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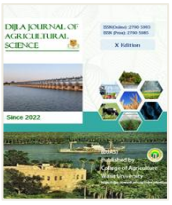

Research article

In Vitro antibacterial activity of ZnO nanoparticles against Klebsiella oxytoca isolated from honeybee (Apis mellifera)

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ABSTRACT

Honeybees (Apis mellifera) are environmentally and economically important insects, although their colonies are infected with a number of bacterial pathogens, including Klebsiella oxytoca, a Gram-negative bacterium that belongs to the Enterobacteriaceae family, that may contribute to the decline in the health of honeybees. The aim of this study was to assess the antibacterial activity of zinc oxide nanoparticles (ZnO NPs) against K. oxytoca, which was isolated from honeybees, in vitro. Samples were collected from a number of beehives from different places in Al-Diwaniyah Governorate, Iraq. Isolates were diagnosed using culture and biochemical tests, in addition to a confirmation diagnosis by the VITEK® 2 Compact System and 16S rRNA gene amplification. The 96-well microtiter plate method was used to evaluate the effectiveness of various ZnO NP concentrations (0, 10, 100, and 500 µg/ml). The results showed that ZnO NPs have effective inhibition on K. oxytoca. The concentration of 500 µg/mL was the most effective, with significant variations at the $P < 0.05$ level. These results show that ZnO NPs could be a good choice instead of antimicrobials used to control diseases caused by bacteria in honeybees.

1. Introduction

Honeybees (A. mellifera) are indispensable pollinators within terrestrial ecosystems (Boğ et al., 2020) and render vital ecosystem services that are integral to sustaining plant biodiversity and improving the quality of horticultural and agricultural produce (Papa et al., 2022). Apiculture is a significant endeavour that fosters sustainable environmental, social, and economic outcomes. The contributions of A. mellifera to humanity are numerous, with 90% of these benefits arising from pollination services and the remaining 10% from apiarian products (such as honey, propolis, and beeswax (Kaya et al., 2023). A. mellifera provides a variety of advantages, notably the production of honey, a natural aliment characterised by numerous health benefits, including immunity enhancement and energy provision (Wood et al., 2020). Furthermore, honey serves as a topical treatment for wounds and burns. In summary, honey constitutes a comprehensive food source abundant in vitamins and minerals, rendering it a nutritious dietary component (Decker et al., 2022). Additionally, A. mellifera produces

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beeswax, which is utilised in the fabrication of wax products, cosmetics, and pharmaceuticals (Wood et al., 2020).

Other products include propolis, which has antibacterial activity, and royal jelly, which is used as a dietary supplement. Moreover, A. mellifera plays a pivotal role in sustaining ecosystem equilibrium by fostering plant growth. In the absence of floral pollination, the production of fruits, crucial for food security, would be compromised. These advantages are significant for both human populations and the surrounding environment (Cunningham et al., 2022). The preservation of these services is fundamental in achieving both environmental integrity and economic viability, thereby ensuring a prosperous future for humankind (Kaya et al., 2023). There is increasing global worry about the declining health of the bee colony. The scientific community recommends several control and prevention methods to reduce the impact of these diseases. In addition, pathogens found in bee colonies around the world affect the economy of food-producing countries and also threaten dozens of local insect species (Matthijs et al., 2020) .

K. oxytoca is a Gram-negative, non-motile, encapsulated bacterium that belongs to Enterobacteriaceae. K. oxytoca is an opportunistic pathogen that impacts honeybees, particularly if their gut microbiota is out of balance. This impact leads to morbidity and mortality. Researchers have found K. oxytoca in dead and sick adult honeybees, showing that it is part of a wide range of bacteria that exist in beehives (Boğ et al., 2020). K. oxytoca has virulence factors that make cytotoxins, including tilivalline and tilimycin, which are related to antibiotic-associated hemorrhagic colitis (AAHC). Also, capsular polysaccharides and membranes (Yang et al., 2022). The gut disorder that can happen when honeybees are exposed to antibiotics is related to their health. This disorder reduces the normal gut microbiome and increases susceptibility to opportunistic pathogens such as K. oxytoca (Raymann et al., 2017). Modern beekeeping encourages the search for new antibacterial agents to ensure safe, highquality, and saleable bee products. Initial (in vitro) evaluation of the effectiveness of disinfectants against specific pathogens of specific bee diseases will ensure increased efficacy of these agents in hives, which is economically efficient for practical beekeeping (Romanishina et al., 2024) .

Nanotechnology is the technology of manipulating matter at the nanoscale to produce new structures, materials, and devices (Shanmugapriya and Sanjay, 2024). These materials are called nanomaterials and are small particles with dimensions ranging from 1 to 100 nanometers, which is about 100,000 times smaller than the diameter of a human hair (Ikeh et al., 2021) . Therefore, they can only be seen with a powerful microscope (Donough et al., 2024; Weerasinghe et al., 2024). Research on nanoparticles has seen increasing interest in the past decade due to their stimulating and antibacterial properties (Oves et al., 2022; Trivedi et al., 2022).

Nanoparticles have revolutionised methods of controlling bacterial infections as antibiotic resistance has increased, offering a promising alternative due to their unique physical and chemical properties that distinguish them from traditional antibiotics. Their high surface area to volume ratio, ability to interact with different molecules, and distinctive optical, electronic, and magnetic properties enable them to exert powerful antibacterial effects. The mechanisms include physically disrupting bacterial membranes, generating reactive oxygen species (ROS), and releasing metal ions that disrupt bacterial metabolism. Furthermore, nanoparticles penetrate biological membranes and bacterial cell walls more effectively than traditional antibiotics and can be precisely targeted to reduce untargeted effects. Most importantly, nanoparticles reduce the development of bacterial resistance by utilizing multiple, simultaneous mechanisms of action, making it difficult for bacteria to adapt through single genetic mutations. As research progresses, nanotechnology holds enormous promise in revolutionizing

antibacterial treatments, offering effective solutions that treat current infections and combat antibiotic resistance worldwide (Akash et al., 2025) .

Because of their unique physical and chemical properties that distinguish them from conventional antibiotics, nanoparticles have completely changed the ways that bacterial infections are treated as antibiotic resistance has grown. They have strong antibacterial effects because of their high surface area-to-volume ratio, capacity to interact with a variety of molecules, and special optical, electronic, and magnetic properties. These characteristics enable them to release metal ions that disrupt bacterial metabolism, cause physical destruction of bacterial membranes, and increase the production of reactive oxygen species (ROS). Furthermore, nanoparticles are able to penetrate bacterial cell walls and biological membranes more efficiently compared to most traditional antibiotics and are capable of being targeted with greater precision to mitigate off-target effects. Crucially, nanoparticles inhibit the ability of bacteria to develop resistance via a variety of simultaneous mechanisms of action, which prevents bacteria from adapting via single-point mutations. Further research could lead to nanotechnology revolutionising antibacterial treatments and providing efficient approaches to treat existing bacterial infections and prevent antibiotic resistance worldwide (Akash et al., 2025). The current study aims to investigate the effect of zinc oxide nanoparticles (ZnO) in controlling bacteria that affect honeybee health.

2. Materials and Methods

2.1 Sample collection

A total of 225 samples were collected from honeybees from several different hives in the Al-Diwaniyah Governorate, including hives at the Al-Diwaniyah Agricultural Preparatory School and in the districts of Sumer, Afak, Shamiya, and Hamza, during the period from October 8, 2024, to January 8, 2025**.**

2.2 Isolation and diagnosis

All the samples were inoculated onto Mannitol agar, McConkey agar and Blood agar plates and incubated at 37°C for 18–24 h. They were then characterized by a macroscopic observation of colony forms, diameters, colors, and ability to produce hemolysis. A Gram staining by microscopy, and several biochemical assays as lactose fermentation, oxidase, catalase, indole and hemolysis were performed (Forbes *et al.*, 2016). The diagnosis was also confirmed by VITEK® 2 Compact System, and molecular identification was performed using PCR technology targeting the gene for 16S rRNA, using DNA extracted with kit Presto™ Mini gDNA (Geneaid Biotech Ltd., Taiwan) Bacteria Kit Quick Protocol. Universal primers were used, and their sequences are shown in Table 1. The PCR amplified with a thermal cycler, and the products were analyzed by agarose gel electrophoresis. Ultimately, the confirmed isolates were preserved under appropriate conditions until their application in subsequent phases of the experiment.

Table 1. Primer sequences used to amplify the 16S rRNA gene

Primers	Direction	Sequence (3' → 5')	Product size (bp)
27F	Forward	AGAGTTTGATCCTGGCTCA	1500
1492R	Reverse	CTACGGCTACCTTGTTACGA	1500

2.3 Antibiotic Sensitivity Test (AST)

According to guidelines of CLSI (2025), This test was carried out for four antibiotics, including ampicillin, amoxicillin, cefixime, and ceftriaxone, using the disc diffusion method (or KirbyBauer method).

2.4 ZnO Characterization Techniques

Field emission scanning electron microscopy (FESEM) was used to observe the surface morphology of the ZnO particles, and FESEM can provide high resolution images that display the size, shape, and distribution of particles.

Also, energy dispersive X-ray spectroscopy (EDS) is used with FESEM to accurately calculate the elemental composition of the sample and help assist with old verification of main components (zinc and oxygen) and purity (Lewczuk and Szyryńska, 2021).

2.5 Antimicrobial activity of ZnO on *K. oxytoca*

2.5.1 ZnO stock solution and concentrations preparation

The approach of Ghosh *et al.* (2020) was used to study the antibacterial activity of zinc oxide nanoparticles (ZnO), purchased from US Research Nanomaterials Inc., USA. A stock solution of concentration of 1000 µg/mL was prepared by dissolving 10 mg of ZnO powder in 10 mL of sterile distilled water made up in a glass beaker 100 mL.

The stock solution was mixed using a vortex mixer for 5 minutes as a first step to evenly distribute the ZnO nanoparticles in the stock solution before bathing the non-uniform nanoparticles in an ultrasonic bath for dispersion or fragmentation using ultrasonic waves.

The concentrations used in this study were achieved by performing serial dilutions of the stock solution (0, 10, 100, and 500 µg/mL) using distilled water for applications to bacterial isolates. This test was performed utilizing the 96-well microtiter plate method. The above concentrations were followed with a positive control group (nutrient broth, bacterial suspension, Ciprofloxacin) and a negative control (nutrient broth). For each well, 100µl of nutrient broth was added.

Then, 100µl of each concentration of nanomaterials were added in triplicate for each concentration with mixing with the nutrient broth utilizing a micropipette. Then, 100µl of bacterial suspension was added to wells containing the various concentrations of nanomaterials. For the positive control well, 100 µl of nutrient medium, 100 µl of bacterial suspension, and 100 µl of antibiotic Ciprofloxacin (5 µg) was added; for the negative control well, 100 µl of nutrient broth was added.

The plate was then covered, taped down securely, and placed in a 37°C incubator for 24 hours. The results were then read with an ELISA reader.

2.6 Statistical analysis

The study results were statistically analysed using SPSS version 27. For each indicator, mean and standard error were determined. One-way ANOVA with least significant difference (LSD) was used to compare the differences among rates. Differences among rates that were statistically significant, occurred at $p<0.05$ (Rahman, 2015).

3. Results and Discussion

3.1 Isolation and identification

Out of 225 samples, 105 growth-positive isolates were obtained, including 30 Gram-positive isolates (13.3%) and 75 Gram-negative isolates (33.3%), as shown in Figure 1. The diagnosis was based on cultural and microscopic characteristics, and some biochemical tests were also used for diagnosis, as shown in Table 2. The high percentage of Gram-negative bacteria as opposed to Gram-positive bacteria isolates indicates that Gram-negative bacteria are abundant in the samples that were assessed, possibly due to multiple environmental conditions to which Gram-negative bacteria may adapt given a wider tolerance range, sources of contamination, or attributes of the samples themselves, as Gram-negative bacteria are typically more resistant to environmental conditions and some attributes, thus greater range of occurrences in certain environments. Results of our isolation study were consistent with necker studies indicating greater isolation of Gram-negative than gram-positive bacteria; for instance, there was Cilia et al. (2023), who isolated 52.3% Gram-negative and 47.7% Gram-positive bacteria, and the study of Tootiaie et al. (2021) with 70% Gram-negative and 27% Gram-positive bacteria. The study by Dokuta et al. (2023) did not match the isolation percentages of the present study, with Gram-negative and Gram-positive bacteria isolations of 35.1% and 59.5%, respectively. The most frequent isolates were selected for further study. *K. oxytoca* isolates were the most common with 30 isolates (28.6%), as shown in Table 3, which 4 isolates were confirmed by the VITEK® 2 Compact System and 16S rRNA gene.

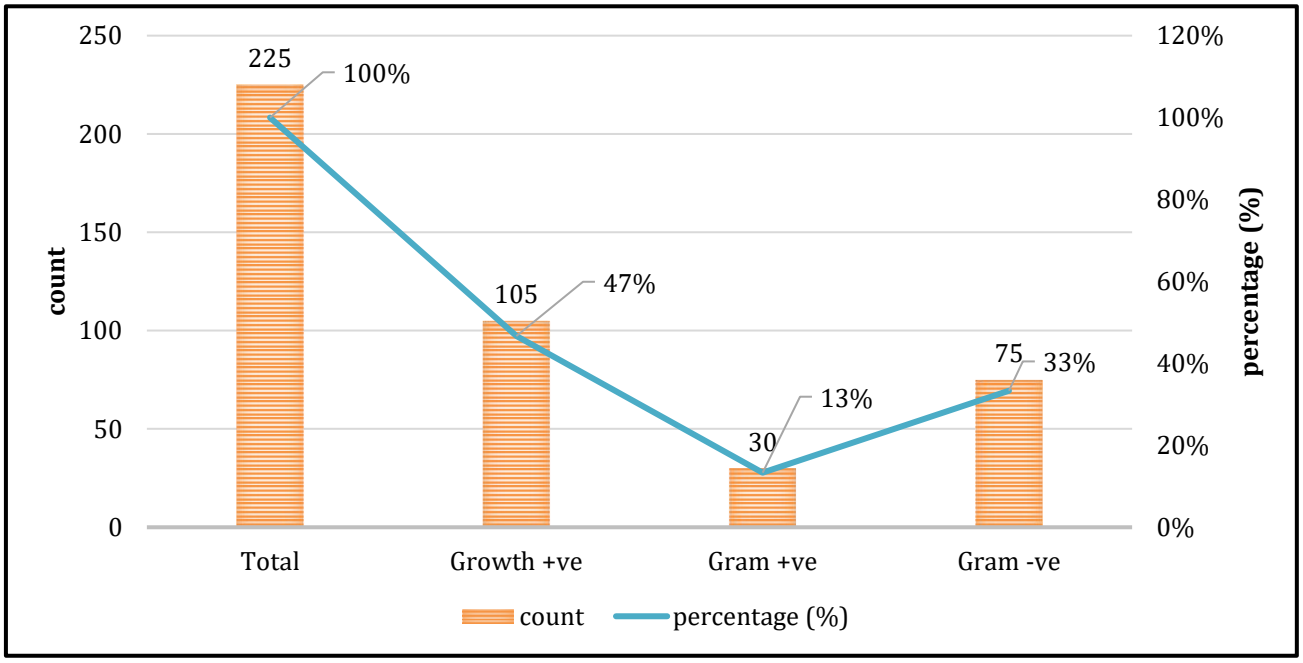

Figure 1. Percentages of bacterial growth.

Table 2. biochemical tests of bacterial isolates.

Bacterial isolates	lactose	Urease	Hemolysis	Indole	Catalase	Oxidase

K. oxytoca	+	+	-	-	-	-
Raoultella ornithinolytica	+	+	-	-	-	-
Pseudomonas aeruginosa	-	-	+	-	+	+
Enterobacter cloacae	+	-	+	-	-	-
Escherichia coli	+	-	-	+	-	-
Staphylococcus aureus	-	-	+	-	+	-
Staphylococcus epidermidis	-	-	+	-	+	-
.Lactobacillus spp	+	-	+	-	-	-

Table 3. Percentage of bacterial isolates.

Bacterial isolates	count	Percentage (%)
K. oxytoca	30	28.6
Raoultella ornithinolytica	15	14.3
Pseudomonas aeruginosa	5	4.8
Enterobacter cloacae	13	12.4
Escherichia coli	12	11.4
Staphylococcus aureus	6	5.7
S. epidermidis	14	13.3
.Lactobacillus spp	10	9.5
Total	**105**	**100**

3.2 Molecular diagnosis of bacterial isolates by detecting the 16S rRNA gene

The results of detecting this gene using the polymerase chain reaction (PCR) technique showed that all four bacterial isolates (100%) contained this gene, and showed the expected amplification size of 1500 bp, which confirms the diagnosis.

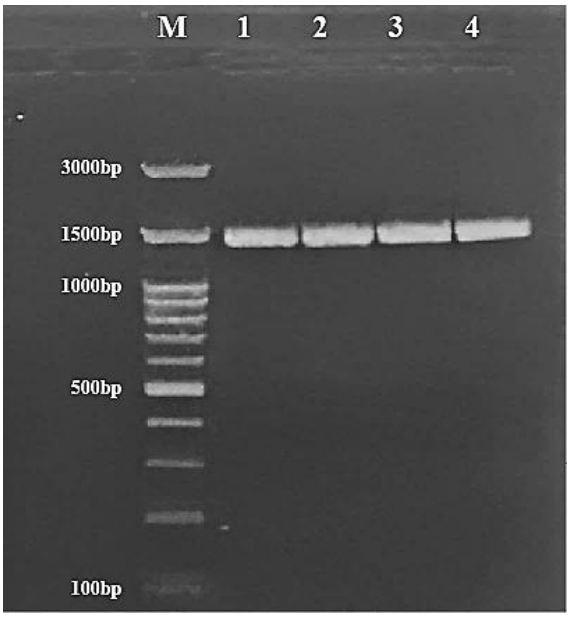

Figure 2. Electrophoresis of agarose gel showing the amplification product of the 16S rRNA gene in *K. oxytoca* bacterial isolates. M refers to the DNA marker ladder with a size of (100-3000 bp), and the wells (1, 2, 3, 4) indicate positive isolates of this gene with a PCR product size of 1500 bp. Electrophoresis conditions: agarose gel (1.5%) stained with ethidium bromide, voltage (100 V), current (80 mA), time (90 min.).

3.3 Antibiotics susceptibility test

The results of the antibiotic sensitivity test in the current study showed that all *K. oxytoca* isolates studied were 100% resistant to Ampicillin and Amoxicillin. In contrast, complete sensitivity (100%) was observed towards Cefixime and Ceftriaxone, indicating the high efficacy of these antibiotics in inhibiting the bacteria, as shown in Figures 3 and 4. The results reveal a clear pattern of resistance to common antibiotics, such as amoxicillin and ampicillin, due to the bacteria's production of β-lactamases that can degrade the β-lactam ring (Bush & Bradford, 2016). On the other hand, the isolates showed complete susceptibility to antibiotics, including cefixime and ceftriaxone, which are more resistant to these enzymes and have better tissue penetration and ability to pass through the cellular membrane (Afreen et al., 2025).

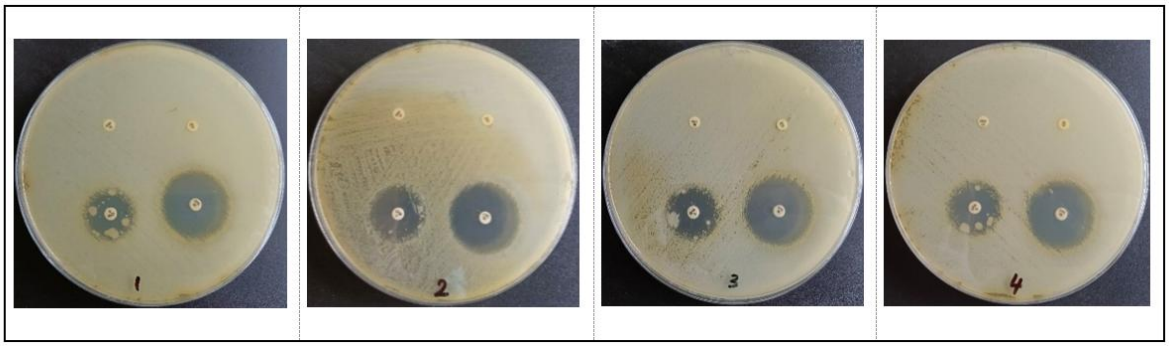

Figure (3) Antibiotic sensitivity testing of four *K. oxytoca* isolates.

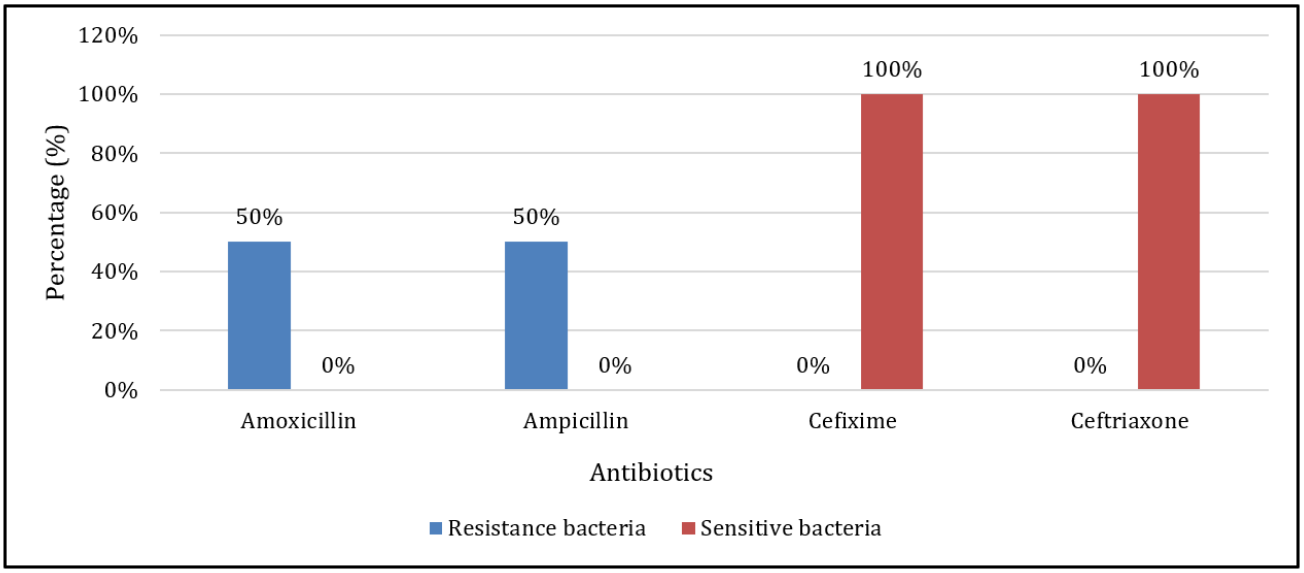

Figure 4. Percentages of sensitivity testing of *K. oxytoca* isolates to a number of antibiotics.

3.4 Field Emission Scanning Electron Microscopy (FESEM) Analysis

Our results of the analysis using FESEM (Figure 5) showed that the zinc nanoparticles were spherical in shape with a smooth surface and clear boundaries, ranging in size from 30 to 70 nanometers. There were no cracks or structural defects on the particles. no obvious agglomerations between the particles, which indicates good preparation quality and enhances their potential physical properties. Our study is

agreeing with study of Dumbrava et al. (2019) which showed that smaller ZnO nanoparticles demonstrate higher antimicrobial activity. Some microorganisms are more sensitive to the shape of nanoparticles, which leads to mechanical destruction of the cell membrane to gain entry, agree with Motelica et al. (2022).

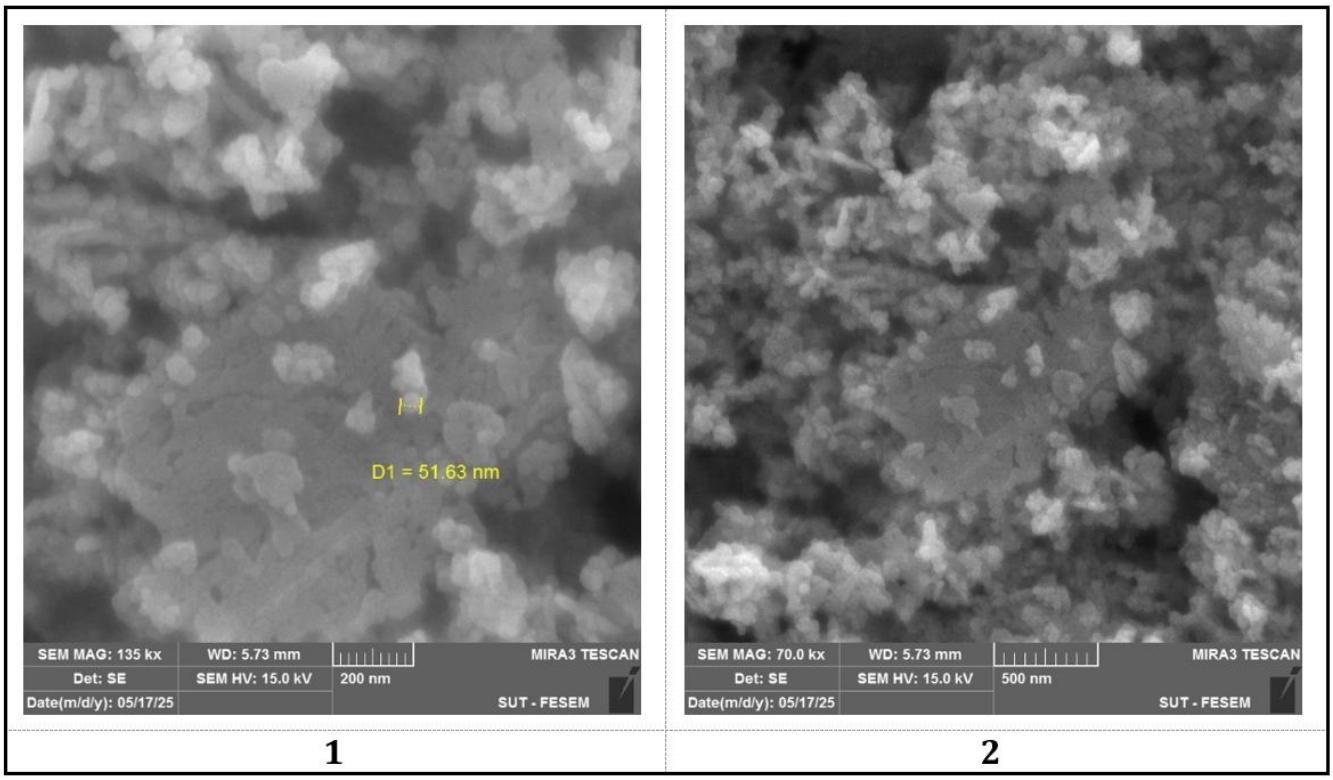

Figure 5. FESEM images of ZnO nanoparticles with a regular spherical shape at a magnification of 150,000X. 1. At a scale of 200 nm. 2. At a scale of 500 nm.

3.5 Energy dispersive X-ray spectroscopy (EDS) Analysis

EDS analysis of the ZnO NPs sample indicated the presence of Zn (82.1 wt %) and O (17.9 wt %), shown in Figure 6, reflecting high purity of the sample and confirming composition of ZnO. The slight excess of Zn in atomic percentage (52.95%) over O (47.05%) agreed with others who reported oxygen vacancies in ZnO nanostructures (Rasmussen et al., 2010; Sirelkhatim et al., 2015). The excess Zn compared to O results in a greater release of Zn^{2+} ions leading to the generation of ROS and compromising membrane integrity, and therefore it contributes to the antimicrobial activity by ZnO nanoparticles. Additional peaks from Au were detected, most likely due to the coating layer applied to improve electrical conductivity during testing, and do not alter the actual composition of the sample. Table 4 below indicates the concentration ratio as well as element detected, in addition to type of spectral line for each respective element. The presence of Au was due to a thin film of gold that formed due to a gold coating during the preparation of the SEM images and therefore does not influence the composition of the sample. The results are consistent with Heu et al. (2019) reports of the antimicrobial potential of ZnO nanoparticles.

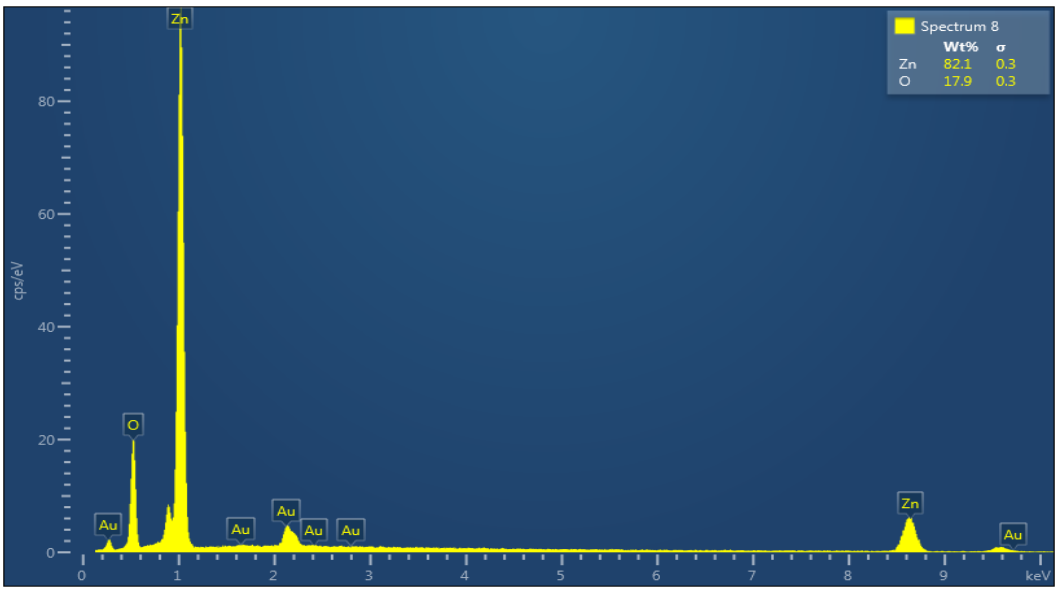

Figure 6. EDS spectrum of ZnO nanoparticle sample

Table 4. EDS analysis data for ZnO sample

Element	Line Type	Apparent Concentration	k Ratio	Wt%	Wt% Sigma	Atomic %	Standard Label	Factory Standard
O	K series	4.86	0.01636	17.87	0.25	47.05	SiO2	Yes
Zn	L series	13.56	0.13558	82.13	0.25	52.95	Zn	Yes
Total				100.00		100.00		

3.6 Antibacterial activity of Zinc oxide nanoparticles

Table 4 presents the effect of ZnO-NPs at several concentrations on the growth of *K. oxytoca*, as measured by absorbance readings at the wavelength of 600 nm. The absorbance values varied significantly across the concentrations evaluated. The highest concentration of 500 µg/mL resulted in the maximum antibacterial activity (0.660 ± 0.16), while concentrations of 10 and 100 µg/mL resulted in lower absorbance values. It is also noteworthy that the negative control exhibited weak activity (0.079 ± 0.01), indicating that in the absence of ZnO-NPs, bacterial growth was uninhibited. The analysis results exhibited significant differences between the treatments (P<0.001) with the least significant difference (LSD) between treatments as 0.218. These results suggest that the particles do exhibit antibacterial characteristics, and this activity increased with concentration, with the most evident effect at the maximum concentration evaluated in this study. The relatively high variability recorded in the negative control group can be attributed to variation in bacterial growth in the absence of nanoparticles. Our findings confirm the antibacterial properties of ZnO nanoparticles, revealing a positive correlation between their concentration and antibacterial efficacy. These results are in agreement with the findings presented by Sirelkhatim et al. (2015), who emphasised that particle size, shape, and concentration have a significant impact on the antimicrobial activity of nanomaterials. They observed that larger concentrations produce stronger inhibitory effects, a pattern that is amply demonstrated in the present investigation. This observation corresponds with the results of Sirelkhatim et al. (2015) and Raghupathi et al. (2011), who showed that ZnO has potent antibacterial properties against a wide range of both Gram-positive and Gram-negative bacteria. They also proposed that the production of reactive oxygen

species (ROS) and the accumulation of nanoparticles on bacterial biofilms or within the cytoplasm may be involved in the antimicrobial mechanism of ZnO nanoparticles. Furthermore, Premanathan et al. (2011) suggested that the fundamental mechanism of this effect is connected to ZnO capacity to produce ROS, which can harm important cellular constituents like proteins and DNA as well as the bacterial cell wall. Zhang et al. (2008) also confirmed that this activity increases with increasing concentrations, which supports the current study's findings and emphasises ZnO potential as an effective antimicrobial agent, particularly given the rise in bacterial resistance to traditional antibiotics.

Table 4. Mean absorbance values of ZnO treatments compared to controls (Mean± SE).

Treatments	Mean concentrations (µg/mL) ± standard error (SE)			
	0	**10**	**100**	**500**
ZnO	02. ± 01.06	0.09 0.705 ±	± 0.030.563	± 0.160.660
PC	± 0.010.118			
NC	± 0.010.079			
P-Value	< 0.001			
LSD	0.218			

4. Conclusion

This study confirms the antibacterial potential of ZnO nanoparticles against *K. oxytoca*, with effectiveness increasing by concentration. The results support their role as a promising alternative to conventional antibiotics, especially given rising resistance. The proposed mechanisms, such as ROS generation and nanoparticle accumulation, likely contribute to their inhibitory effect.

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