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Green Synthesis of ZnO Nanostructures and Their Antibacterial Activity Against Catfish Pathogen

Mazhar Hussain Ibupoto*[1], Safdar Hussain Ujjan[1] and Aneela Tahira[2]
[1]*Department of Zoology, Shah Abdul Latif University, KhairpurMirs, Sindh, Pakistan.*
[2]*Institute of Chemistry, Shah Abdul Latif University, KhairpurMirs, Sindh, Pakistan.*
*Corresponding Author Email: mazhar.hjibupoto@salu.edu.pk
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Abstract
Due to its antibacterial activity against clinical bacterial pathogens at the time of tissue development, zinc oxide (ZnO) is considered one of the most important metal oxide nanostructured materials. In this study, ZnO nanostructures were prepared using Aloe vera gel juice, which is easy, inexpensive, and ecofriendly. The nanostructures of ZnO were characterized using a variety of analytical techniques, including scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and dynamic light scattering (DLS). As a result of the XRD study, the hexagonal phase of ZnO with the structure of wurtzite has been identified. ZnO material with high heterogeneity morphology has been observed by SEM analysis. As-prepared ZnO nanostructures were tested for their antibacterial activity against the newly discovered pathogen of catfish. A ZnO sample prepared with a high concentration of Aloe vera gel possessed an inhibition zone of 13.21±0.04 mm, whereas a ZnO sample prepared with a low concentration of Aloe vera juice had an inhibition zone of 6.23±0.02 mm and a pure ZnO sample had an inhibition zone of 3.31±0.03 mm. Furthermore, we examined the effectiveness of the ZnO nanostructures on bacterial strains based on the type of nanoparticles applied to the commercial strains. In addition to their modified surface properties, small particle size, and highly toxic effects for the killingof bacterial cell growth, the Aloe vera juice assisted ZnO nanostructures demonstrated excellent antibacterial activity against the catfish pathogens.

Keywords: Aloe vera Gel, ZnO nanostructures, Antibacterial activity, Catfish pathogens.

Introduction

Natural or synthetic antibiotics are used as antibacterial agents to inhibit the spread of bacteria. Aquaculture and animal systems use antibiotics in a variety of ways, including prophylactically, therapeutically, and metaphylactically [1-5]. Using antibiotics in aquaculture has the potential to enhance fish growth. Therefore, a wide array of antibiotics are employed for this purpose, including aminoglycosides, quinolones, sulfonamides, tetracyclines, chloramphenicols, macrolides, nitrofurans, polymyxins, and lincosamides [6]. A large number of irregularities exist in the monitoring of antibiotics in developing countries, as a result of which a large amount of antibiotics are being misused [7]. However, there is still a lack of strict regulations regarding the use of antibiotics in areas where laws are effective. Several developing countries face significant challenges in terms of compliance, which are not reliable and intense. A major obstacle to accurate determinations of antimicrobial usage in aquaculture worldwide is the differences in regulatory systems, antibiotic concentration, and farming methods among the countries.

Vietnam is the country that has the largest number of antibiotics authorized (30), followed by South Korea (17) and Chile (19), while the United Kingdom, Brazil, and the United States of America have the lowest number of antibiotics authorized [8]. Oxytetracycline, forfenicol, and sulfadiazine are among the most widely used antibiotics in aquaculture [4, 6]. In several countries, antibiotics have been used without proper regulation by fish farmers, which has led to the spread of infectious fish diseases [9]. Among the most common uses of antibiotics in aquaculture are mixing antibiotics with aquaculture feed and sprinkling or injecting antibiotics into the pond [10]. It has been shown that all methods of administering antibiotics other than injection have severe effects on aquatic life and the environment. Even the antibiotics are well metabolized by fish [6] and approximately seventy-five percent of the antibiotics given to fish are released into the pond water by fish during excretion [11]. A number of studies have demonstrated that antibiotics aggregate and settle at the bottom of pond water, leaving antibiotic residue and adversely affecting animal tissues and cultured aquaculture [12, 8]. Antibiotic sediments could be hazardous to consumers and aggravate the complexity of microflora within the gastrointestinal tract of cultured aquatic species [13]. Aplastic anemia is caused by low amounts of antibiotic residues such as chloramphenicol. As a result, the residuals of penicillin show a negative effect on hypersensitivity, gentamicin, mutagenicity, and nephropathy, as well as sulfamethazine, oxytetracycline, and furazolidone immunopathology and a high likelihood of carcinogenicity [14]. On the other hand, the effluents of aquaculture, which contain antibiotics, are disposed of in aquatic systems such as rivers or farmlands, where they potentially pollute the water and cause long-term health problems. If this occurs, residual antibiotics can enter the food chain and cause unwanted and unfavorable effects. In addition, antibiotic residues can have a detrimental effect on phytoplankton and zooplankton biodiversity [15, 16]. Antibiotics such as quinolones, sulfonamides, and tetracyclines have been identified as the cause of zooplankton at an early stage [15, 17], as well as phytoplankton chlorophyll development. For more than two decades, nanotechnology has been known as the art and science of manipulating existing bulk materials at the nanoscale with excellent properties for a wide range of applications that meet the needs of society today. Biomedical, environmental, industrial, food, and agriculture fields have increased their use of nanostructured materials as a result of their enhanced properties [18-21]. In addition to nanomaterials, various transition metals and their metal oxide phases have been used in a variety of sectors due to their unique characteristics [22]. A number of studies have shown that synthetic methods play a significant role in the final application of materials [23]. To achieve this goal, several fabrication methods have been developed for preparing nanostructured materials. It has been found that green chemical methods have proved to be more effective in recent years due to the fact that they are inexpensive, easy to use, and environmentally friendly [24]. A variety of plant parts have been used to synthesize zinc oxide nanostructures (ZnO), including roots, seeds, stems, peels, and fruits [25-34]. Furthermore, phytochemical sources stabilize and reduce nanostructured materials [35, 36]. Previous studies have demonstrated in detail the processes used to synthesize ZnO nanostructures using extracts from Citrus aurantifolia, Jacaranda mimosifolia, Carissa edulis, Trifolium pretense, and Nepheliumlappaceum flowers [37-40]. By using metabolites, proteins, and surface tailoring agents, phytochemicals can effectively reduce metal ions [41-43]. ZnO nanostructures have been produced by co-

precipitation and hydrothermal processes using Aloe vera [44]. It has a wide range of properties, such as antifungal, antibacterial, antioxidant, anti-inflammatory, enhancement of the immune system, and hypoglycemic properties [45]. A previous study used the base of Aloe vera instead of plant extract of Aloe vera during the hydrothermal process, in order to increase the kinetics of nucleation and metal ion reduction [46]. In addition, the Aloe vera plant extract is also used to accelerate the synthesis of ZnO nanostructures through hydrothermal processes [46]. Prior to this study, no studies have been conducted on the effect of green chemical compounds on ZnO's antibacterial properties. Through a synergetic effect between ZnO and carbon from Aloe vera gel, the Aloe vera gel induces carbon into ZnO during the calcination process, further enhancing its antibacterial properties. Furthermore, the Aloe vera flower extract has not been reported to date, despite its high density of phytochemicals that can be used to tune ZnO's surface properties to fight novel catfish pathogens.

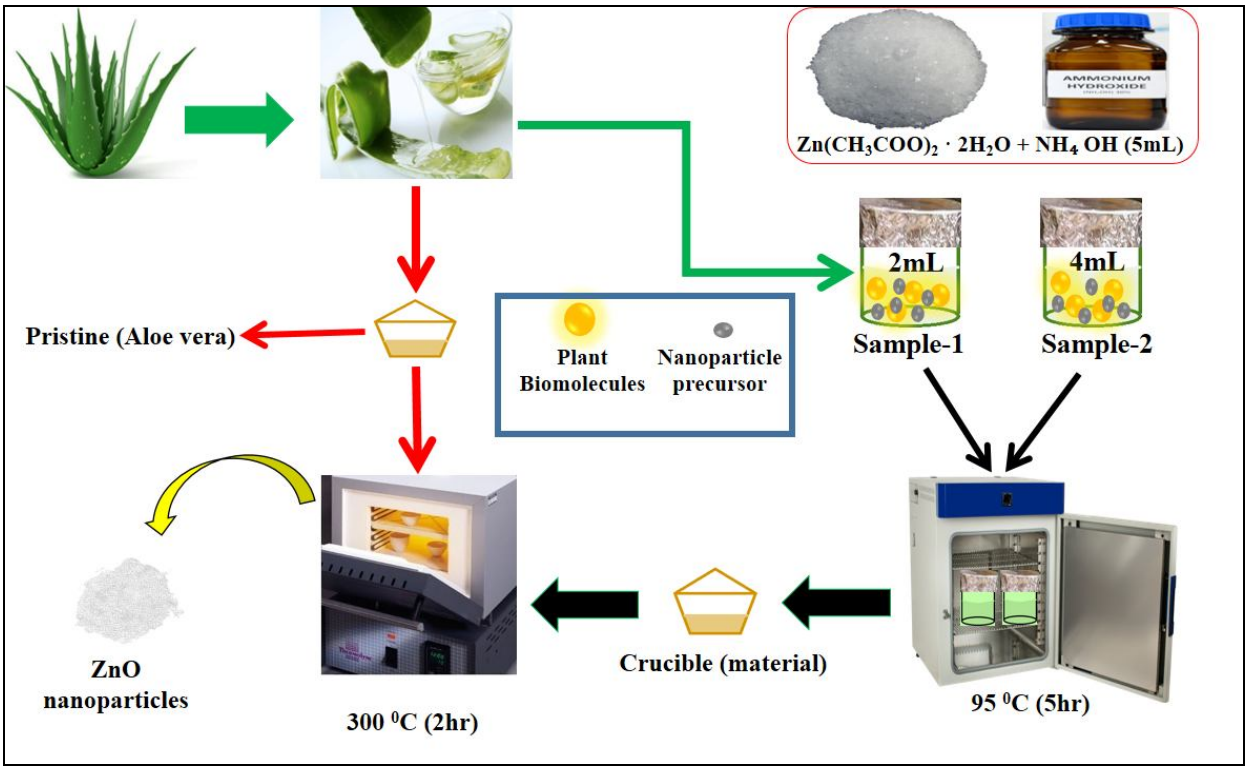
Materials and Methods

Chemical Reagents

Sigma Aldrich, Karachi Pakistan, provided zinc acetate dihydrate (99%), ammonia hydroxide solution (33%), sodium hydroxide (98%), hydrochloric acid (37%), sodium chloride (99%), absolute ethanol (98%), and methanol (99%). The preparation of desired solutions was done in the deionized water. When not in use, aloe vera juice was collected manually and stored at 4 °C in a refrigerator. An infected catfish was obtained from a local fish market in the district of Jamshoro, Sindh, Pakistan. Using newly prepared ZnO nanostructures, the novel fish pathogen SINDH YZB01 F was isolated by bacterial growth in agar. A detailed description of the novel bacteria can be found in the NCBI GenBank database (www.ncbi.nlm.nih.gov).

Synthesis of ZnO Nanostructures Using Aloe Vera Juice Via Wet Chemical Method

Separate beakers were filled with a 50.0 mM solution of zinc acetate dihydrate prepared in deionized water. A solution of zinc precursor and a solution of 33% ammonia was contained in each beaker. Two of the beakers were modified with the addition of 2 and 4 mL of Aloe vera fruit juice, respectively, and were identified as samples 1 and 2. In the presence of Aloe vera juice, the solution turned light greenish. In a third beaker, only zinc precursor and ammonia solution were present. It was labelled as a pure ZnO sample. The aluminum sheet was sealed very tightly after the homogeneous growth dissolution through mechanical stirring. An electric oven was used to heat the growth solutions for five hours at 95 °C. Over the course of four hours, the growth solution became turbid and well-developed precipitates settled at the bottom of each beaker. In the following step, beakers containing grown ZnO nanostructures were removed from the electric oven and allowed to naturally cool at room temperature. Precipitates were obtained on ordinary filter paper and washed several times with ethanol and deionized water. The nanostructured ZnO materials were dried overnight at 60 ^{o}C. For the purpose of eliminating organic contaminants from the juice of Aloe vera, as prepared ZnO samples were annealed at 300 ºC for two hours. In Scheme 1, a brief description of the synthesis process is provided. After that, ZnO samples were characterized in terms of structural, chemical, optical, and antibacterial properties.



Scheme 1: **Synthesis of ZnO nanostructures using Aloe vera fruit juice by hydrothermal method followed by calcination.**

Structural and Functional Group Characterization of Prepared ZnO Nanostructures

ZnO samples were examined optically by a double beam UV-Vis spectrophotometer using a Lamda 25 Perkin Elmer for wavelength ranges 200 to 700 nm for the collection of UV-Vis absorption spectra. Fourier Transform Infrared Spectroscopy (FTIR) studied Zn-O and other functional groups' chemical binding features. In order to conduct the FTIR measurements, we used a PerkinElmer FTIR instrument with a frequency range of 400 to 4000 cm^{-1}. The powder diffraction patterns were measured by X-ray diffraction instrument using X-ray's source from Cu Kα radiation and a two theta sweeping rate at 0.021o s^{-1} for (2θ) from 30º to 80º. The particle sizes and distributions were measured using the Dynamic Light Scattering (DLS) tool. A cuvette was used to prepare aliquots of each sample in deionized water with the dilution factor of times. We conducted DLS experiments using a Malvern Zetasizer, Nano Z500 UK at 25 °C and a scattering intensity of 27193 cps. Using a 20 kV accelerating voltage, scanning electron microscopy was used to examine the morphology of ZnO samples as they were prepared.

As a result of the Aloe vera juice assisted ZnO nanostructures, antibacterial activity was found against the SINDH YZB01 F infected catfish pathogen as well as commercial bacteria such as Salmonella SP and Aeromonas SP. The antibacterial activity against catfish pathogens was performed with the permission and regulations of the biological institutes and laboratories of the University of Sindh Jamshoro, Sindh Pakistan. The antibacterial study was conducted using a diffusion method that has been developed

elsewhere. Bacterial cultures were prepared using agar as a nutrient for 24 hours at 37 °C. The antibacterial experiments were then performed using Mueller Hinton Broth (MHB) inoculum for 12 hours. During non-disturbing conditions, the inoculum was gently distributed onto Mueller Hinton Agar Petri dishes. By applying the inoculums uniform distribution, wells appeared along the sterile cork with a diameter of approximately 6 mm on agar dishes. A 40 µg/mL solution of pure ZnO and Aloe vera-assisted ZnO nanostructures was then added to the wells. ZnO nanostructures were incubated at 36 °C with the above bacteria strains for 24 hours. In addition, after incubation for 24 hours, inhibition zones were observed along the wells for fish pathogens and commercial bacteria.

Results and Discussion

Morphology and Optical Investigations of Modified ZnO Nanostructures with the Aloe Vera Fruit Juice

Fig. 1 shows the characteristic SEM images of pure ZnO and Aloe vera fruit juice assisted ZnO nanostructures. As shown in Fig. 1a, the pure ZnO nanostructures obtained with zinc acetate precursors exhibit typical nanorod-like shapes with non-uniform distribution and heterogeneity in size and alignment. Fig. 1b, 1c illustrates how Aloe vera assisted synthesis of ZnO nanostructures resulted in lumps with a high degree of irregularity in morphology and dimension. As shown in Fig. 1c, the use of an increased amount of Aloe vera extract had no significant effect on the morphology of ZnO nanostructures. Based on the use of Aloe vera fruit juice, ZnO morphologies were found to be very similar to those reported for ZnO prepared from different plant extracts [47, 48]. Fig. 1d illustrates the optical examination of ZnO nanostructured materials as prepared using a UV-visible spectrophotometer. We measured the absorbance of ZnO nanostructures dispersed in water in a quartz cuvette of a path length of 1 cm at room temperature. An important analytical tool for understanding nanoscale information is the UV-visible spectroscopic characterization of nanostructured materials.

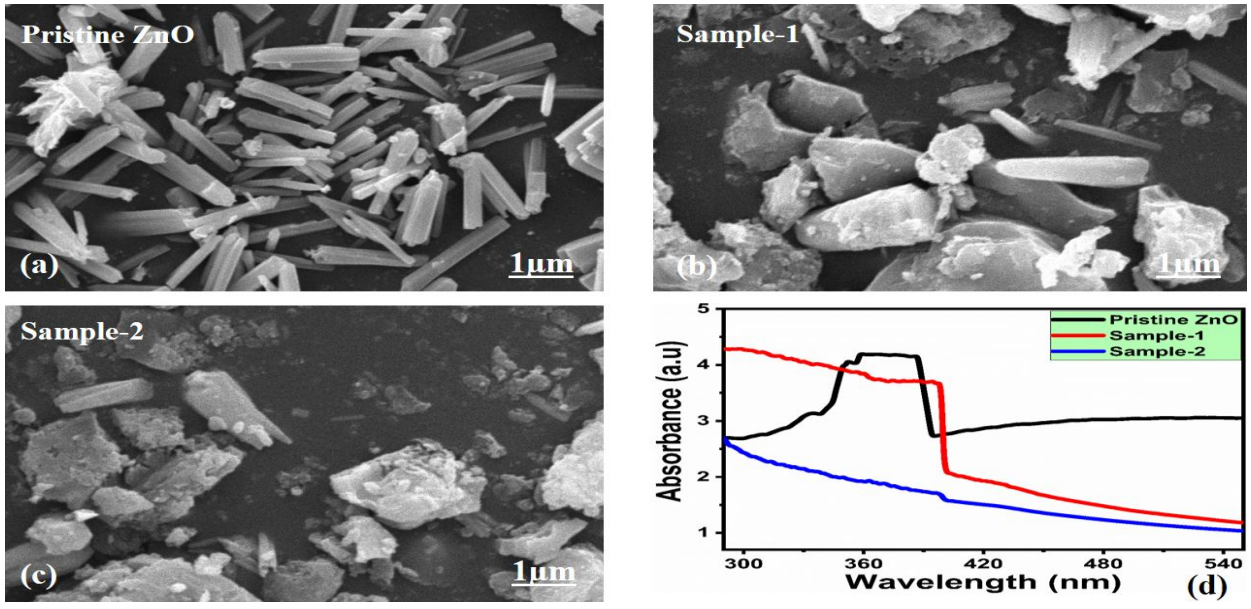


Figure 1. **SEM images (a) pristine ZnO, (b, c) nanostructured ZnO with 2 mL (sample 1) and 4 mL (sample 2) of Aloe vera fruit juice, (d) UV-visible absorbance spectra of various ZnO nanostructures**

A pure ZnO sample was observed to absorb at 382 nm, whereas the absorbance of nanostructures prepared with Aloe vera juice was slightly shifted toward a higher wavelength, indicating that the chemical constituents of Aloe vera fruit juice affect optical properties. Furthermore, Fig. 1d shows relatively better antibacterial effectiveness. In order to obtain information related to the purity and type of material, vibrational spectroscopy of specific substances can be obtained by using FTIR spectra.

As shown in Fig. 2, FTIR spectra were recorded for the frequency range of 4000 - 400 cm^{-1}. Fig. 2 illustrates the FTIR spectra of pure ZnO and Aloe vera fruit juice assisted ZnO nanostructures. As shown in Fig. 2, the typical bands at 4367, 2958, and 2498 cm^{-1} correspond to OH stretching, H-C-H symmetric and asymmetric vibration, and hydrogen bound OH stretching. However, narrower bands were observed at 1441, 887, and 701 cm^{-1}, which were assigned to the C-N stretching band, C-O stretching band, and C-H bending band, respectively. Typical metal-oxygen bonds exhibit a small vibration band at 596 cm^{-1} [49]. According to the presented vibrational information, the synthesized ZnO nanostructures were enhanced by plant extracts [48, 50].

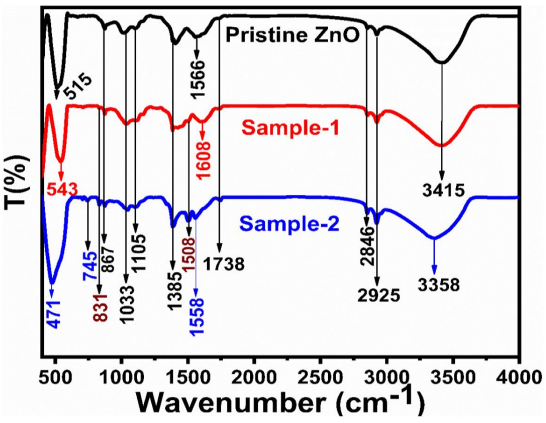


Figure 2. **FTIR vibrational bands of pure ZnO and ZnO nanostructures prepare with 1 mL (sample 1) and 3 mL (sample 2) of Aloe vera fruit juice**

Fig. 3 illustrates the size and size distribution of ZnO samples as prepared using the DLS. As a result of the DLS, it has been established that the narrower and broader spectra are related to the size of particles [47]. In the DLS study, nanostructures with a diameter of around 3230 nanometers were observed. XRD analysis of powdered ZnO nanostructures assisted by Aloe vera fruit juice was used to determine the phase information and verify the nanostructures.

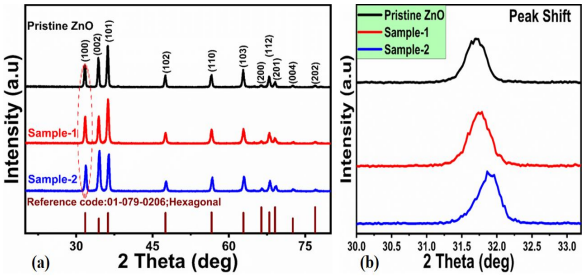


Figure 3. **(a) XRD patterns of pristine ZnO, and the preparaed ZnO nanostructured using 2 mL (sample 1) and 4 mL (sample 2) of Aloe vera fruit juice, (b) Two theta angle shift for the ZnO sample 1 and 2.**

As shown in Fig. 4, pure ZnO nanostructures and green method assisted ZnO nanostructures exhibit different diffraction patterns. The observed 2θ values at 31.8°, 34.44°, 36.29°, 47.57°, 56.61°, 67.96° and 69.07° were indexed to crystal planes of (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 1 2) and (2 0 1), respectively. A face centered cubic structure is indicated by the measured crystal planes, and the recorded diffraction patterns have been confirmed for the hexagonal phase of ZnO [50]. There is a good agreement between all the reflections and the standard JCPDS reference card no. 89-7102. The effect of 2θ could offer new defects in the crystal structure and they can play a positive role in the enhanced antibacterial activity. Aloe vera juice was studied using the recently published green method assisted synthesis of ZnO nanostructures [48]. As prepared ZnO nanostructures with Aloe vera juice, the diffraction patterns were well characterized by

intense reflection peaks. This supports the high crystal quality of the nanostructures. As indicated in equation (1), the average crystallite size of as prepared ZnO nanostructures was calculated using the Scherer equation. Aloe vera assisted ZnO nanostructures were found with an average crystallite size of 55 nm.

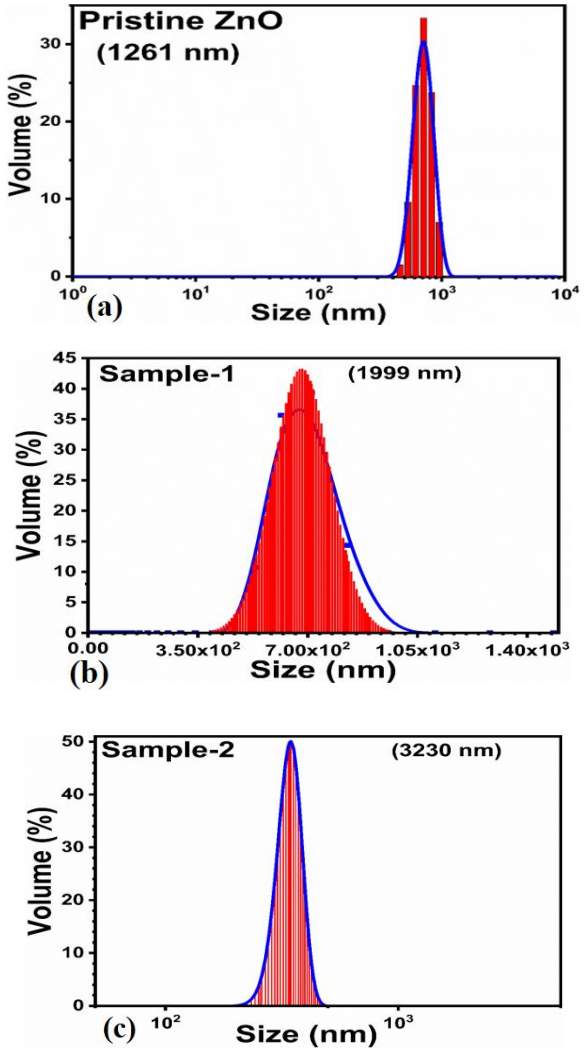


Figure 4. **(a) DLS analysis about the particle size and its distribution of pristine ZnO, and (b, c) nanostructured ZnO with 2 mL (sample 1) and 4 mL (sample 2) of Aloe vera fruit juice**

$$D_p = 0{:}94\lambda \div \beta 1/2 \cos\theta \quad (1)$$

Herein, D_p is showing the average crystallite size, β is representing the line width in radians, θ as Bragg angle, and λ as wavelength of used X-rays during XRD measurements.

The antibacterial activities of different bacterial strains, including the catfish and commercial strains of bacteria, were successfully investigated using ZnO nanostructures assisted by Aloe vera juice. The nanostructured materials based antibacterial agents are highly dependent on the morphology, size, and optical properties of the material to produce the reactive oxygen species which are responsible for the antibacterial performance. According to Fig. 5, ZnO nanostructures were evaluated using the diffusion method for their antibacterial properties. In comparison to pure ZnO and commercial bacteria, ZnO nanostructures synthesized with Aloe vera fruit juice were highly effective against catfish pathogens. The Aloe vera juice assisted ZnO nanostructures experienced a large inhibition zone against catfish pathogens of 13.21±0.04 mm for sample 2 and sample 1 has an inhibition zone against catfish of 6.23±0.02 mm. However, the pure ZnO has revealed an inhibition zone against catfish pathogen about 3.11±0.03 mm. Additionally, the activity of ZnO assisted with Aloe vera juice was compared with commercial bacterial strains, and the obtained effectiveness was lower, as shown in Fig. 5. The relative order of antibacterial of ZnO sample 1, sample 2, and pure ZnO was as 4.11±0.21 mm, 5.54±0.34 mm and 2.22±18 mm, respectively. The antibacterial activity of pure ZnO is limited by the low production of reactive oxygen species which are actually responsible for the killing of bacteria. In the literature survey, it is noted that ZnO nanoparticles affect the survival of microorganisms, such as bacteria, by accumulating on their surfaces and altering the functionalities of lipids, proteins, DNA, and peptidoglycan due to their nanoscale nature and high surface area [51].

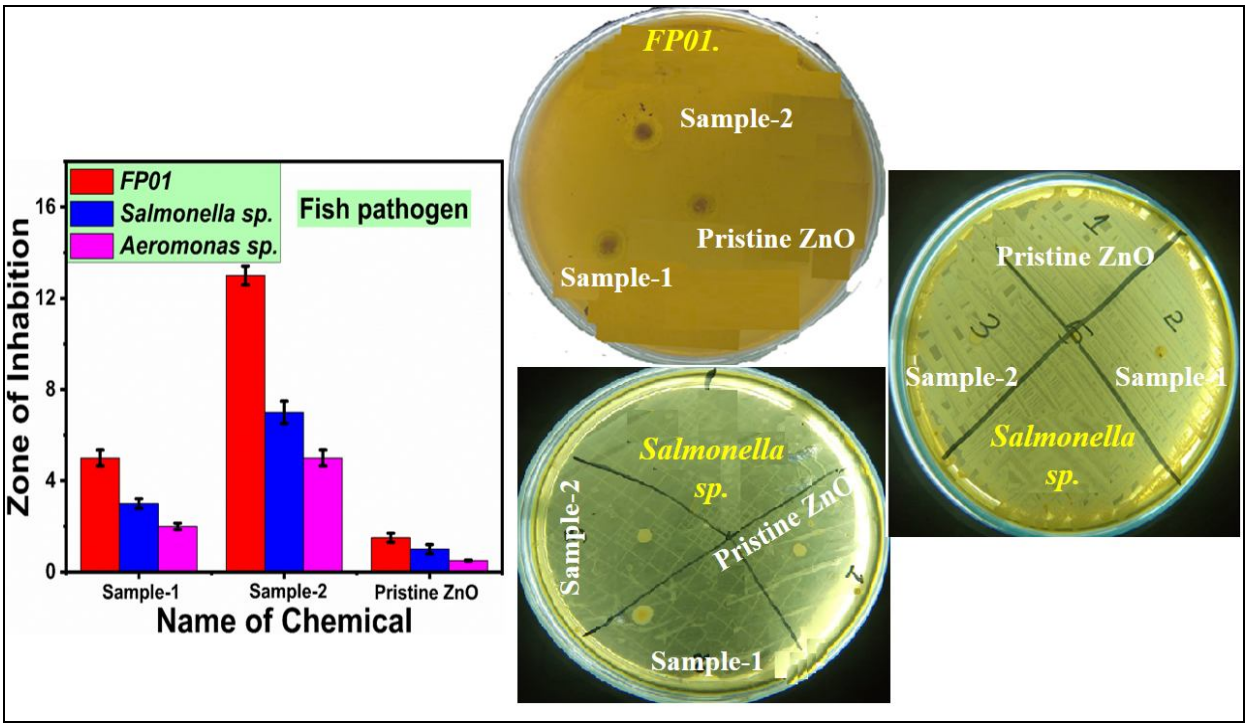
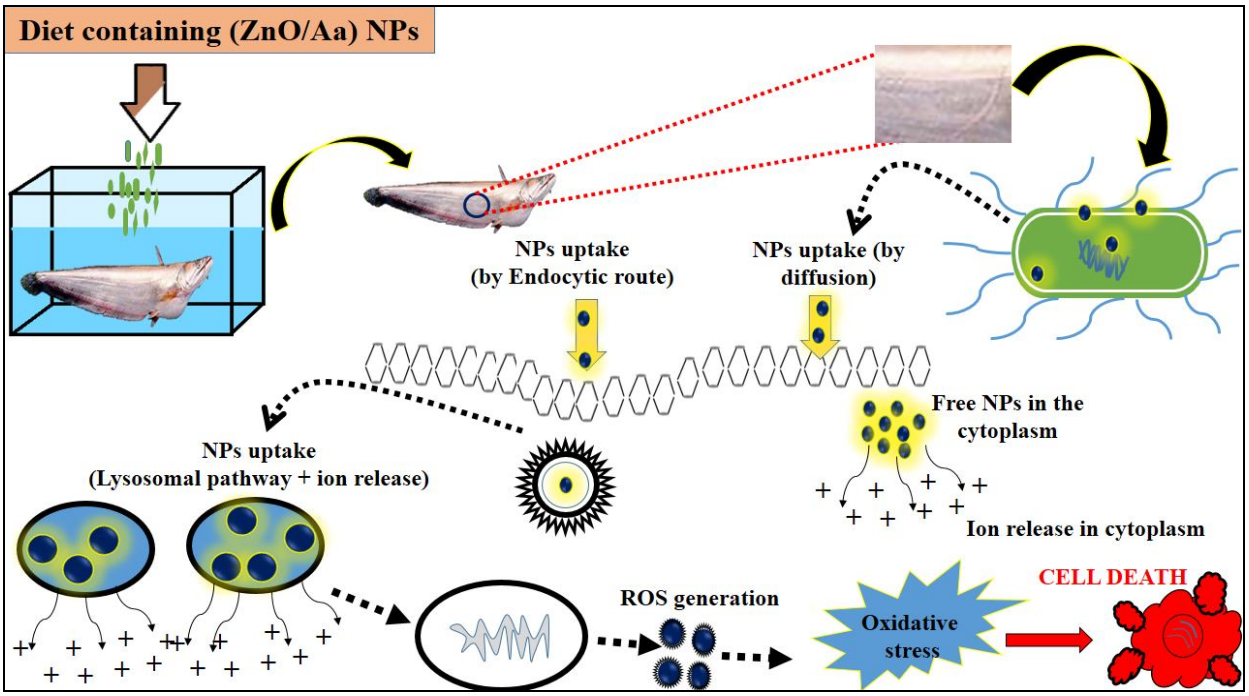


Figure 5. **Different nanostructured ZnO samples like pristine ZnO, sample 1 and 2 with their corresponding antibacterial activity against novel bacterial pathogens and commercial bacterial strains (Inhibition zone in mm)**

It has been established that the thick peptidoglycan of gram-positive bacteria results in a lower sensitivity than that of gram-negative bacteria. A thin peptidoglycan is present in the cell wall of Gram negative bacteria, which facilitates the easy penetration of nanostructured materials inside the bacteria [51]. Nanostructured materials have been reported to possess antibacterial properties against both Gram negative and Gram positive bacteria, and these properties have been attributed to variations in the structure and chemical composition of the particles [25]. A green synthesis using Aloe vera fruit juice possessed optimum zone inhibition of catfish pathogens compared to P. caerulea leaf aqueous extract and C. neilgherrensis leaf aqueous extract [52]. This proves that ZnO nanostructured material is biocidal. ZnO nanostructures prepared with Aloe vera juice showed antibacterial activity that could be considered an effective antimicrobial agent. Aloe vera has shown an influence on the shape and size of the ZnO, consequently an improved antibacterial was noticed. ZnO nanoparticles were observed to exhibit a rapturing property for membranes, which may be caused by the generation of reactive oxygen species, particularly superoxide and hydroxyl radicals [53]. Scheme 2 illustrates an antibacterial mechanism based on ZnO. According to recent studies, ZnO nanoparticles have a positive zeta potential on their surface and this can be of great interest based on the nature of different bacteria's surface. ZnO nanoparticles dose and the nature of the surfactant used during the measurement have been found to significantly influence the antibacterial action. In addition to damaging the bacterial cell membrane, ZnO nanoparticles can also extrude the cytoplasmic levels of the bacteria, ultimately resulting in the death of the bacteria. Based on the literature about antibacterial activity, ZnO nanoparticles inhibit bacterial growth by destroying bacterial membranes and extruding cytoplasmic levels [54].



Scheme 2. **Generalized view of antibacterial activity of ZnO nanostructures against the death of bacteria of catfish.**

Conclusion

In summary, we have utilized Aloe vera fruit juice for the growth of ZnO nanostructures because of its advantages such as its low cost, simplicity, ecofriendly nature, and ability to scale up the synthesis. ZnO nanostructures have characterized with hexagonal phases as confirmed by XRD. SEM study has shown the heterogeneous morphology of various ZnO samples prepared with Aloe vera gel. This study indicates that the prepared ZnO nanostructures with Aloe vera fruit juice have demonstrated significant antibacterial activity against newly discovered pathogens in catfish based on optical, particle size, crystal quality, chemical composition, and morphology results. Sample 2 of ZnO prepared with Aloe vera gel has shown maximum antibacterial activity with an inhibition zone of 5.54±0.34 mm. Further, the effectiveness of ZnO nanostructures against a variety of bacterial strains was carried out. This was to determine the effectiveness of the nanostructures as prepared on the nature of the bacterial strain in question. Based on these findings, it appears that the Aloe vera-assisted ZnO nanostructures may be useful for the development of therapeutic applications because they may be phytogenic towards tissue growth.

Conflict of Interest

The authors declare no conflict of interest in the presented study.

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