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Alternanthera Sessilis Leaf Extract-Derived Green Synthesised Zinc Oxide Nanoparticles Antibacterial Activity Against Infections In Freshwater Fish

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Abstract- Biological reductants continue to be studied worldwide to reduce the harmful effects of toxic chemicals used to create nanoparticles. Utilizing an aqueous leaf extract of *Alternanthera sessilis*, this study analyzed the effects of bio-synthesized ZnO nanoparticles(As-ZnO NPs). Zinc acetate dihydrate was employed as the precursor, and the leaf extract was used as a biological reductant to produce zinc oxide nanoparticles. The synthesis conditions, such as heat, $p^{H,}$ and mixing time, were optimized for the synthesis of ZnO nanoparticles. The obtained nanopowder was characterized using several analytical methods and was kept in a dry state. The As-ZnO nanoparticles were characterized using UV-Vis Spectroscopy, Scanning Electron Microscope, Energy Dispersive X-ray analysis, Powder X-ray diffraction and FT-IR Spectroscopy. The synthesized As-ZnO NPs exhibited a peak at 333 nm in the UV-Vis spectroscopy analysis. According to Scanning Electron Microscope, the structure of nanoparticles showed a hexagonal wurtzite shape. The crystalline nature of the nanoparticles was validated by Powder X-ray diffraction. The synthesized nanoparticles were tested for their antibacterial potential against the main aquatic pathogens such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Aeromonas hydrophilia*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. This study reported an efficient green method for synthesising zinc oxide nanoparticles with antibacterial properties against freshwater fish pathogens.

Keywords: *Alternanthera sessilis*, zinc oxide nanoparticles, anti-bacterial, aquatic pathogens.

Highlights

- Hexagonal wurtzite-shaped Zinc oxide nanoparticles were synthesized using *Alternanthera sessilis* leaf extract.
- *A.sessilis* extract served as both a capping and reducing agent for the bio-synthesis of zinc oxide nanoparticles.
- Characterization of the structure morphology, size, form, and functional group of green-produced zinc oxide nanoparticles was done using UV-Visible spectroscopy, SEM, EDX, XRD, and FT-IR.
- Prepared green ZnO nanoparticles exhibited significant antibacterial effects against both gram-positive and negative bacteria of fish pathogens.

I.Introduction

The rapidly-growing food industry in the world is aquaculture. This is evolving, growing and intensifying in virtually every region of the world. Bacterial, viral, fungal and parasitic fish diseases pose a major threat to the aquaculture industry. Farmers have adopted farming procedures that are harmful to pond water quality in response to rising global demand for aquaculture-produced food. This has increased the susceptibility of cultured animals to various diseases like ulcers, fin-rot, tail-rot, hemorrhagic septicaemia, etc. A fascinating field of nanomaterials at present is the synthesis of metal oxide nanoparticles because of their wide application in optics, biology, and electronics and NPs have proven to be efficient in medicine, pharmaceuticals, tissue engineering, environment, energy, electronics, biomolecules, protein diagnostics and cell engineering. Nanoparticles are classified into different groups based on properties, shape and size [1], [2]. These groups include fullerenes, metallic NPs, ceramic NPs, and polymeric NPs. Zinc oxide is cheap and has relatively low toxicity compared with other nanoparticles. ZnO NPs have excellent biomedical applications such as anti-cancer, drug delivery, antibacterial, diabetic therapy, anti-inflammatory drugs, wound healing and bioimaging [3]–[5]. Also, the biosynthesis of metal nanoparticles utilising biological sources is more and more interesting [6]. Plants sources have been reported to be the most suitable among these creatures for the large-scale production of nanoparticles [7]. Compared to those produced through other microorganisms, nanoparticles generated by plants are more stable and have a greater variety of sizes and shapes [8], [9]. *Alternanthera sessilis* is an aquatic plant, often found in marshes and swamps. It is an edible and medicinal plant that was selected as the target sample for the study [10]. It contains many phytochemicals, including stigmasterol, campesterol, ß-sitosterol, α- and ß-spinasterol, sterol palmitate, etc. It has proven anti-microbial, wound healing, anti-oxidant, antipyretic, hepatoprotective, hematinic, anti-ulcer, hypoglycemic, and anti-diarrhoeal properties, and anti-inflammatory activity [11]–[13]. These studies have mainly concentrated on the synthesis of environmentally friendly ZnO NPs using a biotic substrate and the evaluation of antibacterial activity against fish pathogens.

II.Material and methods

1.1 Plant collection

Alternanthera sessilis (Nattu Ponnanganni) fresh leaves were purchased from a local market in Chinnalapatti, Dindigul District, Tamil Nadu, India and utilized for this experiment.

1.2 Preparatory measures of aqueous leaf extract

Ten grams of leaves were taken and cleaned well with demineralized water to remove dust particles. The washed leaves were air-dried and cut into small pieces to collect the aqueous extract. 100 millilitres of demineralized water was poured into a 250-millilitre borosil beaker along with small pieces of leaves for aqueous extract preparation**.** The mixture was boiled under the hot plate at a constant temperature of 60^{o}C for 20 min. After boiling, it allows it to cool to room temperature and filter through Whatman filter paper No 1. A light green extract was obtained, which was used as a stabilizing and reducing agent for the synthesis of zinc oxide nanoparticles [14].

2.3 Synthesis of Zinc Oxide nanoparticles using *A. sessilis* aqueous leaf extract

Leaf extract (L. extract) and precursor solution were taken at a 1:9 ratio for nanoparticle synthesis. Briefly, 10 millilitres of leaf extract and 90 millilitres of 0.01M of Zinc acetate dihydrate were taken. The leaf extract was added bit-by-bit to the precursor solution by continuous stirring in a magnetic stirrer. To maintain the p^{H} of 12, 1M of NaOH was used with optimum temperature conditions. After setting the p^{H}, the solution was allowed to stir until a precipitate formed [15]. The subsequent colloidal solution was given time to settle down. The final step was centrifuging the resulting solution for 15 minutes at a speed of 6000 rpm. The washing process was repeated several times with demineralized water and ethanol until the impurities were removed. Finally,

the white precipitate was desiccated at 60 °C overnight in a hot air oven. The dried nanopowder was stored for further structural characterization and anti-bacterial evaluation of the selected pathogens.

2.4 Characterization of synthesized As- ZnO NPs

Synthesised As-Zinc Oxide nanoparticles were characterised by UV-VIS, SEM, EDX, P-XRD and FT-IR.

2.4.1 UV-Vis Spectroscopy

The synthesized ZnO NPs absorption spectra were recorded using UV-Vis spectroscopy. The samples were recorded between 200 and 800 nm. The data were gathered and analysed using a Thermo Scientific GENESYS 180 UV-Vis Spectrophotometer [16].

2.4.2 Scanning Electron Microscope

The morphology of As-ZnO NPs was observed using the VEGA 3 system from Tescan (Czech Republic). To conduct this study, a small amount of the sample's thin films were deposited onto a copper grid that had been covered in carbon. The accelerating voltage of 10 kV was used. The photos' contrast and brightness were adjusted to the ideal values for separating the particles from the backdrop. The SEM grid's film was subsequently left to dry for five minutes under a mercury lamp after the excess solution was wiped away with tissue paper [17].

2.4.3 EDX Spectroscopy

The energy-dispersive X-ray analysis (EDX) technique involves activating the nanoparticles and analysing them with an Energy Dispersive X-ray spectrophotometer[18]. This method was carried out using Bruker Nano, GmbH, D-12489 (Germany) and after maintaining the proper instrumental conditions, delivers accurate findings for both element detection and figuring out the concentration of synthesized As- ZnO nanoparticles.

2.4.4 Powder X-Ray Diffraction

Powder X-ray diffraction (P-XRD), a fast analytical technique, is frequently used to ascertain the phase of nanocrystals. Additionally, it can provide details about crystalline materials. To confirm the crystallite structure, P-XRD was performed on ZnO NPs. X-ray diffractometer PANalytical / X pert 3 powder was used to record the sample. The material is homogenised and finely ground during analysis before an average assessment of the bulk composition is made. The system was operated using Cu K radiation in a configuration at 45kv of voltage and 30mA of current. Using Scherer's estimate based on the width of the P-XRD peaks, the crystallite size of the ZnO NPs was identified. The size of the domesticated crystallites was calculated using the Debye-Sherrer equation. ($D=K\lambda/\beta.\cos\theta$). where k is the aspect ratio with a constant value of 0.94, "D" appears to be the average size of the crystallite across the reflecting planes, λ is the wavelength of the radiation Cu k and the Bragg diffraction angle is measured in half - Maximum full-width radians (FWHM) [19].

2.4.5 FT-IR Spectroscopy

Two milligrams of air-dried As-ZnO NPs were combined with KBr and ground into pellets[20]. The pellets were examined by inserting the samples into the sample holder and recording the spectrum in the 400 to 4000 cm^{-1} wavelength region. The Jasco FT/IR - 4700 type ‘A’ was utilized in recording the FT-IR spectrum.

2.5 Anti-bacterial activity against freshwater fish pathogens

Well diffusion assay

Three Gram-positive bacterial strains *Enterococcus faecalis* (NCT337), *Staphylococcus aureus* (NCT45) and *Streptococcus pyogenes* (NCT289) and three Gram-negative bacterial strains *Aeromonas hydrophilia* (NCT337), *Klebsiella pneumonia* (NCT40) and *Pseudomonas aeruginosa* (NCT114.2) were obtained from National Culture Collection Centre (www.ncccc.in), Department of Biotechnology and Microbiology, National College (Autonomous), Trichy, Tamilnadu, India and used for the anti-bacterial activity. A loop of culture was added to 5 millilitres of Muller-Hintons broth to prepare the test organisms, and the composition was then incubated for 24 hrs at 37° C. On each of the triplicate plates, the bacterial inoculums were gently wiped onto the Muller-Hinton agar medium using a sterile cotton swab. The prepared agar plates consisted of six wells punched using a 5mm diameter sterile cork-borer. Each well consists of different concentrations of As-ZnO NPs (25µg/millilitre, 50µg/millilitre, 75µg/millilitre and 100µg/millilitre). The standard antibiotic streptomycin (5 µg /millilitre) was used as the positive control, while dimethyl sulfoxide (DMSO) was used as the negative control. The plates were subjected to incubation for 24 hrs at 37 °C. The diameter of the inhibitory zone was measured and recorded in millimetres [21], [22].

III.Result and discussion

In recent decades, zinc oxide nanoparticles have attracted more attention because of their special properties, and the production of metal nanoparticles and metal oxides through biological mechanisms has increased among scientists due to the environmental friendliness of the synthesis process. This creates an opportunity to create processes on a large scale. The biomolecules contained in plant extracts are responsible for the synthesis of nanoparticles and have the ability to act as stabilizing and reducing factors in this process. In addition, the zinc salt reacts with the bioactive substances of the plant and reduces or forms complexes with the metal.

3.1 Synthesis of ZnO NPs from *A. sessilis* (L.extract)

First, the formation of the nanoparticles was confirmed by visual observation of the colour change. In this study, an aqueous extract of *A. sessilis* leaves was used for the synthesis of ZnO NPs. During synthesis, the colourless solution turns pale green, indicating the formation of nanoparticles. Using UV visible spectra in the 200–800 nm range, showed the characteristic absorption peak of produced ZnO NPs at 333nm [23]. *Alternanthera sessilis* leaves were extracts used to prepare Zinc oxide nanoparticles. This mechanism is illustrated in figure.1. This peak confirms the characteristic absorption band of ZnO nanoparticles.

3.2 Physicochemical properties of As-ZnO nanoparticles

3.2.1 UV-Vis Spectroscopy

The UV-Vis spectra of *A. sessilis* (L.extract) and As-ZnO NPs were shows λ_{max} at 328 and 333 nm, respectively as shown in figure 2. Additionally, the *A. sessilis* L. extract showed an absorption peak at 328 nm. It is clear that the produced NPs are pure as there is no additional absorption peak in the UV-Vis spectra. ZnO nanoparticles based on *Ailanthus altissima* fruit extract showed an absorption peak at 369 nm [24]. NP-mediated ZnO extracts from *Olea europaea*, *Matricaria chamomilla* and *Lycopersicon esculentum* extracts showed peaks at 384, 380 and 386 nm [25]. This is consistent with reports by Ahmad et al. [26] who reported that ZnO NPs based on *Euphorbia hirta* leaf extract showed a peak at 370 nm.

3.2.2 Scanning Electron Microscope

SEM analysis revealed the presence of hexagonal wurtzite shapes of ZnO nanoparticles, and the particle size of the As-ZnO NPs on average was found to be 43.5 nm as shown in figure 3. SEM images clearly showed the structure of As-ZnO NPs and the purity of the NPs produced by plant extract *A. sessilis*, which also showed that they are remarkably good at producing ZnO NPs. The nanoparticles are more or less spherical in shape, grouped together, and appear to have a rough surface from a topographical

perspective [27]. Under SEM analysis, it was noted in the current study that there were agglomerated NPs with a hexagonal wurtzite shape.

3.2.3 EDX analysis

The qualitative elemental composition of the As-ZnO NPs showed that the elements zinc and oxygen were present in amounts of 69.26% and 30.74%, respectively as illustrated in figure 4. Ahmad et al [28] stated that the structure of the NPs was agglomerated with irregular morphology and Ashkarran et al [29] demonstrated that particles were agglomerated and had uniform particle distribution. The weight percentage of ZnO nanoparticles is displayed in the EDX pattern. On the basis of the EDX report, Zinc was found at 69.26% and oxide was found at 30.74%. Using EDX, it was shown that cyclic groups of heterocyclic biomolecules coexisting with ZnO nanoparticles cause the presence of other elements. Similar findings were reported by Anand et al. [30], who found that employing *Prunus dulcis* resulted in weight percentages of Zn and O of 69.28 and 30.72, respectively.

3.2.4 Powder X-ray Diffraction

Powder X-Ray Diffraction was used to analyze the crystalline structure of the As-ZnO NPs. The peak positions are 31.70°, 34.28°, 36.14°, 47.47°, 56.52°, 62.73°, and 68.09°, which represent the lattice planes at (100), (002), (101), (102), (110), (103), and (200), respectively. These different peaks indicate the hexagonal wurtzite shape of the synthesized As-ZnO NPs, as shown in figure 5. The synthesised ZnO NPs had a 20.3 nm crystalline size. According to Mohamad Sukri et al., the average particle size of ZnO NPs produced using *Passiflora foetida* was 58 nm. [32] reported that *Punica granatum*-mediated ZnO NPs with increasing annealing temperatures S1, S2, S3 and S4 with respect to 400, 500, 600 and 700 ºC obtained crystalline sizes was 22.39, 30.08, 32.39 and 57.36 nm respectively.

3.2.5 FT-IR Spectroscopy

The bioreduction process of As-ZnO NPs occurred due to the involvement of biomolecules and was identified using a Fourier transform infrared spectroscopy (FT-IR) instrument. The groups identified are shown in figure 6. Thus, As-ZnO NPs showed peaks at 3453, 2964, 2469, 1996, 1678, 1430, 1067, 857, 640 and 460 cm^{-1}. The broader peak at 3453 cm^{-1} represents the O-H stretch representing the moisture captured from the atmosphere, and the shorter peak at 2964 cm^{-1} is due to the O-H stretching of the carboxylic acid [33]. The peak at 2469 cm^{-1} represents the O-H stretching of alcohol [34]. The peak at 1678 cm^{-1} represents the stretching of C=C alkenes [35]. The high and strong peak at 1430 cm^{-1} was due to C-N stretching of the amines. The peak at 1067 cm^{-1} indicates the stretching of the C-N alkenes [36]. Sharp and smaller peaks at 857, 640 and 460 cm^{-1} represent zinc oxide metal bonds [37].

3.3 Anti-bacterial activity

The antibacterial effects of As-ZnO NPs on three gram-positive bacteria, including *Enterococcus faecalis, Staphylococcus aureus, and Streptococcus pyogenes,* as well as three gram-negative bacteria, including *Aeromonas hydrophila, Klebsiella-Pneumonia,* and *Pseudomonas aeruginosa*, were assessed. The zones of inhibition are shown in figure 7. For each pathogen, the zone of inhibited diameter was calculated in millimetres illustrated in Table 1. Good antibacterial activity was observed in both gram-positive and negative strains. However, inhibition is stronger against gram-positive *E. faecalis* (23.66 ± 0.28) and gram-negative *A. hydrophilia* (23.83 ±1.04). The results showed that As-ZnO NPs have a strong potential to inhibit the pathogens of the studied fish. ZnO nanoparticles possess incredible antibacterial activity because of the formation of ROS (Reactive Oxygen Species) and loss of cell integrity owing to the interaction between ZnO materials and cell walls. It has been suggested that the internalization of ZnO NPs and the release of Zn2+ ions are the four mechanisms of action that give zinc oxide particles their antibacterial properties [38]. These mechanisms explain the reason why ZnO NPs adhere to the surfaces of both gram-positive and gram-negative bacteria. Teichoic acid from the peptidoglycan layer and lipoteichoic acid from the membrane provides the cell

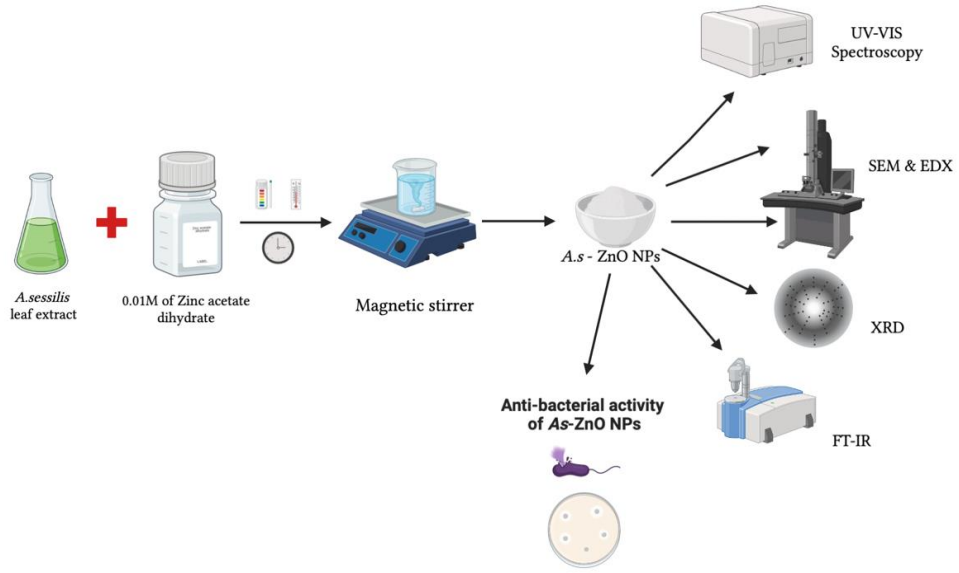
surface negative charges. An electrostatic gradient difference results from electrostatic interactions between the positive charges on ZnO NPs and the positive charges on the cell surface [39].

There was good antibacterial activity observed against gram-positive (*Staphylococcus aureus Enterococcus faecalis, and Streptococcus pyogenes*) and gram-negative (*Klebsiella pneumonia, Aeromonas hydrophilia, and Pseudomonas aeruginosa*) bacteria. Kadiyala et al. recently investigated the antibacterial mechanism of ZnO NPs against methicillin-resistant *Staphylococcus aureus* [40]. In this study, ROS toxicity was not an important mediator of antibacterial activity, in contrast to previous studies showing that antibacterial activity depends on ROS or Zn ions.

IV.Conclusion

The results of the present study demonstrated that a green, low-cost, and environmentally friendly technique is better suited for the synthesis of hexagonal As- ZnO NPs with strong antibacterial activities against gram-positive and gram-negative freshwater fish pathogens. The physicochemical properties of synthesized materials were characterized to know them by UV-Vis Spectroscopy, SEM, EDX, FT-IR, and P-XRD. It has been demonstrated that biosynthesized As-ZnO nanoparticles are more potent at eliminating pathogenic germs than traditional antibiotics and plant extracts. This can be seen by its antibacterial activity against six different fish pathogens. As a result, the incorporation of biological and oxide nanoparticles can effectively prevent infections in fish caused by microorganisms. This research has a significant societal impact because it develops a cost-efficient, non-toxic substance that can be used in antibacterial formulations.

Graphical abstract



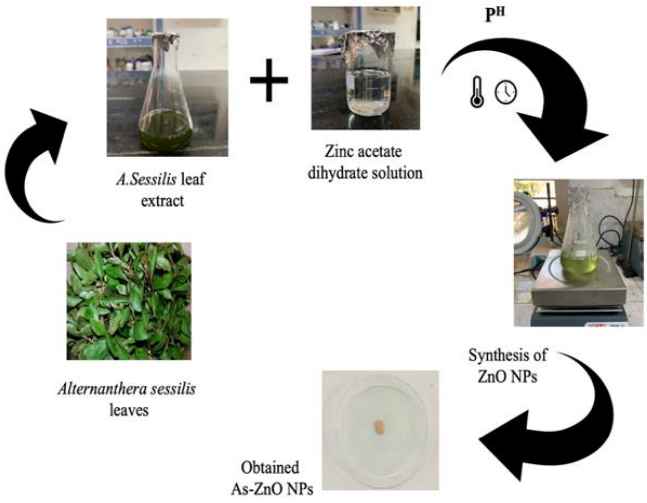


Figure 1: Synthesizing process of As-ZnO NPs

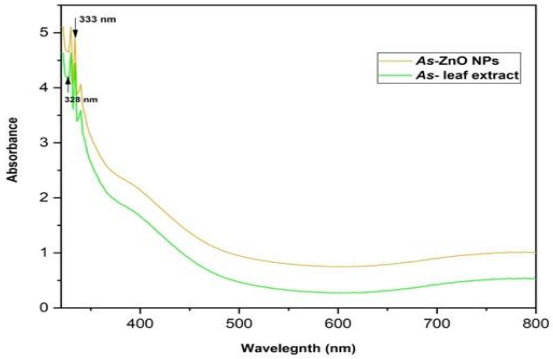


Figure 2: UV-Vis absorbance spectra of *A.sesslis* and As-ZnO NPs

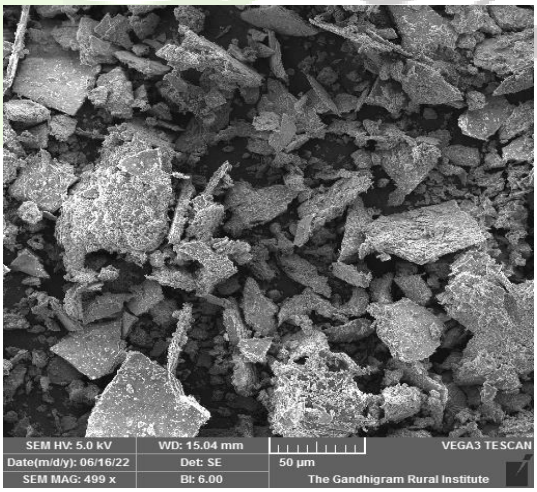


Figure 3: SEM micrograph of As-ZnO NPs

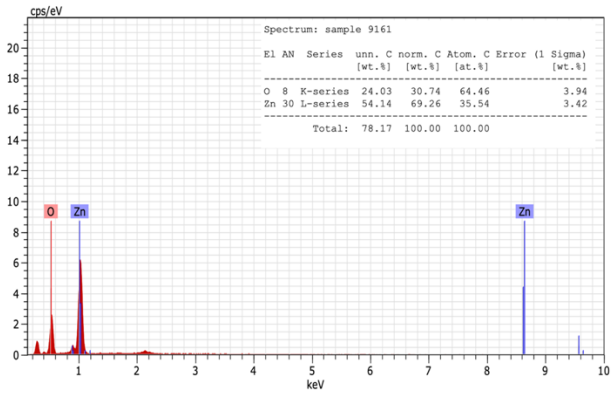


Figure 4: EDX analysis of As-ZnO NPs

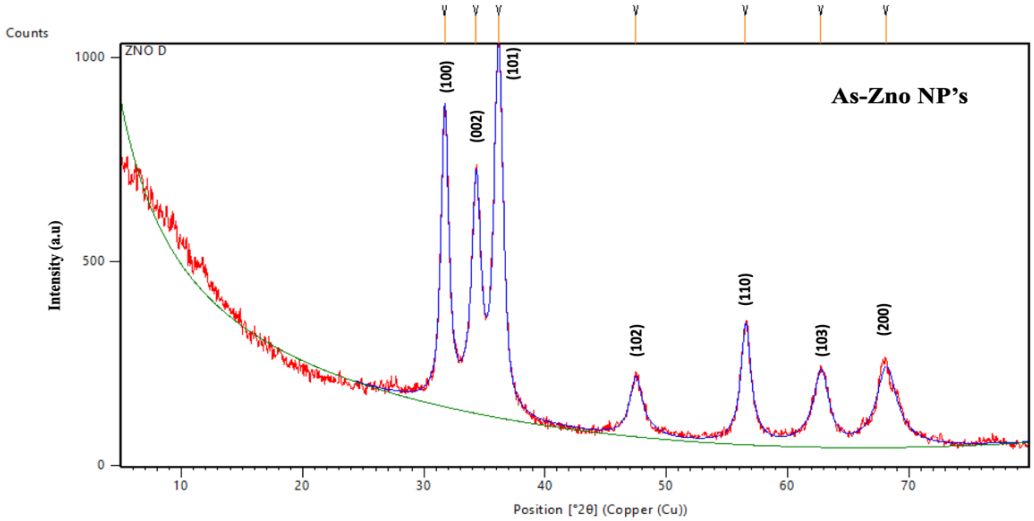


Figure 5: P-XRD spectrum of As-ZnO NPs

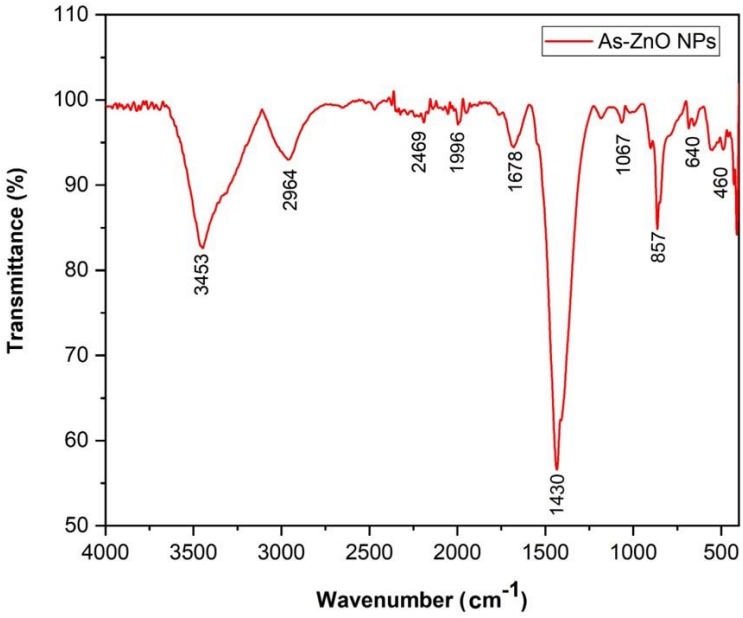


Figure 6: FT-IR spectrum showing the different functional groups of As-ZnO NPs

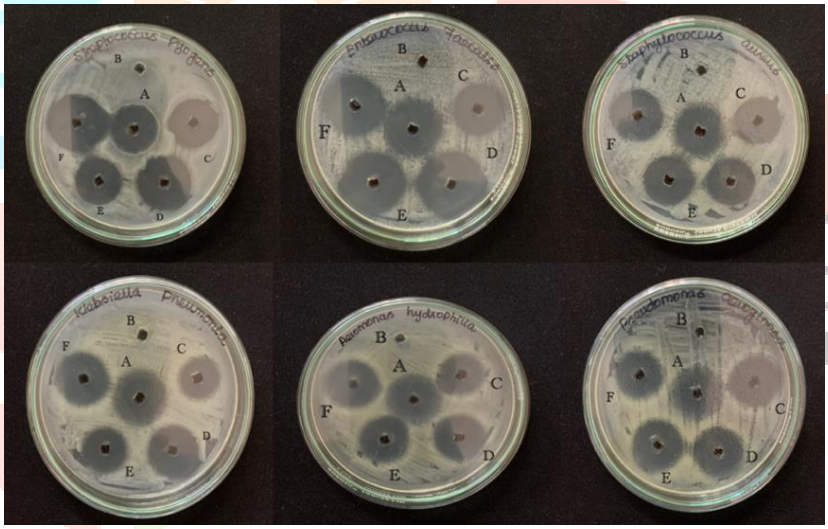


Figure 7: Anti-bacterial effect of As-ZnO NPs

Table 1: Zone of inhibition in diameter (in mm)

Pathogens	Positive control (A)	Negative control (B)	25ug/ml (C)	50ug/ml (D)	75ug/ml (E)	100ug/ml (F)
S. pyogens	21.33±0.28	0	19.5 ± 0.5	20.66 ± 0.57	21.66 ± 1.25	22.66 ± 1.04
E. faecalis	25.5±0.5	0	19.5 ± 1.32	20.66 ± 0.5	22.16 ± 0.76	23.66 ± 0.28
S. aureus	22.83±1.25	0	19.83 ± 0.28	20.5 ± 0.5	21.5 ± 0.5	22.16 ± 0.28
K. pneumonia	24.76±0.40	0	17.9 ± 0.65	19.76 ± 0.68	21.16 ± 0.28	23 ± 0.5
A. hydrophilia	23.16±1.16	0	20.33 ± 0.57	21.66 ± 0.57	22.83 ± 0.28	23.83 ± 1.04
P. aeruginosa	24±1	0	18.83 ± 0.76	19.83 ± 0.28	21.5 ± 0.5	22.83 ± 0.28

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during this study.

Competing interests

The authors declare that they have no conflict of interest.

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Authorship

The contributions of the first authors, Murugeshwaran Dayana Senthamarai and Muthuswami Ruby Rajan, to this work are equal. Both authors have read and approved the final manuscript.

Corresponding author

1- Ms M. Dayana Senthamarai Worked on the following – The design of the work, the acquisition, analysis, and interpretation of data.

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