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ANTIBACTERIAL ACTIVITY OF ZINC OXIDE NANOPARTICLES AGAINST BACTERIA CAUSING SECONDARY INFECTION IN CUTANEOUS LEISHMANIASIS

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Abstract

Antibiotics remain the gold standard for treating bacterial infections; however, the rise of antibiotic resistance underscores the urgent need for alternative therapies. This study evaluates the antibacterial activity of zinc oxide nanoparticles (ZnO NPs) against bacterial isolates recovered from cutaneous leishmaniasis (CL) lesions. Wound samples were cultured, and bacterial identification was performed using biochemical tests. ZnO nanoparticles were synthesized using the wet chemical method, and their size and morphology were characterized using scanning electron microscopy (SEM), which revealed spherical particles ranging from 10 to 20 nm. X-ray diffraction (XRD) confirmed the hexagonal wurtzite crystal structure, while UV-Visible spectroscopy displayed absorption peaks between 350 nm and 370 nm, confirming the formation of ZnO NPs. The minimum inhibitory concentrations (MICs) of ZnO NPs were 200 µg/mL for Staphylococcus aureus, 400 µg/mL for Escherichia coli, and 400 µg/mL for Klebsiella pneumoniae. Zeta potential analysis indicated a positive surface charge of 1.04 mV, supporting nanoparticle stability. These results demonstrate that ZnO NPs possess strong antibacterial properties and could serve as an effective alternative to traditional antibiotics for the treatment of secondary bacterial infections in cutaneous leishmaniasis lesions.

Keywords: *Cutaneous leishmaniasis, Nanomedicine, Secondary bacterial infections, Zinc oxide nanoparticles*

INTRODUCTION

Cutaneous leishmaniasis (CL) is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania*, transmitted through the bite of infected phlebotomine sandflies. It is the most prevalent form of leishmaniasis, with approximately 1.5 million new cases annually and 350 million individuals at risk worldwide (1, 2). According to the World Health Organization (WHO), over 90% of CL cases occur in endemic regions such as Afghanistan, Iran, Iraq, Algeria, Pakistan, Turkey, and Peru, often affecting socioeconomically disadvantaged populations (3). CL typically begins as erythematous papules at the bite sites, which evolve into nodular plaques and ulcerative lesions within 4–12 weeks. However, the disease is complicated by secondary bacterial infections, commonly caused by *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, and others(4, 5). These infections, which occur due to environmental exposure and poor hygiene, exacerbate tissue damage and delay healing (6).

Although antibiotics are the standard treatment for secondary infections, the growing prevalence of antimicrobial resistance (AMR) has become a critical public health concern. This resistance is driven by the overuse of antibiotics, particularly in resource-limited settings where culture and susceptibility testing may be unavailable (7, 8). Therefore, new therapeutic strategies are urgently needed.

Nanomedicine, particularly metal oxide nanoparticles (e.g., Ag_2O, TiO_2, CuO, and ZnO), has garnered attention for its potential in drug delivery and antimicrobial therapy (9, 10). Zinc oxide nanoparticles (ZnO NPs) are particularly promising due to their broad-spectrum antimicrobial properties

and biocompatibility (11, 12). Notably, ZnO NPs display selective toxicity, demonstrating minimal cytotoxicity to human cells while exerting potent leishmanicidal effects against both amastigote and promastigote forms of *Leishmania* parasites, as well as broad-spectrum antibacterial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria. This antimicrobial action is primarily mediated through the generation of reactive oxygen species (ROS) on nanoparticle surfaces, which induce lipid peroxidation in the membranes of prokaryotic and eukaryotic cells, thereby overcoming microbial resistance mechanisms (13, 14). This study aims to explore the antibacterial potential of ZnO NPs against bacteria isolated from CL lesions.

MATERIALS AND METHODS

STUDY DESIGN

This multicenter study was conducted over six months at the Outpatient Department of Dermatology at Lady Reading Hospital, Khyber Teaching Hospital, and the Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan. The study protocol was approved by the Institutional Review Board of the Institute of Basic Medical Sciences (IBMS), Khyber Medical University (Approval No: KMU/IPDM/IEC/2023/19). Informed consent was obtained from all participants.

SAMPLE COLLECTION AND BACTERIAL IDENTIFICATION

Wound samples from patients diagnosed with cutaneous leishmaniasis were collected using sterile cotton-tipped applicators. The samples were transported under aseptic conditions to the microbiology laboratory for further processing. Bacterial identification was carried out using a series of biochemical tests, including catalase, coagulase, triple sugar iron (TSI), citrate utilization, and sulfide indole motility (SIM) tests. The bacterial isolates were cultured on appropriate agar media, including Blood Agar, MacConkey Agar, Eosin Methylene Blue (EMB) agar, and Mannitol Salt Agar (MSA) for the selective isolation of Staphylococcus aureus.

SYNTHESIS OF ZINC OXIDE NANOPARTICLES

ZnO NPs were synthesized using the wet chemical method with modifications (15). In brief, 0.2g of sodium hydroxide (NaOH) was dissolved in 100 mL of distilled water to form a 0.050 M NaOH solution. The solution was stirred at 400 RPM for 30 minutes at 50°C. Then, 0.10g of zinc acetate dihydrate ($Zn(CH3CO2)2H2O$) was dissolved in 25 mL of isopropanol, achieving a 0.018 M concentration, and this solution was stirred for 2 hours at 400 RPM at 60°C. Upon complete dissolution, the NaOH solution was added dropwise into the zinc acetate solution while stirring continuously, allowing the reaction to proceed at room temperature for 2 hours. The mixture was allowed to settle overnight, after which the supernatant was discarded. The resulting ZnO nanoparticles were then separated by centrifugation at 6,000 rpm for 10 minutes, washed thrice with ethanol to remove impurities, and finally dried at 70°C. The dried nanoparticles were ground into a fine powder using a mortar and pestle (16-18).

CHARACTERIZATION OF ZINC OXIDE NANOPARTICLES

The synthesized zinc oxide nanoparticles (ZnO NPs) were characterized using several analytical techniques to confirm their size, morphology, and crystallinity. UV-Visible spectroscopy was employed to assess the formation and stability of the nanoparticles. The UV spectra showed distinct absorption peaks between 350 nm and 370 nm, which are indicative of the characteristic band gap of ZnO NPs, confirming their successful synthesis. X-ray diffraction (XRD) analysis was conducted to study the crystallinity and phase structure of the nanoparticles. The XRD pattern exhibited strong diffraction peaks at $2\theta \approx 31.8°$, 34.4°, 36.3°, 47.5°, 56.6°, 62.9°, among others, which corresponded to the hexagonal wurtzite structure of ZnO. These results confirm the crystalline nature of the nanoparticles. Additionally, scanning electron microscopy (SEM) was used to observe the morphology and size of the ZnO NPs. SEM images revealed that the nanoparticles were spherical in shape and ranged in size from 10 nm to 20 nm, consistent with the desired nanoscale. To further evaluate the stability and surface charge of the nanoparticles, zeta potential analysis

was performed. The analysis revealed a positive surface charge of 1.04 mV, indicating that the nanoparticles were stable in suspension, which is critical for their application in biological systems. These combined techniques confirmed the successful synthesis and the desirable characteristics of ZnO NPs for antibacterial applications.

ANTIBACTERIAL ACTIVITY TESTING

The antibacterial activity of ZnO NPs was evaluated against three bacterial isolates commonly associated with secondary infections in cutaneous leishmaniasis lesions, namely Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae. The minimum inhibitory concentration (MIC) values of ZnO NPs were determined using the broth microdilution method.

A stock solution of ZnO nanoparticles was prepared by dissolving 20 mg of ZnO NPs in 10 mL of dimethyl sulfoxide (DMSO) to yield a concentration of 2000 µg/mL. Serial dilutions of the stock solution, ranging from 12.5 µg/mL to 900 µg/mL, were prepared for testing.

Bacterial colonies were suspended in Mueller-Hinton broth to prepare bacterial solutions with a final concentration of approximately 1 x 10^6 CFU/mL. A 96-well microtiter plate was used to determine the MIC values. In each well, 100 µL of bacterial suspension was combined with various concentrations of ZnO nanoparticles. The plates were incubated at 37°C for 24 hours. The MIC was determined by visual inspection for bacterial growth, with the lowest concentration showing no visible growth being recorded as the MIC.

Amoxicillin was used as a positive control. The MIC values of amoxicillin were determined using the same procedure as for ZnO NPs for comparison. Fig. 1 illustrates the methodology flowchart depicting the steps from bacterial isolation to ZnO-NPs antibacterial activity evaluation.

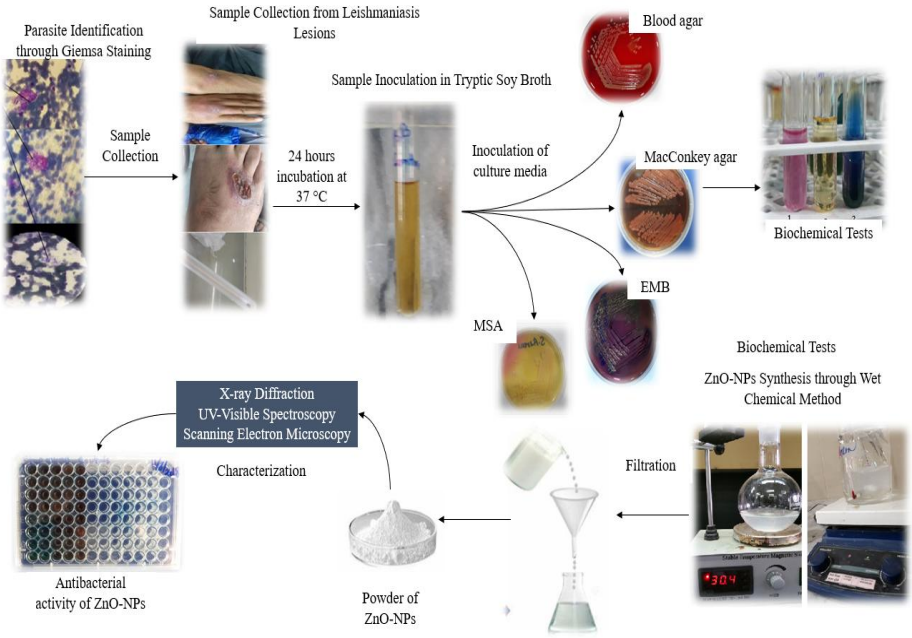

Fig. 1. Flowchart illustrating the isolation and identification of bacterial pathogens from cutaneous leishmaniasis lesions, synthesis and characterization of ZnO-NPs, and evaluation of their antibacterial activity

STATISTICAL ANALYSIS

The results were analyzed using descriptive statistics. The MIC values were calculated based on the lowest concentration of ZnO NPs that inhibited bacterial growth. The effectiveness of ZnO NPs was compared with that of amoxicillin, the standard antibiotic, to assess the potential of ZnO NPs as an alternative antibacterial agent

RESULTS

BACTERIAL ISOLATES AND PREVALENCE

Out of 100 patients diagnosed with cutaneous leishmaniasis (CL), 74 patients (74%) exhibited secondary bacterial infections. The bacterial isolates identified through culture and biochemical testing were

predominantly Staphylococcus aureus (68%), followed by Escherichia coli (22%) and Klebsiella pneumoniae (10%). The prevalence of bacterial isolates is summarized in Table I.

Table I. Bacterial Isolates from cutaneous leishmaniasis lesions

Bacterial isolate	Number of isolates	Prevalence (%)
Staphylococcus aureus	24	68
Escherichia coli	11	22
Klebsiella pneumoniae	5	10

CHARACTERIZATION

The synthesized zinc oxide nanoparticles (ZnO NPs) were characterized using several analytical techniques to confirm their size, morphology, and crystallinity. UV-Visible spectroscopy showed absorption peaks between 350 nm and 370 nm, which are characteristic of the ZnO NPs' band gap, confirming their successful formation. X-ray diffraction (XRD) analysis revealed a hexagonal wurtzite structure, with prominent diffraction peaks at $2\theta \approx 31.8°$ (100), 34.4° (002), 36.3° (101), 47.5° (102), 56.6° (110), 62.9° (103), and others. Scanning electron microscopy (SEM) confirmed the spherical morphology of the ZnO NPs, with particle sizes ranging from 10–20 nm. The zeta potential of the nanoparticles was measured at 1.04 mV, indicating their stability in suspension, which is critical for their biological application. The various techniques used for characterization, along with their results, are summarized in Table II.

Table II. Characterization of Zinc Oxide Nanoparticles

Technique	Results
UV-Visible Spectroscopy	Absorption peaks observed between 350 nm and 370 nm, confirming ZnO NPs formation
X-ray Diffraction (XRD)	Peaks at $2\theta \approx 31.8°$, 34.4°, 36.3°, and others confirming the hexagonal wurtzite structure of ZnO
Scanning Electron Microscopy (SEM)	Spherical shape with a size ranging from 10–20 nm
Zeta Potential	Positive surface charge of 1.04 mV, indicating good nanoparticle stability

ANTIBACTERIAL ACTIVITY OF ZNO NANOPARTICLES

The antibacterial activity of ZnO nanoparticles was tested against Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae. The Minimum Inhibitory Concentration (MIC) was determined using the broth microdilution method. The results of MIC are illustrated in Table III.

Table III. Comparison of MIC of ZnO-NPs and amoxicillin against bacterial isolates from cutaneous leishmaniasis lesions

Bacterial isolates	MIC of ZnO-NPs	MIC of amoxicillin
Escherichia coli	400 µg/mL	600 µg/mL
Staphylococcus aureus	200 µg/mL	400 µg/mL
Klebsiella pneumoniae	400 µg/mL	800 µg/mL

ZnO nanoparticles exhibited the lowest MIC value against Staphylococcus aureus (200 µg/mL), indicating a potent antibacterial effect. The MIC for Escherichia coli and Klebsiella pneumoniae was observed to be 400 µg/mL for both, showing moderate antibacterial activity. A comparison of MIC values between ZnO-NPs and the standard antibiotic amoxicillin demonstrated that ZnO NPs showed comparable antibacterial efficacy, especially against Staphylococcus aureus, a major pathogen involved in secondary infections of cutaneous leishmaniasis lesions.

DISCUSSION

Bacterial infections represent a significant global public health concern, posing a serious threat to human health. Both Gram-positive and Gram-negative bacterial strains contribute substantially to this challenge. Historically, antibiotics have been the primary therapeutic agents for managing community-

acquired and hospital-associated infections. However, their widespread and indiscriminate use has led to the emergence of resistant bacterial strains, diminishing therapeutic efficacy, complicating disease management, and contributing to elevated mortality rates. A study conducted in Ethiopia revealed that over 80% of bacterial isolates recovered from cutaneous leishmaniasis lesions exhibited multidrug resistance (MDR) to two or more antibiotics. Notably, *Staphylococcus aureus* demonstrated particularly high resistance rates to methicillin (83.9%), penicillin (86.4%), and tetracycline (47.5%) (7). Similarly, surveillance data from Ghana indicated near-universal multidrug resistance among bacterial isolates, further increases the demand for alternative antimicrobial strategies (19).

Recent advancements in nanotechnology have enabled novel applications across diverse disciplines, with particular emphasis on the utilization of nanomaterials as antimicrobial agents targeting viral, bacterial, and other pathogenic microorganisms(20). These nanomaterials not only allow targeted delivery of drugs but also show greater efficiency with lower toxicity in a very small dosage compared to conventional drugs(20). Metal oxide nanoparticles have emerged as particularly promising candidates in this domain due to their exceptional stability, biocompatibility, and potent biological activity (21). Among these, zinc oxide nanoparticles (ZnO NPs) have attracted significant research attention owing to their cost-effectiveness and broad-spectrum antimicrobial properties (22). The antimicrobial mechanism of ZnO NPs is multifactorial, involving: (1) disruption of bacterial cell wall integrity and membrane permeability, (2) generation of reactive oxygen species (ROS) leading to oxidative damage of cellular proteins and nucleic acids, and (3) interference with DNA replication processes. These combined effects contribute to their potent bactericidal activity (21).

Various methods are employed for ZnO-NPs preparation, including hydrothermal synthesis, chemical vapor deposition, precipitation, and sol-gel techniques. However, in this study, we employed the wet chemical method due to its simplicity, cost-effectiveness, and widespread applicability. The antibacterial efficacy of the synthesized ZnO NPs (10–20 nm) was evaluated against three clinically relevant bacterial isolates obtained from cutaneous leishmaniasis lesions, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* using a broth microdilution assay. The minimum inhibitory concentrations (MICs) were determined to be 200 µg/mL for *S. aureus* and 400 µg/mL for *E. Coli and K.pneumoniae*. This was in contrast to a study conducted by *El-Khawaga et al.* demonstrated that bio-synthesized ZnO NPs (using *Saccharomyces cerevisiae*) exhibited significantly lower MIC values against *S. aureus* (0.000625 mg/mL) and *E. coli* (0.00125 mg/mL). This enhanced activity was attributed to biomolecular capping, which improves NP stability and promotes targeted interactions with bacterial membranes (23). *Chamkouri et al.* reported MIC values of 0.125 mg/mL for *S. aureus* and 0.0625 mg/mL for *E. coli*, further highlighting the variability in efficacy based on synthesis routes. In addition to this, the antibacterial performance of NPs is influenced by other factors such as particle size, morphology, surface charge density, and strain-specific microbial susceptibility profile (24). *Wazir et al.* assessed ZnO NPs (0.1–0.2 mg/mL) via the agar well diffusion method, with the highest concentration (0.2 mg/mL) producing the largest inhibition zones: 24 mm for *E. coli*, 18 mm for *S. aureus*, and 15 mm for *K. pneumoniae*(25). In our study, comparable efficacy was observed, with equivalent MICs for *E. coli* and *K. pneumoniae* (0.4 mg/mL) and superior activity against *S. aureus* (0.2 mg/mL). This difference in performance can be attributed to the dissimilarities present in the structure and composition of their cell membranes. In Gram-negative bacteria, the outer membrane is composed of a lipopolysaccharide (LPS) layer that acts as a barrier against nanoparticles. The findings of our study align with existing literature, reinforcing the potential of ZnO NPs as a promising antimicrobial agent.

Further investigations are warranted to evaluate the antibacterial efficacy of ZnO-NPs using in vivo models, including preclinical animal studies and clinical trials in humans. We evaluated the antibacterial activity of ZnO-NPs only against three isolates: *S. aureus, E. coli, and K. pneumoniae* due to their high prevalence in leishmania-associated co-infections and clinical significance in complicating disease. Their antimicrobial spectrum should be comprehensively assessed against a wider range of pathogenic bacteria, fungi, and viruses to determine broad-spectrum applicability and to ensure biosafety and potential clinical translation, cytotoxicity profiling in relevant mammalian cell lines and animal models is essential. Although

there are other techniques used for characterization of nanoparticles, such as Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FT-IR), which provides additional insights, but due to funding constraints we were limited to SEM, XRD, and UV-Visible. Future work with expanded resources should incorporate these techniques to refine characterization. Furthermore, comparative studies with established antimicrobial agents are needed to benchmark efficacy, elucidate mechanistic pathways, and optimize formulations for commercial viability.

CONCLUSION

The antibacterial efficacy of zinc oxide nanoparticles (ZnO-NPs) was assessed against three clinically relevant bacterial isolates, *Staphylococcus aureus* (Gram-positive), *Escherichia coli*, and *Klebsiella pneumoniae* (Gram-negative), commonly associated with secondary infections in cutaneous leishmaniasis lesions, using the broth microdilution method. ZnO-NPs demonstrated notable antibacterial activity, with the most excellent efficacy observed against *S. aureus* (MIC = 0.2 mg/mL), followed by *E. coli* and *K. pneumoniae* (MIC = 0.4 mg/mL for both). In light of increasing antibiotic resistance, these in vitro results suggest ZnO-NPs hold potential as alternative antimicrobial agents. However, further in vivo studies and comprehensive toxicological evaluations are necessary before considering their suitability for clinical or pharmaceutical applications.

Authors' contribution:

AA & YK Performed the experiments and data collection; MK Designed the study and developed the models; RUK Data curation; AS, YA & FR; Editing and manuscript writing; MK Project design.

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