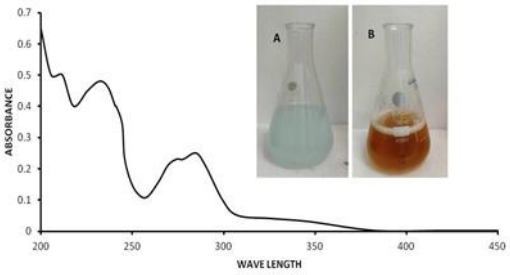

Fig. 4: UV spectra of the green synthesized CuNPs with polyphenol extract.

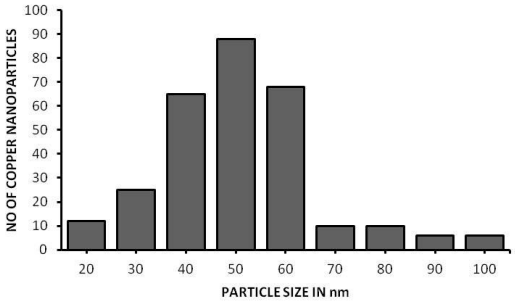

Fig. 5: Histogram showing the distribution of copper nanoparticles obtained from the green synthesis.

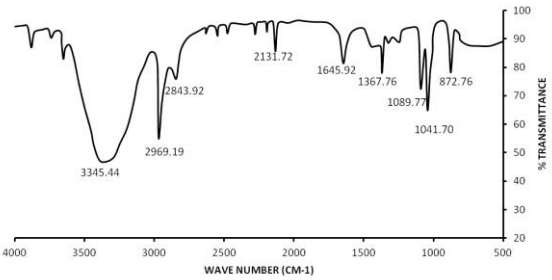

Fig.6: FTIR spectra of Synthesised NPs

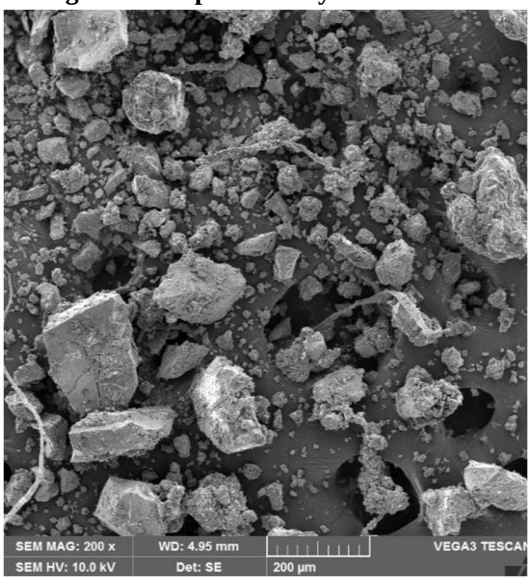

Fig. 7: SEM image of the green synthesized Copper NPs.

Agar well diffusion method: From our results, we found NPs to be highly antibacterial in nature when compared to positive control. NPs exhibited significant antibacterial activity when compared to positive control (12.13 ± 0.502mm) ($p<0.05$). NPs showed the highest antibacterial activity against *R. solanacearum* with a mean zone of inhibition of (11.67 ± 0.68mm).

Fig. 8: Agar well diffusion method: NC: Negative control, PC: Positive control; Cu: $CuSO_4$

E: Extract, NPs: Green synthesized NPs.

Broth dilution assay: Our results are in accordance to the agar well diffusion assay. From the broth dilution assay, we found the green synthesized NPs showed a greater reduction in the optical density value compared to the control. OD was found to reduce to 0.16 ±0.09 from 1.56 ±0.12 at 400µg/ml. This was highly significant when compared to positive control. The positive control showed reduction from 1.49±0.12 to and 0.11±0.04 at 200µg/ml.

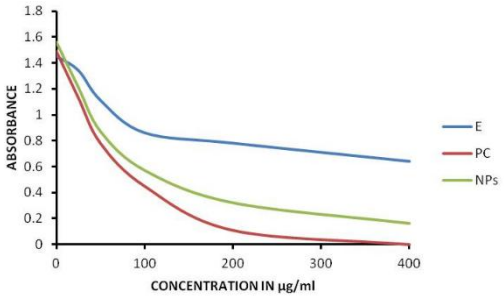

Fig. 9: Broth dilution assay: C: Control, PC: Positive control; E: Extract, NPs: Green synthesized NPs.

Biofilm inhibition assay: From our results, we found biofilm inhibition of the NPs was found to be highly significant in terms of positive control. Though it was time dependant the effect was significantly seen in NPs, when compared to the extract (E). The results are in accordance to broth dilution assay. We found the percent of inhibition for NPs and E to be 84 and 41% respectively at 15 hrs of incubation. On the other hand, positive control showed 94% inhibition at 15 hrs incubation. This confirms that NPs is highlysignificant when compared to positive control.

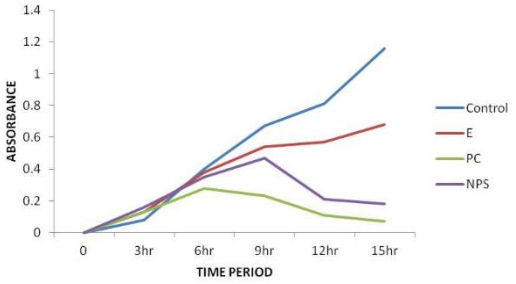

Fig. 10: Biofilm inhibition study: C: Control, PC: Positive control; E: Extract, NPs: Green synthesized NPs.

4. DISCUSSION

Numerous acteria, including Gram-positive and Gram-negative, as well as fungi, are susceptible to the antibacterial activity of polyphenols. Of the more than 10,000 chemicals that can be taken into consideration, a wide range of classifications have been published. There are ten main classes of polyphenols, which can be categorized, for instance, into families based on the number and arrangement of carbon atoms in their structure [19]. Polyphenols have the structural characteristic of having at least one aromatic ring with one or more hydroxyl groups. Simple phenols can undergo oligo/polymerization to produce some of these chemicals. Furthermore, a lot of these substances combine with mono- or polysaccharides to form glycoconjugates, which produce useful byproducts like methyl esters and esters [20].

In this study, we extracted polyphenols from fermented fox tail millets. From our data, we found the total polyphenol content was found to be 47.08102 ± 3.61, 278.7178 ± 7.11 and 223.2112 ± 10.78 GAE equivalents respectively at 3, 7 and 14 days. We were successful in conjugating the polyphenols to the copper based nanoparticles as evident from the FTIR characterization.

The conjugated nanoparticles were used for the antibacterial efficacy against the plant pathogen *Ralstonia solanacearum*.

The synthesized NPs showed a significant inhibition of the bacterial growth as confirmed from agar well and MIC assay. By compromising cell membrane integrity, preventing the development of biofilms, and decreasing the

expression of genes linked to virulence, polyphenols-especially those included in plant extracts-show encouraging antibacterial action against *Ralstonia solanacearum*, the causative agent of bacterial wilt [21]. Studies by Dahham *et al*., [22] also confirmed of the active role of polyphenols against plant pathogens.

Most probably, the bacterial cell membranes may react with polyphenols, changing the permeability of the membrane and eventually causing cell lysis. This could be the possible explanation of the antibacterial efficacy role of the polyphenols [10]. The production of biofilms by *R. solanacearum* contributes to its persistence and disease development; polyphenols can prevent the formation of biofilms, which facilitates the pathogen's control [10].

To sum up, polyphenols extracted have the potential to be an antibacterial agent in plants, which makes it a vital treatment for bacterial wilt in the field in the future. By significantly preventing the production of biofilms and eliminating the bacteria, exposure to polyphenol conjugated copper nanoparticles may shield plants from *R. solanacearum*. As a result, our work offers efficient and eco-friendly methods for the investigation and creation of agents to prevent vascular or bacterial wilt, and it may eventually be expanded to include the management of other plant diseases.

5. CONCLUSION

Using copper sulphate and the polyphenol extract from fermented millets, main conclusions of the study point to a quick, easy, and economical way to create CuNPs in an environmentally friendly manner. A number of common methods, such as UV-Vis, XRD, NTA, and SEM microscopy, verified that CuNPs were successfully formed. CuNPs have an antibacterial effect on Ralstonia solanacearum, according to antibacterial research. Furthermore, inhibition zones and biofilm inhibition assays showed that the CuNPs had strong antibacterial action. While considering all the aspects, multifunctionality of the synthesized CuNPs indicates their encouraging potential for a variety of future uses.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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