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Effectiveness of Zinc-Oxide (ZnO) Nanoparticles Using Achyranthes Aspera Leaf Extract and Their Potent Biological Activities Against the Bacterial Poultry Pathogens

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Abstract

Nanotechnology is one of the most significant areas of research worldwide because of its tremendous applications linked to the high surface area to volume ratio, improved pharmacokinetic profile and targeted drug delivery. In the current study, zinc oxide nanoparticles (ZnO NPs) were synthesized from Achyranthes aspera leaf extract, characterized by UV-visible spectroscopy, XRD, SEM, FTIR, AFM and evaluated for antibacterial efficacy against poultry pathogenic bacterial strains. UV-visible absorption peak was found at 370 nm. XRD showed hexagonal wurtzite structure of ZnO NPs while SEM results indicated an average size less than 100 nm with a minimum and maximum size of 28.63 and 61.42 nm, respectively. Further analysis of synthesized nanoparticles by FTIR showed stretching frequency at 3393.14 cm⁻¹, 2830.99 cm⁻¹, 2285.23 cm⁻¹, and 2108.78 cm⁻¹. The antibacterial activity of synthesized nanoparticles was investigated against common poultry pathogens Salmonella gallinarum and Salmonella enteritidis by the agar well diffusion method. The zone of inhibition with a diameter of 31 mm was observed against S. enteritidis and 30 mm against S. gallinarum was greater than the antibiotic (tetracycline) used. The minimum inhibitory concentration (MIC) was 0.195 and 0.390 mg ml⁻¹ for different bacterial strains. Characterization with different techniques showed a uniform and stable synthesis of ZnO NPs. Furthermore, the findings confirm the higher antibacterial activity of nanoconjugate in comparison to leaf extract and pure drug against pathogenic bacteria.

1. Introduction

Nanoparticles are atomic or molecular aggregates having size less than 100 nm and they act as active carriers towards their targeted site. In recent years, the synthesis and use of nanoparticles increased rapidly in every field of life including healthcare, cosmetics, electronics, etc. The fabrication of nanoparticles has been attempted by using several chemical and physical methods. Similarly, an array of biological systems and natural products such as bacteria, yeast, fungi, and plants bioactive compounds have been used in the synthesis of nanoparticles [1]. Among the various nanoparticles, synthesis of zinc oxide nanoparticles (ZnO NPs) by using green technology (natural products) has progressively emerged as an area of interest because of its efficient synthesis by using inexpensive natural products as reducing agents in comparison to other conventional methods of synthesis [2].

Plant extracts are rich in phytochemicals that act as reducing and stabilizing agents for synthesis of ZnO nanoparticles [3]. The ZnO NPs, like other metal oxides, exhibit significant antibacterial activities against a broad spectrum of bacterial strains. The nanoparticles get in direct contact with the cell wall and as a result, destruct the integrity of bacterial cell. In this context, the present study is planned to synthesize ZnO NPs by
using leaf extracts of *Achyranthes aspera* (*A. aspera*) plant. *A. aspera* belongs to family Amaranthaceae and is distributed in many tropical and temperate regions. The plant is an annual or perennial herb and has several common names such as chaff-flower, prickly chaff flower, Chircha, Kutri and Grootklits. It is a medicinal plant and has been an important part of herbal medicine system throughout the Asian and African countries. This herb has numerous pharmacological activities such as anti-inflammatory, analgesic, and antipyretic activities. It is used to treat a lot of disorders such as piles, fever, cough, digestive problems, dysentery, paralysis, spleen enlargement, control of fertility and post-partum bleeding. It has been employed for the treatment of bites of mad dogs and snakes. It is also used to treat oral diseases as some people used the root of this plant as a toothbrush [4].

The world is facing an issue of undiscriminating use of antimicrobials in the poultry industry that has accelerated the increase of antimicrobial resistance in pathogens. Antimicrobial resistance in poultry pathogens has resulted in financial loss, due to the expenditure on less efficient antimicrobials, as well as a lot of untreated poultry diseases [5]. The poultry industry is a dynamic and flourishing area contributing 5.76%, 26.9%, and 1.27% to the total meat production, farming sector and total GDP, respectively. The poultry sector in Pakistan has flourished with tremendous output within the last few years and functioned as protein rich nutrient source for general public. Poultry meat consumption is progressively increasing worldwide and has extended to 14.2 kg/person/year [6].

In developing countries, poultry is the fastest rising sub-sector of food industry. The poultry sector is expanding across the globe as the production of meat and eggs is constrained by growing population and urbanization [7]. In this perspective of increasing consumption and production, the microbial safety of poultry meat products is one of prime concern. Ingestion of microbial infected poultry meat and eggs has shown to be the major source of foodborne outbreaks in the USA between 1998 and 2012 [8]. The present study was designed to synthesize ZnO nanoparticles from *A. aspera* leaf extract, their characterization by various advanced spectral techniques and then to evaluate their antibacterial activity against the most common poultry pathogens *Salmonella gallinarum* and *Salmonella enteritidis* to combat with the issue of microbial resistance and flourish the poultry industry worldwide.

2. Materials and methods

Synthesis of ZnO NPs with *A. aspera* leaf extract was performed at Department of Chemistry, UMT Lahore-Pakistan and antibacterial activity was carried out at the Institute of Biochemistry and Biotechnology, UVAS, Lahore-Pakistan.

2.1. Chemicals

The chemicals used in the study were purchased from Sigma-Aldrich while distilled water was obtained from the laboratory of the university.

2.2. Collection and processing of leaves of *A. aspera*

Fresh leaves of *A. aspera* plant were collected from the University of Punjab, Quaid-e-Azam campus, Lahore. Identification of plant was done by the Department of Botany, GC University, Lahore. The collected leaves were washed with distilled water properly and then dried in an oven at 60 °C for 1 h. The dried leaves were ground to acquire the powdered material [9]. Extract was prepared by adding 20 g of leaves powder in 200 ml distilled water and heating for 20–30 min on the heating mantle. The obtained extract was filtered using Whatman filter paper No. 1 and kept in the refrigerator for further experimentation [10].

2.3. Preparation and characterization of zinc oxide nanoparticles

For the synthesis of nanoparticles, 10 ml of aqueous extract obtained from leaves of *A. aspera* was mixed with 0.1 mM zinc acetate [Zn (CH₃COO)₂] solution and placed on a magnetic stirrer. After 30 min, NaOH (2 g/50 ml) was added dropwise. This mixture was stirred continuously for 4 h and centrifuged at 10,000 rpm for 30 min. The supernatant was discarded and the pellet was centrifuged with deionized water followed by absolute ethanol three to four times. Finally, pellet was placed in hot air oven at 60 °C for 24 h to obtain the zinc oxide nanoparticles. Characterization of zinc oxide nanoparticles was carried out by using UV-Visible spectrophotometer of Shimadzu, UV-1280, X-ray diffraction (XRD) of Rigaku, XtalAB mini II, Fourier transform infrared spectroscopy (FTIR) of Shimadzu, Prestige-21, scanning electron microscopy (SEM) of Agilent Technologies, TV 1001 SEM and atomic force microscopy (AFM) of CoreAFM [11].
2.4. Preparation of bacterial culture and stock solutions
The characterized bacterial strains of *Salmonella gallinarum* and *Salmonella enteritidis* were obtained from the Department of microbiology, UVAS Lahore, Pakistan. Bacterial culture was maintained on a nutrient agar plates at 4 °C. The stock solution of ZnO nanoparticles was prepared by adding 100 mg of ZnO/ml of DMSO. Similarly, a solution of pure antibiotic was prepared by adding 100 mg of antibiotic (tetracycline) in 1 ml of distilled water.

2.5. Preparation of inoculum
The inoculum was prepared by direct colony suspension method that was picked from an 18 to 24 h agar plate. Normal saline solution (10 ml) was taken in test tubes and autoclaved at 121 °C, 15 psi for 15 min. A loopful culture of *S. enteritidis* and *S. gallinarum* was inoculated in two different test tubes containing autoclaved normal saline tubes. To standardize the inoculum density, the turbidity of inoculums were compared with 0.5 McFarland standard having 1.5 × 10^8 CFU (prepared by adding a 1.175% solution of BaCl2, 2H2O and 1% solution of H2SO4) [12].

2.6. Assessment of antibacterial activity of ZnO NPs by Well diffusion method
For antibacterial assay, a sterile cotton swab was immersed in bacterial inoculum and was evenly swabbed on the nutrient agar plate in 3 to 4 planes. In the last, the edge of agar was swabbed and left the plates undisturbed for 10 to 15 min. Then the wells of 5 mm diameter and 4 mm depth were made on inoculated nutrient agar plates using a sterile glass borer. The wells were sealed with molten agar. Nanoparticles suspension (50 µl) from solution (100 mg ml⁻¹) was dispensed into respective well. Tetracycline was used as a positive control whereas DMSO and plant extract as the negative control. All the experiments were run in triplicate to get précised and accurate results. The plates were incubated for 24 h at 37 °C, and the zone of inhibition was measured in mm produced around the colonies [13].

2.7. Determination of minimum inhibitory concentration by broth micro-dilution method
After the well diffusion method, MIC was determined by broth micro dilution method to confirm the concentration of ZnO nanoparticles that inhibited the bacterial growth after overnight incubation. In 96 well micro titre plate, 50 µl of nutrient broth was added in all wells using sterile micropipette. Then 100 µl of prepared bacterial inoculum was added up to 11th well. ZnO nanoparticles solution (50 µl) containing 2500 µg ml⁻¹ nanoparticles was added in first well and mixed with media. By using micropipette, 50 µl was transferred from 1st well to 2nd well to perform two-fold serial dilution. The procedure was continued up to 10th well to obtain the concentrations of 1250, 625, 312.5, 156.25, 78.125, 39.06, 19.53, 9.76 and 4.88 µg ZnO NPs/ml. In the positive control (11th well), only the organism was grown without NPs nanoparticles while negative control (12th) contained only nutrient media. These steps were repeated for each bacterial strain under test. All the tubes were incubated at 37 °C for 24 h. Finally, the absorbance was taken at 630 nm by means of an ELISA reader. The minimum concentration of extract that showed least growth was termed as MIC value and reported [14].

2.8. Statistical analysis
All experiments were run in triplicate. Data were analyzed by using descriptive analysis mean and standard deviation on SPSS-20 [13, 14].

3. Results

3.1. UV visible spectroscopy
The UV/visible spectroscopy was utilized to indicate the presence of ZnO nanoparticles. When light hits nanoparticles, its electromagnetic field oscillation produces a collective oscillation of metal’s electrons, which induce charge separation with respect to the ionic lattice. At a specific frequency, amplitude of oscillation was maximum, which is called surface Plasmon resonance (SPR) [15]. UV-visible analysis of synthesized ZnO nanoparticles showed surface Plasmon resonance absorption peak (SPR) of ZnO nanoparticles at 370 nm (figure 1) that was a characteristic peak of ZnO nanoparticles.

3.2. X-Ray diffraction analysis
X-ray diffraction pattern of biosynthesized ZnO nanoparticles is given in figure 2. It indicated the hexagonal wurtzite structure of ZnO nanoparticles. The average crystalline size of ZnO nanoparticles calculated by Debye Scherer’s formula was 34 nm.
3.3. FTIR analysis
FTIR analysis was done to reveal the surface chemistry of biosynthesized ZnO nanoparticles. It was carried out in the range of 500 to 4000 cm$^{-1}$. The spectrum of ZnO NPs had absorption bands at 3393.14, 2830.99, 2285.23, 2108.78, 1506.13, 1416.46 and 701.96 cm$^{-1}$ (figure 3).

3.4. Atomic force microscopy
AFM was performed to find topological appearance, size, height distribution and morphology of nanoparticles of biosynthesized ZnO nanoparticles. The 2D and 3D images of ZnO nanoparticles were obtained and the average roughness revealed. The results showed hexagonal shape of ZnO NPs as depicted in figure 4.

3.5. SEM analysis
SEM was carried out to find the structure, size and shape of ZnO NPs. SEM images proved that the synthesized particles were in nano range with hexagonal arrangement and uniform distribution. Figure 5 revealed the particle size distribution of synthesized nanoparticles that concludes the average size ranged from 28.63 to 61.42 nm.

3.6. Antimicrobial potential of zinc oxide nanoparticles
Antibacterial activity of biosynthesized ZnO nanoparticles was observed against S. enteritidis and S. gallinarum. The results of the zone of inhibitions (mm±S.D) are displayed in figure 6 and table 1.
3.7. Determination of MIC by broth dilution method

MIC was done to check the minimum inhibitory concentration of ZnO nanoparticles that inhibit the growth of bacterial. The standard protocol was followed to observe the MIC of nanoparticles for *S. gallinarum* and *S. enteritidis* that was $0.390 \pm 0.00$ mg and $0.195 \pm 0.00$ mg respectively as indicated in figure 7 and table 1.

![Figure 3. FTIR analysis of zinc oxide nanoparticle produced by green synthesis using leaf extract of *A. aspera*.](image)

![Figure 4. AFM images of ZnO NPs produced by leaf extract of *A. aspera*.](image)
4. Discussion

Microorganisms, especially bacteria, cause many health issues and chronic infections. Antibiotics are used as effective agents against bacterial infections due to their cost-efficacy and powerful consequences. Antibiotic resistance against Gram-negative bacteria is a major problem because they have double-layered cell membranes making it more difficult for the drugs to penetrate. Organisms such as *S. gallinarum* and *S. enteritidis* are poultry pathogens that cause infection in humans as well as in poultry animals. Various nanoparticles have been synthesized and their antimicrobial activity has been determined but this is the first study on preparation of ZnO NPs by using *A. aspera* and their utilization against the poultry pathogens. In the present study, *A. aspera* leaf extract was used as reducing agent to produce zinc oxide. The synthesized nanoparticles were characterized using UV-visible spectroscopy, XRD, FTIR, SEM and AFM. UV-Visible analysis confirmed the presence of ZnO nanoparticles in a colloidal solution. The absorption by ZnO nanoparticles produced a specific peak at 370 nm (figure 1). As indicated in literature, ZnO NPs display characteristic peak between 320 nm—380 nm [14, 16].

FTIR analysis of ZnO nanoparticles showed absorption band at 3393.14 cm⁻¹ that indicate O–H stretches, while bands at 2830.99 cm⁻¹ is due to C–H stretches. The bands at 2285.23 cm⁻¹ and 2108.78 cm⁻¹ is due to the
Table 1. Antimicrobial activity and MIC of ZnO NPs produced by A. aspera leaf extract against pathogenic strains.

| Microbial strains | Aq. extract of A. aspera leaves (100 mg ml\(^{-1}\)) | ZnO NPs of A. aspera leaves (100 mg ml\(^{-1}\)) | Tetracycline +ve control (100 mg ml\(^{-1}\)) | MIC ± SD |
|-------------------|---------------------------------|---------------------------------|---------------------------------|----------|
| *Salmonella enteritidis* | No zone | 31 mm ± 0.37 | 28.8 mm ± 0.62 | ≥0.195 mg ± 0.00 |
| *Salmonella gallinarum* | No zone | 30 mm ± 0.26 | 27.6 mm ± 0.47 | ≥0.390 mg ± 0.00 |
stretches of C≡N and C≡C. The two bands at 1506.13 cm⁻¹ and 1416.46 cm⁻¹ designate the carbonyl groups of amides. The band at 1096.33 cm⁻¹ show C–O stretching in amino acid (figure 2). The results indicated the existence of organic biomolecules such as proteins and enzymes on surface of nanoparticles that are accountable for reduction and stabilization of ZnO nanoparticles. Presence of hexagonal phase ZnO nanoparticles was indicated by a weak band at 701.96 cm⁻¹. Findings of Surendra et al[17] and Irshad et al[14] had similar results and revealed that protein stabilized ZnO nanoparticles form clusters during the green synthesis. Datta et al[10] also reported similar results of the FTIR study of ZnO NPs, with the presence of an intense band at 3458.48 cm⁻¹ that signified OH bond stretching. The bands at 1643.51 cm⁻¹ showed the presence of primary amines and the band at 2245.22 cm⁻¹ indicated the nitriles and alkyls.

XRD of biosynthesized ZnO nanoparticles was used to calculate its crystalline size. The peaks in the ZnO nanoparticles spectrum appeared at diffraction angles 31.4°, 34.07°, 35.9°, 47.21°, 56.3°, 62.57°, 66.05°, 67.67°, 68.75°, 76.61°, 89.36°, 92.69°, 95° and 98.33° (figure 3). The average crystalline size of ZnO nanoparticles was 34 nm, which was calculated by the Debye–Scherrer equation. The result of present study was compatible with the literature as Getie et al[18] showed a similar XRD diffractogram with an angle at 31.77, 34.42, 36.25, 47.16, 56.53, 62.84, 67.83, and 69.15°. Rai et al[19] also reported the synthesis of ZnO nanoparticles using aqueous leaf extract of Cochlospermum religiosum where average crystalline size was less than 100 nm that supports present results. AFM describes the roughness and shape of ZnO nanoparticles. It was observed by tip corrected AFM measurement that ZnO nanoparticles were hexagonal in shape. Gupta et al[20] also narrated similar results of AFM of nanoparticles. Nagarajan et al[21] showed 2D and 3D images of ZnO NPs using a line profile. The result revealed size of 36 nm and has a spherical hexagonal shape.

SEM analysis of ZnO nanoparticles revealed the size and shape of sample. The current study showed that ZnO NPs have spherical and radial shape. The maximum and minimum particle size of ZnO NPs was 28.63 nm and 61.42 nm, respectively. Present results were in good agreement with Chauhan et al[22] that documented green synthesis of ZnO NPs from Cassia siamea leaves extracts. Whereas Datta et al[10] stated that biosynthesized nanoparticles were radial, cylindrical and spherical accumulated in small clusters. Yung et al[23] showed that ZnO NPs have size in the range of 50–70 nm that is closely related to results presented in current investigation.

Siddiqi et al[24] and Jones et al[25] stated that zinc oxide nanoparticles have a significant antimicrobial effect on a number of Gram-positive as well as on Gram-negative bacteria. The antibacterial activity of biologically synthesized ZnO nanoparticles was studied against S. enteritidis and S. gallinarum in current research (table 1). ZnO nanoparticles showed better antibacterial activity as compared to antibiotic and plant extract. The diameter of zone of inhibition produced by ZnO nanoparticles (100 mg ml⁻¹) against S. enteritidis strain was 31 mm ± 0.37 while against S. gallinarum was 30 mm ± 0.26. The results indicated that use of nanoparticles is a better option to control the problem of antibiotics resistance. The ZnO NPs act by penetrating the bacterial cell wall and cause the lipid peroxidation by generating reactive oxygen species. ZnO nanoparticles also disturb the bacterial cell integrity that causes the death of the bacterial cell. The results of antibacterial activity of ZnO nanoparticles against different bacterial strains as reported in literature are compared in table 2.
Irshad et al [14] also reported similar trend in results as, the aqueous extract of Camellia sinensis leaves did not showed any antimicrobial activity against different microbial strains under study, while zinc oxide showed maximum zone of inhibition in comparison to standard antibiotic (gentamycin) and plant extract at same concentration. Although, Yadav et al [4] reported the antimicrobial activity of A. aspera against Streptococcus sp, but it was not effective antimicrobial agent against the poultry pathogens tested in present report. The reason is that both the plant extract and zinc oxide nanoparticles produced from it have different mode of action on bacteria. The ZnO NPs act by penetrating the bacterial cell wall and cause the lipid peroxidation by generating reactive oxygen species. ZnO nanoparticles also disturb the bacterial cell integrity that causes the death of the bacterial cell.

In present research work, the antibacterial activity of ZnO nanoparticles against Salmonella enteritidis and Salmonella gallinarum was tested that is not reported yet in literature. MIC of ZnO nanoparticles for S. gallinarum and S. enteritidis was $\geq 0.390$ mg ± 0.00 and $\geq 0.195$ mg ± 0.00, respectively. The comparison of results of MIC of ZnO nanoparticles against different bacterial strains as reported in literature are stated in table3.

### Table 2. Comparison of antibacterial activity of ZnO NPs against different bacterial strains reported in literature.

| Bacterial strain | Zone of inhibition | References |
|------------------|--------------------|------------|
| S. enteritidis and S. gallinarum | S. enteritidis: 31 mm ± 0.37 | Current research |
| | S. gallinarum: 30 mm ± 0.26 | |
| Gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) and Gram-negative bacteria (Salmonella paraatyphi and Escherichia coli) | S. aureus: 10 mm E. coli: 12 mm | Suresh et al [3] |
| Klebsiella pneumoniae and Salmonella typhi | S. paratyphi: 17 mm B. subtilis: 12 mm for both the strains | Joel et al [26] |
| E. coli, S. aureus and Aspergillus niger | E. coli: 36.15 mm ± 0.304 | Irshad et al [14] |
| | S. aureus: 40.05 mm ± 0.137 Aspergillus niger: 40.10 mm ± 0.050 | |

### Table 3. Comparison of MIC of ZnO NPs against different bacterial strains reported in literature.

| Bacterial strain | MIC | References |
|------------------|-----|------------|
| S. gallinarum and S. enteritidis | S. gallinarum: $\geq 0.390$ mg ± 0.00 | Current research |
| | S. enteritidis: 0.195 mg ± 0.00 | |
| 15 clinical isolates of P. aeruginosa | 158–325 µg ml$^{-1}$ | Sangani et al [27] |
| P. aeruginosa | 300 µg ml$^{-1}$ | Saadat et al [28] |
| S. aureus and E. coli | 6.25 µg ml$^{-1}$ | Al Bukhatty et al [29] |
| A. bamanii and S. marcescens | A. bamanii: 1250 µg ml$^{-1}$ | Kareem et al [30] |
| | S. marcescens: 2500 µg ml$^{-1}$ | |
| E. coli and S. aureus | E. coli: 19.531 µg ml$^{-1}$ | Irshad et al [14] |
| | S. aureus: 9.765 µg ml$^{-1}$ | |

Irshad et al [14] also reported similar trend in results as, the aqueous extract of Camellia sinensis leaves did not showed any antimicrobial activity against different microbial strains under study, while zinc oxide showed maximum zone of inhibition in comparison to standard antibiotic (gentamycin) and plant extract at same concentration. Although, Yadav et al [4] reported the antimicrobial activity of A. aspera against Streptococcus sp, but it was not effective antimicrobial agent against the poultry pathogens tested in present report. The reason is that both the plant extract and zinc oxide nanoparticles produced from it have different mode of action on bacteria. The ZnO NPs act by penetrating the bacterial cell wall and cause the lipid peroxidation by generating reactive oxygen species. ZnO nanoparticles also disturb the bacterial cell integrity that causes the death of the bacterial cell.

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### 5. Conclusions

The utilization of nanoparticles is the best alternative against antimicrobial-resistant pathogens. Biosynthesis of ZnO NPs through aqueous extract of leaves of A. aspera is a safe, effective and ecofriendly approach to fight with infectious microbes. The characterization techniques like UV–vis, FTIR, SEM, AFM, and XRD are rapid and sensitive techniques to identify and explain stability, shape, roughness, and integrity of synthesized nanomaterial. The excellent results of antimicrobial activity of ZnO NPs against poultry pathogens in comparison to standard drug encourage the use of these nanoparticles as antibacterial agents. Further in-vivo testing is required to apply it in pharmaceutical formulations.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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