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Biogenic Synthesis and Characterization of Zinc Oxide Nanoparticles by using Green Machinery: Antibacterial and Antibiofilm Potential

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Abstract

In the field of biomedicine, the green synthesis of Zinc Oxide Nanoparticles (ZnONPs) utilising plant extracts has piqued interest. The reduction nature of herbal extracts has recently aided in the production of spherical ZnONPs of various potentials from zinc salt. In this study, fresh leaf (aqueous) extracts of *Cannabis sativa* were used as reducing and stabilising agents in a rapid, environmentally friendly approach for the synthesis of ZnONPs. UV–VIS and Fourier transform infrared spectroscopy, as well as transmission electron microscopy, were used to analyse the biosynthesized CNS-ZnONPs (TEM). The antibacterial and antibiofilm properties of produced CNS-ZnONPs were also studied in vitro. The presence of a prominent absorption peak at 380 nm, which corresponds to the CNS-ZnONPs' Surface Plasmon Resonance (SPR) band, indicated the creation of CNS-ZnONPs. The produced CNS-ZnONPs were spherical in shape, with an average particle size of 16.25 nm, according to TEM examination. The synthesised CNS-ZnONPs also showed significant antibacterial activity against a variety of Gram-positive and Gram-negative microorganisms. Furthermore, the biosynthesized CNS-ZnONPs significantly reduced biofilm formation. *Cannabis sativa* leaf extracts may be utilised to easily synthesise ZnONPs, which can be employed as a natural source of antibacterial and antibiofilm agents.

Keywords: Antibacterial, Antibiofilm, *Cannabis sativa*, Green Synthesis, Zinc Oxide Nanoparticles (ZnONPs)

1. Introduction

The term "Green Chemistry" encompasses environmentally friendly materials that are suitable for biomedical and therapeutic applications, in which the lethal element/compound does not use in the process of synthesis. The different types of micro-organisms have been employed to synthesize diverse metallic nanoparticles (NPs). That has an advantage over other conventional chemical processes like minimal cost, energy saving, as its green chemistry. Biocompatibility of bio-inspired NPs opens up a lot of possibilities in biomedicine and other sectors[1,2]. The inorganic nanoparticle demands have been increasing enormously and manufacturing is expected to reach $13.7 billion by 2026[3–6]. The applications of nanomaterials in the various field have contributed vastly to macroeconomic industries[7]. Antibacterial properties are seen in a variety of inorganic NPs, including Ag, Au, Cu, CuO, TiO_2, and ZnO. Including the other inorganic NPs, particular ZnONPs have great attention because of the simple and easy preparation method, inexpensive and harmless for humans as well as animals. Along with being widely employed in the preparation of health care items[8,9]. Additionally, ZnONPs have a great potential in biological uses such as gene delivery, biological sensing, biological tagging, drug delivery, and nanomedicines. Cotton textiles were used to synthesize stable nanoparticles as well[10,11]. From the antimicrobial source, the green synthesized nanoparticles have additional features of self-functionalization of molecular medicine on the nanoparticles, as seen by their improved antibacterial efficacy. Vijayakumar et al., in the year 2018, employed the study that indicated the production of improved antibacterial nanoparticles. The various article demonstrated that synthesized ZnONPs form the green routes extracts obtained from diverse plants which produce NPs of different sizes and shapes. ZnONPs have different shapes like nanoflowers, nanosphere, hollow sphere, hexagonal wurtzite, and nanorods were described by Pachaiappan et al.,[12–14]. While ZnO is hydrophilic, hydrophobic films are effective in preventing biofilm adherence. Meanwhile, one study found that ZnO-coated surfaces significantly reduced biofilm

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development and that the formation of hydroxyl radicals, rather than the presence of zinc ions, played a critical role in antibiofilm activity[15,16]. Furthermore, ZnO composite films may be employed in a variety of applications to prevent biofilm development, including aquatic product preservation[17,18]. Furthermore, ZnO composite films may be utilized to prevent biofilm formation in a variety of industries, and they have a promising future in the preservation of aquatic products. As a result, the hydrophobic characteristics of ZnO films must be improved[19]. *Cannabis sativa* belongs to the *Cannabis binaceae* family, which is also known as weed hemp, Ganja, Hashish, reefer, marijuana, grass. Mainly found in Central Asia, cannabis is one of the oldest psychoactive plants known to man, along with that it is also used worldwide either as a medicinal plant or as a good source of fibers and food[20,21]. Therefore, surprisingly, leaves and seeds are mainly employed to prepare extracts in most research assessing antibacterial activities of cannabis. *Cannabis sativa* has multiple bioactive compounds. Cannabinoids, flavones, and terpenes are the main components are present in this plant. Article published by Khan et al., (2016) revealed that secondary metabolites, such as terpenoids, play an important role a reducing agent in the synthesis of NPs.[22] AIDS, cancer, Post-Traumatic Stress Disorder (PTSD), multiple sclerosis, anorexia, nausea, cachexia, neuropathic pain, drug addiction, neurological and other mental disorders have all seen an upsurge in interest in legalizing cannabis for therapeutic purposes[23,24].

During this study, we used *Cannabis sativa* leaf extract to synthesize CNS-ZnONPs via green pathways, with a focus on NP development at an optimum temperature. UV-visible (UV-VIS) spectroscopy was used to determine the optical characteristics of the synthesized nanoparticles. Fourier transformed infrared spectroscopy (FTIR) and Transmission Electron Microscopy (TEM) was used to characterize NPs. Further the purified and characterized ZnONPs were showed antibiofilm and antimicrobial properties against *B. subtilis* (MTCC 441), *Escherichia coli* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), and *B. pumilis* (MTCC 160).

2. Material and Methods

2.1 Chemicals

The media and chemicals were procured from Sigma Aldrich (St. Louis, USA) and HiMedia, India. The Multi-Drug Resistant (MDR) strain *Escherichia coli* (NCIM 2571), *B. pumilis (MTCC 160), Klebsiella pneumoniae (ATCC 13883)* and *B. subtilis (MTCC 441)* were purchased from NCIM, Pune (India). All bacterial strains were cultured in MHB (Mueller Hinton broth) g/L: Casein hydrolysate, 17.5; Starch 1.5; dw, 1000 mL and pH 7.3 at 37°C for 24 h.

2.2 ZnONPs Synthesis with Plant Extract

For the preparation of plant extract, the *cannabis sativa* leaves were thoroughly washed, dried and weighed. The leaves were crushed with the help of pestle mortar and tris buffer was added in it. Take some ice cubes in the polypropylene moulded tray and place pestle & mortar in it with plant extract and leave it for some time. Then again crush the extract and filter it with the help of Whatsman filter paper in the centrifuge tube and then the tubes were placed in centrifuge at 6000 rpm at 4°C for 10 min. Then remove the pellet from the extract and take the supernatant in another centrifuge tube. Extract is stored in refrigerator for future purposes. 3 ml of the prepared plant extract was taken in 20 ml of centrifuge tube and 3µl of zinc sulphate salt was added to the plant extract. Keep the reaction tube in incubator at about 37°C for 48 hrs. After 48 hrs. Then the sample was taken for UV-VIS spectroscopy and O.D values were recorded, sample was stored in the refrigerator for further experiments.

2.3 Characterization of Synthesized CNS-ZnONPs

For the characterization of the sample UV–VIS spectroscopic technique was used and set to a resolution of 1 nm in the quartz cuvette. Further TEM (Transmission Electron Microscopy) of the sample was accomplished by TECNAI G2 Spirit BioTWIN operated at an 80 kV accelerating voltage by the FEI Company's Transmission Electron Microscope. FTIR of CNS-ZnONPs was performed by the method used by baker et al., in year 2020, with few modifications[25].

2.4 Antibacterial Screening

To test the antibacterial activity of the CNS-ZnONPs, well diffusion method was used[26]. The antibacterial potential of biosynthesized NPs was estimated against some pathogenic bacteria, i.e., MDR strain of *B. subtilis* (MTCC 441), *Escherichia coli* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883) and *B. pumilis* (MTCC 160). The Antibacterial activity was performed as described earlier by Bano et al., in year 2021, with few modifications and the final prepared plates were incubated for 24 h at 37C. Subsequently, the zones of inhibition were measured in mm[27].

2.5 MIC and IC50 Value Determination

The determination of minimum inhibitory concentration was done by using the microdilution method in 96-microwell plates[28,29]. The antibacterial potential of CNS-ZnONPs was intended by their MICs against *B. subtilis* (MTCC 441), *Escherichia coli* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883) and *B. pumilis* (MTCC 160). The bacterial strain was collected in the mid-logarithm phase and diluted to $2x10^5$ CFU/mL in 0.03% of Luria-Bertani (LB) broth in PBS. The 100 µL of LB medium consist of CNS-ZnONPs were serially diluted in 96 well plates. The lowest concentration of NPs at which 25%, 50%, 75% growth of microbes was inhibited is defined as MIC25, MIC50, and MIC75, respectively. For negative control, Milli-Q water was used for each experiment.

2.6 Antibiofilm Potential of ZnONPs

The formation of biofilm was usually pathogenic in nature and can cause many nosocomial infections. According to the National Institute of Health (NIH) approximately 65–80% of all infections occur due to biofilm-forming microorganisms[30–33]. CNS-ZnONPs have the unique potential to destroy the biofilms of a variety of harmful bacteria types. The biofilms of *B. subtilis* (MTCC 441) *Escherichia coli* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883) and *B. pumilis* (MTCC 160) were produced using the method reported earlier, with minor alterations[34,35]. Biofilm's viability was resoluted using the crystal violet colorimetric assay[27]. The calculation of Percent attachment was done by the following equation:

$$\text{Attachment \%} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The following equation was used to calculate the percent inhibition of the biofilm.

$$\text{Biofilm \% Inhibition} = \frac{\text{Control OD 490nm}}{\text{Test sample OD 490nm}} \times 100$$

2.7 Statistical Analysis

The mean differences between groups were examined using a one-way Analysis of Variance (ANOVA) with a post-hoc Tukey HSD test. The statistical analysis was carried out with the Origin 6.0 software (US)[36]. All tests were declared statistically significant at $p \leq 0.05$[27].

3. Results

3.1 Biosynthesis of Zinc Oxide Nanoparticles and Characterization

Synthesis of ZnONPs via green routes using *Cannabis sativa* needed initial techniques by UV–vis spectroscopy. During the process of synthesis *Cannabis sativa* act as both capping as well as reducing agents. By reporting the distinctive Surface Plasmon Resonance (SPR) spectra of CNS-ZnONPs at a wavelength of 380 nm, the synthesis of CNS-ZnONPs was verified (Figure 1(a)). According to the Mie theory, the size was shown to be 16 nm, which was further validated by TEM analysis (Figure 1(b)) using the Gatan Digital Micrograph. The TEM micrographs reported well-defined, monodispersed CNS-ZnONPs of identical size. Pure extract (Figure 1(c)) had peaks at 3550 to 3300 cm1 (–NH), 2900 cm1 (–CN), 1632 cm1 (amide I), and 1065 cm1 (C–O) in the FTIR spectra. The –OH peak was missing in CNS-ZnONPs (Figure 1(d)), indicating that –OH was involved in the interaction with NPs during encapsulation.

3.2 Antibacterial Screening using Well Diffusion Method

The antibacterial action of CNS-ZnONPs against both gram-positive and gram-negative bacterial strains was found to be satisfactory. Using the agar well diffusion method, the antibacterial potential of CNS-ZnONPs was evaluated against normal and MDR strains of *B. subtilis* (MTCC 441), *B. pumilis* (MTCC 160), *Klebsiella pneumoniae* (ATCC 13883), and *Escherichia coli* (ATCC 25923). The antibacterial potential was confirmed by a clear zone of inhibition surrounding the inoculated region (Figure 2). The maximum zone of inhibition was found against *Escherichia coli* (Figure 2(d)).

3.3 MIC and IC50 value determination

The MIC of bioactive compounds produced by ZnONPs was used to determine their antibacterial activity. After incubation at 37 °C for 24 to 48 hours, the inoculated plates were analyzed. The ZnONPs' MIC value was determined to be around 31.2 10 g/mL. The IC50 values for *B. subtilis* (MTCC 441), *Escherichia coli* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), and *B. pumilis* (MTCC 160) were reported to be 23.8, 31.2, 33.1, and 33.8 g/mL, respectively (Figure 3).

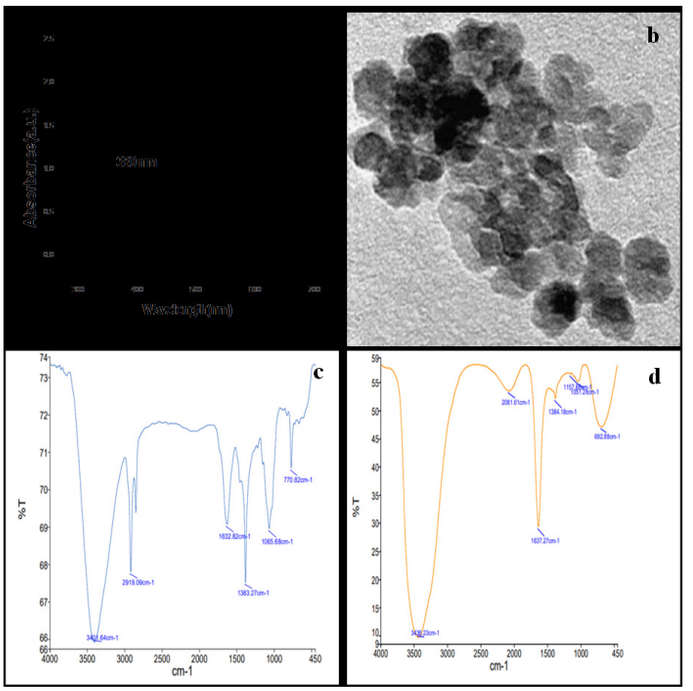

Figure 1. Characterization of *Cannabis sativa* mediated Zinc oxide nanoparticles (CNS-ZnONPs). **(a)** UV–VIS spectroscopy. **(b)** Transmission electron microscopy. **(c)** Fourier transform infrared (FTIR) spectrum of Extract. **(d)** CNS-ZnONPs.

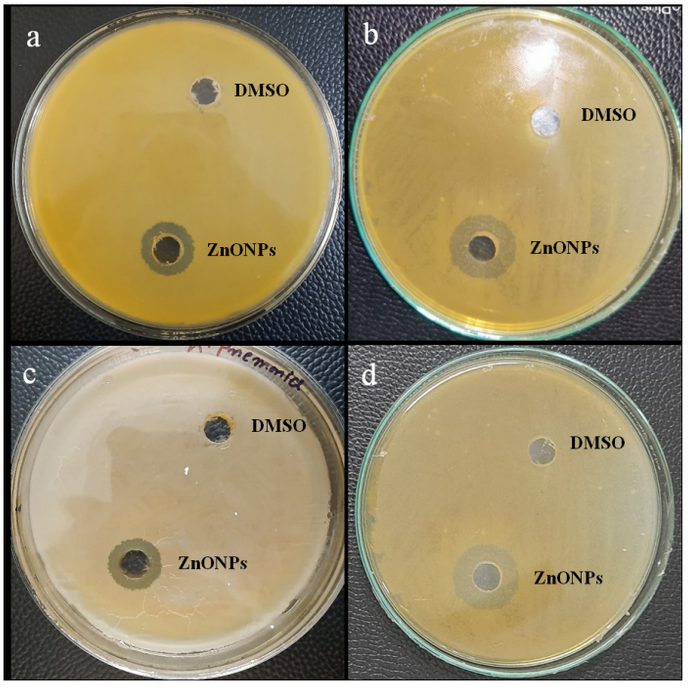

Figure 2. Qualitative assessment of antibacterial activity of CNS-ZnONPs. Müeller–Hinton (MH) agar plates were seeded with standardized suspensions (equivalent to the 0.5 McFarland) of. **(a)** *B. subtilis* (MTCC 441). **(b)** *B. pumilis* (MTCC 160). **(c)** *Klebsiella pneumoniae* (ATCC 13883). **(d)** *Escherichia coli* (ATCC 25923).

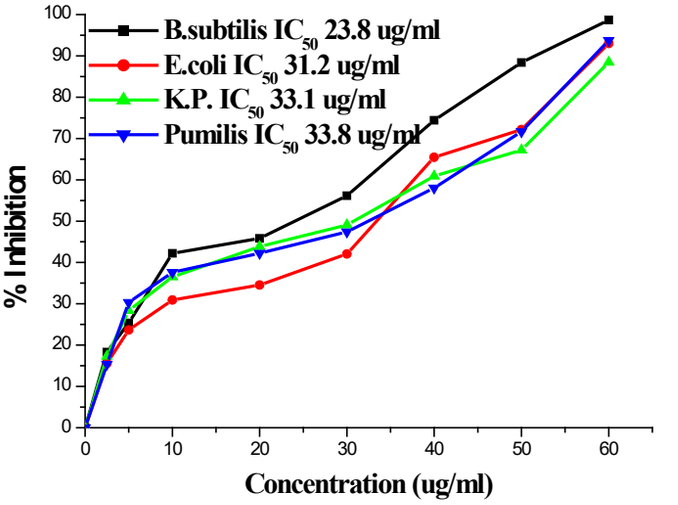

Figure 3. Determination of minimum inhibitory concentration (MIC) of CNS-ZnONPs against *B. subtilis* (MTCC 441), *Escherichia coli* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), and *B. pumilis* (MTCC 160).

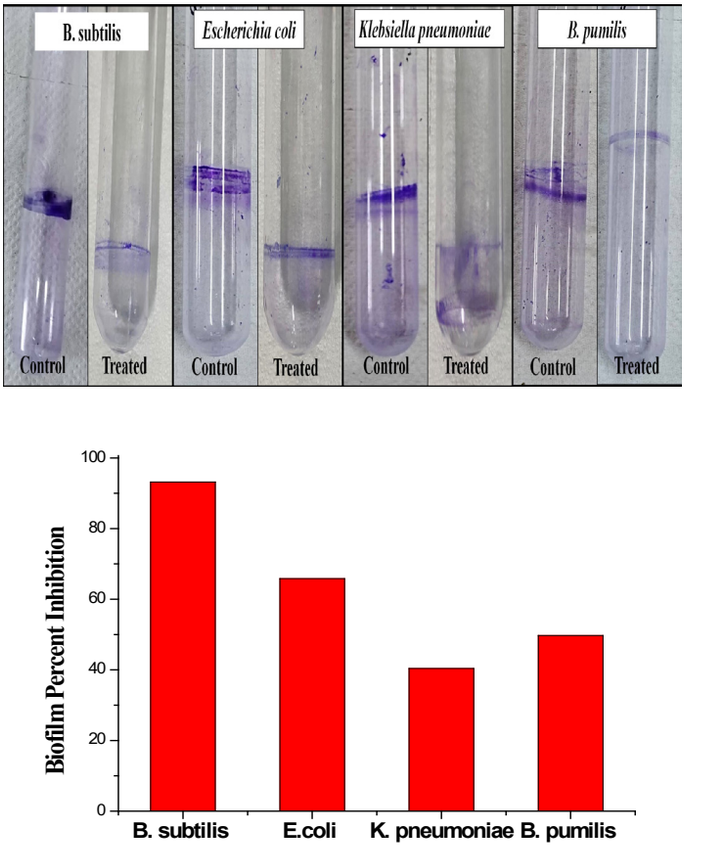

Figure 4. Image and graph showing inhibition of biofilm formation of CNS-ZnONPs.

3.4 Antibiofilm Potential of CNS-ZnONPs Biofilms

The crystal violet staining technique was used to investigate the anti-biofilm potential of ZnONPs. The bacterial strains were cultured in test tubes with and without extract. The biofilm development was significantly reduced (70-80%) when CNS-ZnONPs were used at IC50. CNS-ZnONPs was shown to be a more effective antibiofilm agent than the other extract. In the case of *B. subtilis* and *E. coli*, the decrease was more than threefold. In the positive control, there was no reduction in biofilm formation (without ZnONPs treatment) (Figure 4).

4. Discussion

Cannabis sativa was utilised as a reducing and stabilising agent in this work, and zinc sulphate was used as a precursor. The synthesis of CNS-ZnONPs is thought to be triggered by aqueous leaf extract's reducing enzymes and capping agents, such as secondary metabolites, which work together to decrease zinc sulphate[14]. UV–VIS spectra indicated the synthesis of CNS-ZnONPs, and TEM confirmed the particle size distribution profile of the synthesised CNS-ZnONPs. Nonetheless, as compared to ZnONPs, silver nanoparticles showed to have higher antibacterial action against a variety of harmful microorganisms. The inherent antimicrobial characteristics of silver ions, as opposed to zinc ions, may explain the higher antibacterial activity of silver nanoparticles. ZnONPs were tested against a variety of bacteria types to determine that they had antibacterial properties[35]. ZnONPs inhibited a wide range of microorganisms in the current investigation, owing to different mechanisms. Due to the limited therapeutic choices for certain illnesses, antimicrobial resistance is one of the most challenging global public health challenges. Many studies address the problem of bacterial resistance by repurposing or renewing the therapeutic applications of medicinal plants. Furthermore, novel techniques to increase antimicrobial medication distribution, penetration, targeting, and pharmacokinetics have been discovered, one of which is drug nanoparticle compositions. Metallic nanoparticles were used to efficiently distribute antimicrobials, resulting in a significant increase in targeting and pharmacokinetics[37]. Furthermore, metallic nanoparticles were found to have antibacterial properties and effectively synergized with natural product antimicrobial activity. Since ancient times, zinc has been regarded as the best inorganic antibacterial agent for fighting infections and spoilage[9]. ZnONPs inhibited a wide range of microorganisms in the current investigation, owing to different mechanisms. ZnONPs target the bacterial cell membrane, cell wall, DNA, and proteins, in addition to having a strong penetrative power. ZnONPs have been shown to cause pits in bacterial membranes and cell walls on numerous occasions. ZnONPs have been demonstrated to target subcellular regions of cell membranes, causing pits and cellular breakdown and death. Furthermore, ZnONPs break the link between the glycans N-acetylglucosamine and N-acetylmuramic acid and form a link between the peptide surface and the cell wall's glycan ports, leading to pit formation. ZnONPs have been shown to target more bacterial targets, such as respiratory chain dehydrogenases and bacterial chromosomes, in addition to targeting of cellular membrane and cell wall[13]. Furthermore, metallic nanoparticles' propensity to release Reactive Oxygen Species (ROS), which impede the oxidation of released zinc ions, confers a biocidal function.

5. Conclusion

We have developed a green protocol for synthesizing zinc nanoparticles by using the *Cannabis sativa* leaf extract. The given zinc nanoparticles were found to boost the potential of *Cannabis sativa* leaf in different bioactivities and showed effective antibiofilm, antibacterial activities. The mode of internalization and interaction with bacterial can be a subject of further studies. The toxicity studies of silver nanoparticles have also been a matter of concern. Therefore, a detailed study of toxicity of these particles can also be a good prospect of further studies.

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8. Conflicts of Interest

The authors declare no conflict of interest.

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