



Antibacterial Effect of Biosynthesized Zinc Oxide Nanoparticles against *Erwinia amylovora* and Induction of Crop Growth

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Objectives: Zinc oxide nanoparticles (ZnO-NPs) are widely recognized as one of the most promising types of materials in a wide range of applications, including agriculture. Modern systemic efforts have identified several therapeutically active microalgae-derived compounds, including phenols, flavonoids and others. The antibacterial properties of the phenolic substances were demonstrated. Hence, the present study aims to exhibit the antibacterial activity of the bioactive compound capped silver nanoparticles under *in vitro* conditions.

Methods: Bioactive compound separated by Solid-phase Extraction method. Dispersible Zinc oxide nanoparticles synthesized using the bioactive compound as the major capping agent. Zinc nitrate was used as starting material and its reduction was carried by phenolic components of *Spirulina platensis* aqueous extract from Zn²⁺ to ZnO. The synthesized Zinc oxide nanoparticles are characterized by H1 NMR spectroscopy. Conjugated nanoparticles are characterized physically by Scanning Electron Microscopy (SEM) analysis. SEM demonstrated particle sizes in the range 10–15 nm. ZnO nanoparticles demonstrated antibacterial activity against an isolated plant pathogen *Erwinia amylovora*. Time kill determination assay was done.

Findings: Phenols obtained after Solid Phase Extraction. Hence, this was regarded as the

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maximum quantified bioactive compound of *Spirulina platensis*. H1 NMR spectroscopy analyses showed the presence of phenolic compounds and alcohols groups of long chain were also detected. In SEM analysis, the mean diameter of spherical Phenols-ZnOPs is less than 15 nm surrounded by the capping agent. In given time periods of 4, 8, 16, and 24 hour cells, concentrations of 1000µg/mL were 42 %, 33 %, 20 %, and 18 %. At 500 µg/mL of extract concentration, *Spirulina platensis* inhibited 50% bacterial proliferation (IC50) of *Erwinia amylovora*. A significant inhibitory effect ($p < 0.0001$) was seen against the plant pathogenic strain.

Novelty: In addition to their antibacterial activities, biosynthesized ZnO-NPs are thought to show promise efficacy as growth accelerators. The most dangerous bacterial disease of pear and apple trees is fire blight, caused by *Erwinia amylovora*. Phenolic capped ZnO-NPs have been found to be efficient plant pathogen antagonists.

Keywords: Zinc oxide nanoparticle; *Spirulina platensis*; x-ray diffraction; scanning electron microscope; phenols; *Erwinia amylovora* and fourier transform infrared spectroscopy.

1. INTRODUCTION

Metal oxide (MNPs) may be produced in a variety of ways (chemical, physical, and biosynthetic) and have a diverse set of characteristics and uses. Green synthesis covers the synthesis from algae, fungus, plants, bacteria, and other microorganisms. They enable the large-scale manufacturing of ZnO-NPs devoid of contaminants. Microalgae have been utilized to generate ZnO-NPs because of the specific bio compounds they produce. Natural extracts of Algal components offer a low-cost and environmentally beneficial alternative to using intermediary base groups [1].

Secondary Algal compounds found in crude algal extracts function as both reducing agents and capping or stabilizing agents. Metal ions or metal oxides are reduced to zero valence metal NPs in bioreduction with the aid of Algal-secreted bio compounds such as polyphenolic and phenolic compounds, alkaloids, polysaccharides, amino acids, vitamins, and terpenoids [2]

Algae also have the ability to grow without the help of any addition of outside chemicals or fertilizers. Microalgae grow extremely Quickly, and on average, double their mass 10-fold faster than higher plants. It is known that various species of microalgae reduce metal ions. The results clearly showed that the size of produced NPs reduces as the content of an algal extract increases. All tests revealed NPs with spherical and hexagonal disc shapes, as verified by XRD and SEM analyses. Furthermore, these synthesized NPs were also proved efficient in various biomedical applications like antimicrobial, anti-cancer, anti-diabetic, and antioxidants [3].

Recently, there has been a lot of interest in microalgae as a source of new, physiologically active compounds such phycobilins, phenols, terpenoids, steroids, and polysaccharides. However, the presence of phenolic chemicals in blue-green algae is less well established than in higher plants. Algal phenolic chemicals have been identified as a possible contender for combating free radicals, which are detrimental to human bodies and plant systems. Manipulation of growing conditions for biomass production and productivity, on the other hand, is commonly utilized in the commercial manufacture of potentially valuable chemicals such as carotenoids and phenolics [4].

Here, we disclose the bio-assisted synthesis of ZnO-NPs through an eco-friendly approach using aqueous extracts of *Spirulina platensis* as an efficient oxidizing/reducing and capping agent. The biosynthesis of ZnO-NPs has already been reported; however, their diverse biological properties including antibacterial activities in plants have been less exposed. The aim of the present study is therefore to investigate the biological effects of phenolic capped ZnO-NPs. H NMR spectroscopy and SEM were used to characterize the ZnO-NPs. The well-characterized ZnO-NPs were examined for their biological activities in plant pathogens. The *in vitro* antibacterial potential against *Erwinia sp* was examined for their possible application in the crop field.

2. MATERIALS AND METHODS

The experimental organism *S. platensis* was isolated from Kodai road, Dindugal, Tamilnadu (India) and cultivated in Zarrouk's medium under $30 \pm 2^\circ\text{C}$ temperature and illuminated with white

fluorescent lamps at a light intensity of 2,000 lux (Sharma et al 2014) [5].

Zinc nitrate and other reagent were purchased from Sisco Research laboratory, Chennai.

2.1 Preparation of Microalgal Extract

In an Erlenmeyer flask, 5 g (dry weight) *S. platensis* biomass was suspended in 100 ml double distilled sterile water and heated for 15 minutes at 100°C [5]. The mixture was boiled, then cooled and centrifuged for 15 minutes at 10,000 rpm. The supernatant was collected and kept at 4 degrees Celsius for further analysis.

2.1.1 Solid phase extraction

Solid-phase extraction [phenols from *Spirulina platensis*] is carried out and the filtrates are collected in a Petri plate each of 10 ml quantity and the solvent is evaporated at atmospheric pressure in a water bath. After drying the residual weight is noted down and subtracted from the initial empty weight of the Petri plate, and the amount of phenolic compounds being extracted is calculated [6].

2.2 H¹ NMR Spectroscopy

To analyze the BAC composition and purity within the limits of detection of the method.

2.2.1 Equipment

¹H NMR spectrometer (e.g., Bruker-400 (400 MHz) with Avance II console and Top-spin processing software) [7].

2.2.2 Procedure

The samples were dissolved through dimethyl sulfoxide-d₆ (DMSO-d₆) as a solvent. A volume of 20 μL of tetramethylsilane (TMS) was added as the internal reference. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) expressed in δ units, and spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), t (triplet), or m (multiplet).

2.2.3 Synthesis of bio active compound capped silver nanoparticles

BAC from *Spirulina platensis* was dissolved in 1 mL methanol, and 50 mL volume was made up with deionized water to make a stock solution of

5 mM. BAC – conjugated Zinc oxide nanoparticles were synthesized by the reduction of Zinc nitrate solution using sodium borohydride in the presence of phenols. One milliliter of 1 mM Zinc nitrate solution was magnetically stirred with 1 mL of 1 mM phenols before 5 μL of freshly prepared 5 mM sodium borohydride was added and stirring was continued for 2 h [8].

2.2.4 Physical analysis - SEM analysis

SEM experiments were performed to characterize the size and shape of bio-reduced Zinc oxide nanoparticles. Purified nanoparticles were sonicated for 15 min to make it uniform distribution and SEM measurements were performed at Madurai Kamraj University, Madurai. A samples was placed on the sample holder and were scanned at a magnification of 17000x, 28000x, 40000x, 50000x and 60000x [9].

2.2.5 In vitro antimicrobial assay of ZnONPs against multidrug-resistant bacterial strains

The weakness of ZnONPs against multidrug-safe bacterial strains was resolved utilizing Kirby-Bauer's plate dissemination and agar well dispersion technique as per CLSI (Clinical Laboratory Standard Institute) Guidelines (2009). Bacterial strain *Erwinia sps* was utilized. About 50μL of the test (grouping of ZnONPs 2 mg/50 μL DMSO) was utilized against each strain cleaned on supplement agar plates followed by brooding at 37°C for 24 hrs. DMSO was utilized as negative control while 50μL watery concentrate of *S.platensis* was utilized to assure control. Antibacterial activity was assessed using a zone of inhibition (Zoi) measured after the incubation period against tested bacteria.

2.3 Time–kill Determination

To investigate possible antibacterial activity and thus minimize potential toxicity and resistance problems, mixtures of nanoparticles were also tested. ZnO at sub-MBC concentrations was used in killing assays against *Erwinia sp*.

At time zero, ca. 5×10⁷ CFU/mL of bacteria was added to the nanoparticle suspension at a dilution of 1 in 80. Incubation was then carried out in a shaking incubator (200rpm at 37 °C in air for up to 4 h) Inoculated nano particle-free suspensions in PBS were used as negative controls. Growth was assessed by plating serial

dilutions of each nanoparticle/bacterial suspension at different time points onto tryptone soy agar plates. Plates were then incubated at 37 °C in air with CO₂ for 24 h [10].

2.4 Influence of Biosynthesized ZnO-NPs on *Vicia faba* Seed Germination

The influence of biosynthesized ZnO-NP concentrations on the percentage of germination in the various concentrations over five days. 50 micro litre of *Erwinia sp* was inoculated in the soil.

2.5 Statistical Analysis

The examination was acted in sets of three, Mean ± S.D was contrasted and the standard was utilized and dissected measurably utilizing chart paid instant Dataset1, One way ANOVA. Estimations of $p < 0.05$ were considered statistically significant. IC₁₀ and IC₅₀ esteem in addition to their 95% certainty spans were determined utilizing the probit examination in SPSS variant 13.

3. RESULTS

3.1 Phenolic Extraction

3.3.1 Solid-phase extraction

Phenolic chemicals are separated from the crude extract of *Spirulina platensis*. However, there are a variety of different chemicals, such as alkaloids produced in larger proportions. As a result, the crude extract was shaken with water. After solid-phase extraction, alkaloids dissolved in water and phenolic-rich fraction are obtained.

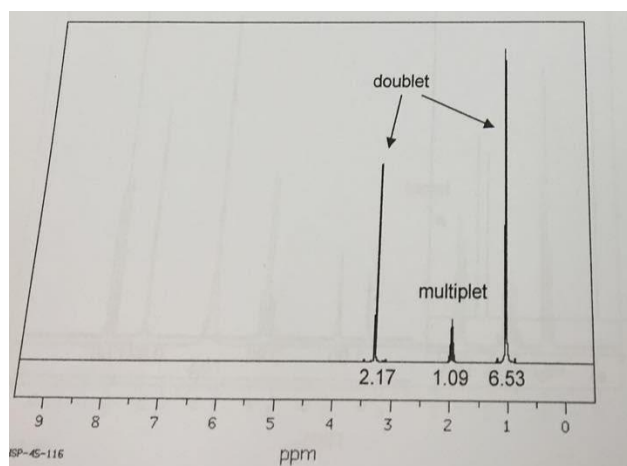
Isolated BioActive Compound [BAC] was further confirmed by H¹ NMR spectroscopy

3.3.2 H¹ – NMR spectroscopy

Reactions of bioactive compounds were carried out in the presence of chloroform. All the reaction mixtures were studied by 1 H - Nuclear Magnetic Resonance Spectroscopy (1 H-NMR). Differences in the composition of the reaction mixture of the BAC by H¹-NMR analysis were found. During the solid phase extraction, phenols are found as major products.

We came to know the highest peak [2977.7] was phenols. Hence solubilization was done with water because water is a poor solvent to dissolve alkaloids. Though other compounds were referred to simultaneously and show no specific pharmaceutical activities. Therefore, phenols was separated further and again checked for NMR reading. Comparing the 1H-NMR spectra of phenols before and after solubilization meaningful differences were found (Graph: 1). The phenolic spectrum only showed the corresponding signals of the protons with chemical shifts between δ 2.13-6.5 ppm and the corresponding signals of the rest of the protons, below δ 2.0 ppm showed other compounds. On the other hand, 1H-NMR spectra of solubilized mixtures obtained under all the experimental conditions described above were identical (from a qualitative point of view).

In Graph: 1, a representative spectrum of phenol is shown. Finally, we found that phenol is a bioactive compound, and it is capable of interacting with the metal ions, by inter and intramolecular reactions.



Graph 1. Spectrum of oleic acid

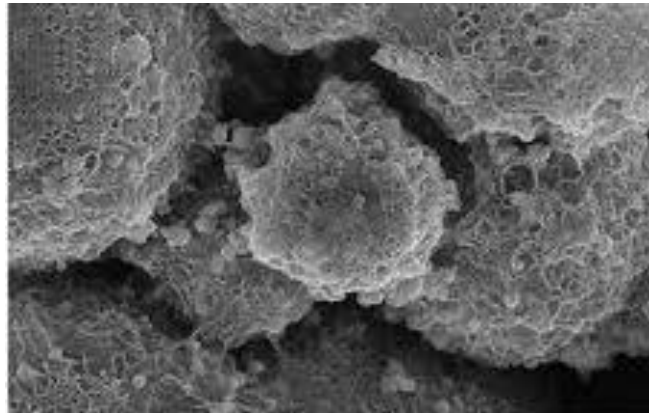


Fig. 1. SEM Analysis



Fig. 2. Colony morphology

3.2 SEM analysis Biosynthesized ZnO Nanoparticles

The morphology of ZnO-NPs was spherical. The mean diameter of spherical NPs was 12.6 ± 3.8 nm [crystalline size less than 20 nm surrounded by the capping agent]. Combination therapy was the most potent therapy for plants. [Fig. 1]

3.2.1 Isolation of *Erwinia amylovora* in King's B media

Colonies of *E. amylovora* on King's media are pale violet, circular, high convex to domed, smooth and mucoid, and they grow more slowly than on King's B media. [Fig. 2]

3.2.2 Minimum bactericidal concentration and time-kill determinations

Using time-kill tests, *Erwinia sp* was decreased by 68 % within 2 hours in the presence of 1000 $\mu\text{g/mL}$ micro ZnO. This was enhanced to 88 % ($p < 0.05$; independent t-tests for students). In the presence of 1000g/mL micro ZnO, these overgrown bacteria were reduced to zero in 4 hours. *Spirulina platensis* inhibited *Erwinia amylovora* bacterial multiplication by 50% (IC50) at a concentration of 500 $\mu\text{g/mL}$ of extract. Against the plant pathogenic strain, there was a considerable inhibitory impact ($p < 0.0001$). [Fig. 3]

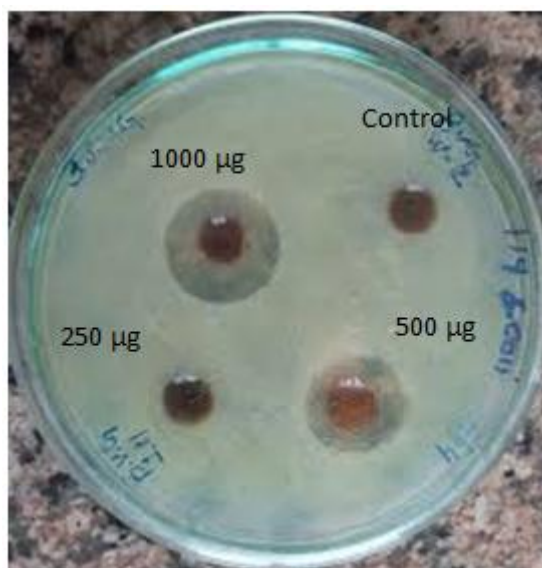


Fig. 3. Well diffusion assay

Seed germination began after one day, with a percentage ranging from 28 under control conditions to 50% with 500 µg/mL ZnO-NP. Seed germination began on the first day as well, but with a low percentage (less than 20% in all the treatments except at 1000 µg/mL concentrations, After five days of harvesting, better results were noticed in 1000µg/mL

ZnO-NP treatments influenced both root and shoot lengths. Root and shoot elongation was stimulated by low-moderate levels of ZnO-NPs, reaching a height of 7.6 ± 0.8 and 6 ± 0.7 cm, respectively, with 12.5 µg/mL of ZnO-NPs, following which the roots steadily decreased at higher concentrations of 100 and 200 µg/mL to 4 ± 0.3 and 4 ± 0.45 cm, respectively.

There was no substantial difference in root lengths between low and moderate levels compared to control, but they decreased drastically at high levels and registered the shortest lengths among all treatments at 100 and 200 base fluid dilutions (2.8 ± 0.2 and 2 ± 0.33 cm, respectively).

4. DISCUSSION

Notably, as multi-drug resistance bacteria have evolved, ZnO-NPs have emerged as promising antimicrobial agents. This is mostly owing to their enhanced ability to tackle a wide range of infections. Furthermore, zinc is recognized as a necessary trace element for the majority of biological functions in the animal's body. As a

result, the use of ZnO-NPs has been shown to greatly improve the health and productivity of plant pathogens [11].

Phenols obtained by Solid Phase Extraction. As a consequence, it was determined that this was the most measured bioactive component of *Spirulina platensis*. H^1 NMR spectroscopy investigations revealed the presence of phenolic chemicals, as well as long-chain alcohol groups. In SEM investigation, the mean diameter of spherical Phenols capped ZnONPs enclosed by the capping agent is less than 15 nm. Concentrations of 1000g/mL were 42 percent, 33 percent, 20 percent, and 18 percent in cells with time periods of 4, 8, 16, and 24 hours. *Spirulina platensis* inhibited *Erwinia amylovora* bacterial multiplication by 50% (IC50) at 500 g/mL extract concentration. Against the plant pathogen, a considerable inhibitory impact ($p < 0.0001$) was observed.

In contrast to our study, Rajendran et al., 2021 revealed the biosynthesis of zinc oxide nanoparticles (ZnO NPs) from crude extracts and phytochemicals has recently received a lot of interest [12]. Green NP synthesis is a cost-effective, environmentally friendly, and promising alternative to chemical synthesis. This research concerns the manufacture of ZnO NPs utilizing *Rubus fairholmianus* root extract (RE) as an effective reducing agent. The RE-ZnO NPs were found to be spherical in form with clusters (1–100 nm) according to SEM examination. The NPs' antibacterial efficacy against *Staphylococcus*

aureus was evaluated using agar well diffusion, minimum inhibitory concentration, and bacterial growth assays. The phytochemicals of *R. fairholmianus* aid in the formation of stable ZnO NPs and demonstrate antibacterial activity.

This might be owing to ZnO NPs' damaging effect on cells and increased formation of highly reactive oxygen species (ROS) such as OH, H₂O₂, and O₂⁻ which causes cell death. After attaching to the surface of the cell membrane, ZnO nanoparticles cause a disruption in the cell's respiration due to interactions with enzymes in the bacteria's respiration chains, as well as increased permeability through the bacterial cells, resulting in the loss of the cell's transport mechanism [12]. This might be because ZnO NPs have a better penetrating capacity proved by Kalaba et al., 2021 [13].

Sarcophyton trocheliophorum 1H NMR revealed three aromatic protons at 7.30, 7.07, and 6.58, corresponding to the 1,2,4-trisubstituted aromatic residue [14].

Low doses of ZnO-NPs aided seed germination and seedling growth in *Vicia faba*, while higher concentrations (1000 and 2000µg/mL) caused phytotoxicity. These findings are similar to those of Youssef and Elamawi [15]. Furthermore, Studies discovered that ZnO-NPs improved rice seed germination, which is similar to our findings [16,17]. Sharaf [12] investigated the effects of 20-nm ZnO-NPs at concentrations of 0, 10, 20, 30, and 40 mg/L on onion root length (*Allium cepa* L).

Hence the present study attributes the mechanism behind the bactericidal activity of Combined nanoparticles. We prove that phenol compound disturbs the cellular membrane of pathogenic bacteria and nanoparticles conjugated with the bioactive compound act as reactive oxygen species, which destroy the genetic material of the pathogen. However the entire study resembles hypothetically, Tiwari et al. [18] explained the mechanism of antibacterial activity of zinc oxide nanoparticles against carbapenem-resistant *Acinetobacter baumannii*.

5. CONCLUSION

This research work is the ongoing portion of the previously biosynthesized ZnO-NPs using an aqueous extract of *Spirulina platensis*, a well-known plant for its medicinal importance. The presence of bioactive compounds has been

confirmed by H¹ NMR spectroscopy analysis. SEM analysis determined morphology and vibrational modes, while apparent charge and steadiness were determined by DLS. The produced Phenolic capped ZnO-NPs have shown good antibacterial capabilities. Phenolic capped ZnO-NPs have shown an effective bactericidal activity at a higher concentration and showed >50% inhibition against plant pathogen *Erwinia amylovora*. Our findings suggest that the above-mentioned Phenolic capped ZnO-NPs might be used in agriculture due to their antibacterial properties. Because of the manner of action of ZnO-NPs and phytochemicals, synergy and additive behavior of ZnO-NPs-phytocompounds arises. Because bacteria cannot acquire resistance to this consortium, this conjugation is always advantageous.

6. RECOMMENDATIONS

Future research in this topic should be in the treatment of different diseases such as tumor causes and other inflammatory disorders in plants. More study on BAC capped ZnO-NPs is needed to investigate their biological properties in vitro and in vivo. Further experiments should be performed, including in vivo measurements, and the side effects of ingesting this compound should be thoroughly investigate.

7. LIMITATIONS

No toxicity assay were analyzed in the present study with the use of BAC capped ZnONPs.

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Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

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