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Antibacterial Activity of Metallic Nanoparticles against Multidrug-Resistant Pathogens Isolated from Environmental Samples: Nanoparticles/Antibiotic Combination Therapy and Cytotoxicity Study

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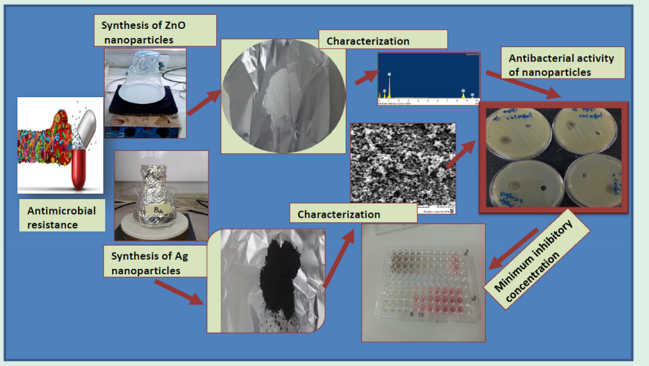
ABSTRACT: Multidrug-resistant organisms have increased the prevalence of infectious diseases and have become the leading source of death globally. The adverse effects associated with conventional antibiotics cannot be underestimated, and as a result, the quest for antibacterial agents has received great attention over the years. Therefore, the current research was designed to synthesize and examine the antibacterial properties of two metallic nanoparticles, silver nanoparticles (AgNPs) and zinc oxide nanoparticles (ZnONPs), as well as their antibiotic combination therapy against multidrug-resistant bacteria. AgNPs and ZnONPs were synthesized by the coprecipitation method and characterized. Thereafter, their antibacterial activity against multidrug-resistant bacteria was investigated using the microdilution technique. Subsequently, the interactions between the synthesized nanoparticles and antibiotics were evaluated by checkerboard assay. Time-kill assays were carried out to assess bacteriostatic or bactericidal effects, and the cytotoxicity study was carried out by MTT assay. The SEM analysis of AgNPs and ZnONPs were spherical with an average size of 21.03 and 43.37 nm, respectively. FTIR analysis showed the characteristics of the metal−oxygen vibrational band for both materials around 450 cm^{-1}, which indicated the successful synthesis of these antibacterial agents. The EDX characterization revealed Zn and O with 77.89% and 18.24% abundance in ZnONPs and Ag with 95.65% abundance in AgNPs. UV−vis absorption spectra of AgNPs was obtained around 400 nm. ZnONPs showed a moderate antibacterial effect against *Enterococcus* species with a MIC range of 2.5−5 mg/mL, while AgNPs demonstrated a strong antibacterial effect against the tested bacterial strains with a MIC range of 0.078−0.039 mg/mL. The ZnONPs were found to be cytotoxic against Vero cell lines at the tested concentrations, whereas AgNPs had no cytotoxic effect at lower concentrations. Their combination activities showed synergetic and additive effects. These findings revealed that these synthesized materials could serve as alternate antibacterial agents against multidrug-resistant *Acinetobacter baumanni* and *Enterococcus* species.



KEYWORDS: *rate of kill, synergistic effect, enterococci, Acinetobacter baumanni, Vero cell lines, drug resistance*

1. INTRODUCTION

Multidrug-resistant (MDR) organisms are becoming a growing public health concern and are making many healthcare-related infections difficult to treat with topical antibiotics.[1] Infections caused by these pathogens are a growing cause of disease and death worldwide.[2] The development of new drugs necessitates a significant financial and labor investment and considerable time.[3] High doses of antibiotics will be administered for these infections, which may result in intolerable toxic and adverse effects, which necessitates the development of alternative strategies.[2] The MDR *Enterococcus faecalis* and *Acinetobacter baumannii* are important healthcare-acquired pathogens.[4] *A. baumannii* causes diseases such as urinary tract infections, pneumonia, endocarditis, meningitis, dermal and soft tissue infections, and peritonitis.[4,5] Enterococci are responsible for infections in the bloodstream, urinary tract, and biliary tract and endocarditis in adults and meningitis in neonates.[6] The two bacteria are curative difficulties since the outgrowth of MDR strains has posed a significant threat to the lives of several patients in various nations globally.[4] *E. faecalis* is the most significant of the entire enterococci species in dentistry

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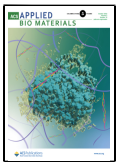

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because of its contribution to dental disorders, which comprise endodontic diseases, dental caries, and periodontitis.[7,8] Nevertheless, because of this organism's capability to exist and survive in harsh habitats and the emerging resistance regarding Enterococcus species, numerous studies have aimed at discovering viable alternative prevention strategies or extermination of *E. faecalis* from the root canal system.[7] Amid these approaches, nanoparticles of typical size in the range of 1−100 nm demonstrated promising results as new antibacterial agents. Their large surface-to-volume ratio surges the contacts between these nanocompounds and bacteria, thereby enhancing their inhibitory impacts.[9]

The use of nanoparticles (NPs) offers a potential strategy for managing multidrug organisms' infections[10] because of their unique physical and chemical properties, such as a large surface area, as well as their electronic and optical properties compared with their major components.[11] The large surface-to-volume ratio substantiated the distinctive qualities of nanoparticles (NPs) as a catalyst.[12] Antibacterial NPs can target multiple biomolecules and have the potential to reduce or eliminate MDRO evolution.[13] When compared with the action of antibiotics in clinical practice, the combination of antibiotics with nanoparticles is more effective in increasing antibiotic efficacy. The combination reduces bacterial resistance development, treatment duration, and antibiotic dose requirements.[14]

In recent studies, nanocomposites have been used in many areas to solve problems such as the green synthesis of Co_3O_4/ZnO nanocomposites for the removal of pollutants from water as reported by Hassanpour et al.[15] Likewise, the synthesis of SiO_2 showed a remarkable effect on the catalytic performance of the Nd_2O_3 photocatalyst for decomposition and magnetically retrievable $CoFe_2O_4$@SiO_2@$Dy_2Ce_2O_7$ nanocomposites as photocatalyst for highly efficient degradation of organic contaminants.[16,17]

ZnONPs have been one of the extremely utilized inorganic metal oxide nanoparticles.[18] This is due to their resistance to extreme conditions, widespread antibacterial qualities, and minimal toxic effect on humans.[18,19] In addition, ZnO has been listed as "Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA 21CFR182.8991)" because of its nonpoisonous nature.[20] Several studies have revealed that ZnONPs have promising biological potential, primarily as antibacterial agents[21] and also in preventing the growth of a broad range of disease-causing organisms,[22,23] which might substitute conventional antibiotics.

Silver is an intrinsic or raw antimicrobial agent.[24] Hence, AgNPs display enormous potential as a new "bactericidal at lower concentrations, showing an oligodynamic result via the presence of toxic metal ions."[25] In addition to their antibacterial characteristics, AgNPs are also described as antiangiogenesis,[26] "anti-inflammatory,"[27] antimalarial,[28,29] and antiplatelet.[30] Therefore, AgNPs are usually used for medical purposes.[31] The principal characteristic that makes them very efficient at fighting bacteria is the aptitude of silver to ionize in solution; they have a low-toxic effect on animal cells but are detrimentally harmful to microorganisms through interactions with bacteria cellular membranes, which impede noxious pathogenic microbes' propagation.[32] AgNPs are also efficient water disinfectants.[33] Basic water-disinfecting products, including free chlorine, could create damaging disinfection byproducts (DBPs), a number of which are well-known to be mutagens, teratogens, and carcinogens.[34] The bactericidal action of AgNPs is long-term and persistent but slow, which impedes pathogens that exist in safe water and prevents the production of dangerous DBPs.[35]

It is imperative to note that while there have been some reports on the antibacterial activity of AgNPs and ZnONPs against bacterial pathogens, few studies have been conducted to evaluate the time-dependent killing potential of these nanoparticles with antibiotics using the known CLSI standards. Furthermore, the effect of these nanoparticles on enhancing the activity of various antibiotics belonging to different antimicrobial classes, particularly antibiotics of last resort, is still being investigated. Therefore, the purpose of this study was to determine the antimicrobial activity of synthesized silver and zinc oxide nanoparticles alone and in combination with antibiotics against MDR Gram-positive and Gram-negative pathogens isolated from environmental sources and to evaluate their cytotoxicity effects.

2. MATERIALS AND METHODS

ZnONPs and Ag NPs were synthesized with procured chemicals with analytical grades including zinc nitrate hexahydrate (Sigma-Aldrich, purity 98%), silver nitrate (Sigma-Aldrich, purity 98%), trisodium citrate (Sigma-Aldrich, purity 98%), glucose (Sigma-Aldrich, purity 98%), and sodium hydroxide (Sigma-Aldrich, purity 98%).

2.1. Bacteria and DNA Extraction of Bacterial Strains. The glycerol stock of MDR *Enterococcus feacium*, *Enterococcus faecalis*, and *Acinetobacter baumanni* were obtained at −80 °C from the AEMREG (Applied and Environmental Research group) culture collection at the Department of Microbiology, Faculty of Science and Agriculture, University of Fort Hare, South Africa. MDR *Enterococcus feacium*, *Enterococcus faecalis*, and *Acinetobacter baumanni* isolates were inoculated into the nutrient broth and Luria Bethani broth depending on which organism was selected and placed in a shaker incubator at 37 °C for 24 h. Genomic DNA extraction was carried out using the boiling method described by Garrido-Maestu et al.[36]

2.2. Synthesis of Metallic Nanoparticles. *2.2.1. Synthesis of Silver Nanoparticles (AgNPs).* Silver nanoparticles were prepared using the chemical reduction method according to Wang et al.[37] with minor changes. Succinctly, silver nitrate solution (A) was prepared with the addition of 100 mM of $AgNO_3$ into 20 mL of sterile distilled water. Solution B, which contained 20 mM of $Na_3C_6H_5O_6{\cdot}2H_2O$ (trisodium citrate dihydrate), 20 mM of $C_6H_{12}O_6$ (glucose), and 20 mM of NaOH (sodium hydroxide), was prepared by dissolving these salts in 60 mL of distilled water under controlled heating. Thereafter, the mixture was heated up to 60 °C under vigorous shaking, and solution A was added to solution B dropwise and further heated for 6 h and stirred for 24 h. The reaction was conducted in the dark. The particles were separated with centrifugation for 10 min at 4400 rpm. Distilled water was used several times to wash the particles and remove any impurities.

2.3. Preparation of Zinc Oxide Nanoparticles (ZnONPs). The synthesis of ZnONPs was conducted using the coprecipitation technique, as reported by Wu et al.[38] The "alkali solution of zinc was prepared by dissolving 14.87 g of zinc nitrate $[Zn(NO_3)_2{\cdot}6H_2O]$ and 4 g of NaOH in deionized water to form a 100 mL solution (Zn^{2+} = 0.5 M, OH^- = 1.0 M). Subsequently, the NaOH solution was heated up to a certain temperature under continuous agitation, and the zinc nitrate solution was added in drops for 30 min. After a 2 h reaction, the white precipitate deposited at the bottom of the flask was collected and washed several times with absolute ethanol and distilled water. In the end, the ZnONPs samples were obtained by centrifugation and dehydration of the precipitate in a vacuum at 60−70 °C." The evenly mixed solution was calcined in a muffle furnace at 550 °C for 30 min.

2.4. Characterization of the Synthesized ZnONPs and AgNPs. Fourier transform infrared spectrophotometer analysis (PerkinElmer Universal ATR 100 FTIR spectrometer) was performed to investigate the major functional groups in the samples. This was done by placing a small amount of dried nanoparticle powder on the

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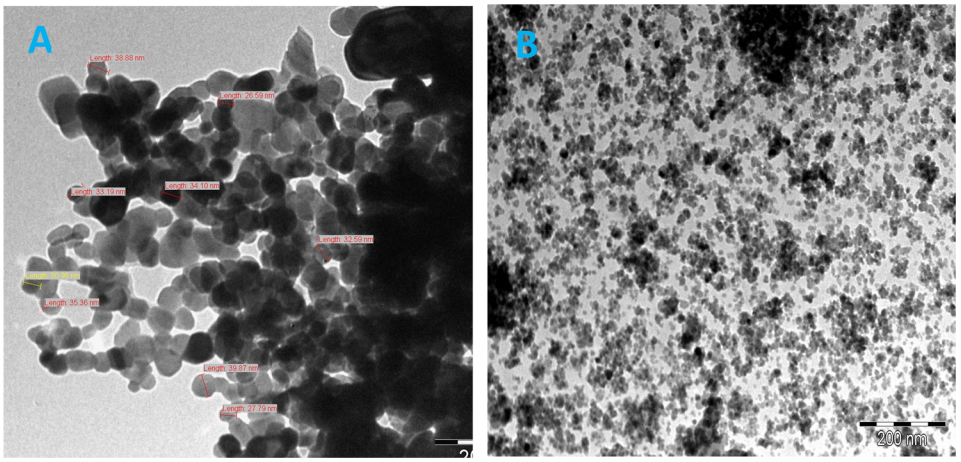

Figure 1. Transmission electron micrographs of (A) ZnONPs and (B) AgNPs.

sample holder of the instrument, and measurements were taken between 400 and 4000 cm^{-1}. A scanning electron microscope (SEM) coupled with an energy dispersive X-ray spectrometer (JEOL JSM-6390) was employed to study the morphological arrangement and identify the elemental compositions in the samples. SEM was carried out by placing a small amount of the samples on a stub previously decorated with a double-sided carbon tape. The size and morphology of the prepared nanoparticles were evaluated using a transmission electron microscope (TEM) (JEOL JEM 1210 l) by dissolving a small amount of the samples in DMSO and placing them on a sample grid for 12 h to dry while their specific absorption spectrum was measured by ultraviolet−visible (UV−vis) spectrophotometry (PerkinElmer).

2.5. Assessment of Antibiotic Susceptibility. Antibiotic susceptibility tests were performed against the studied pathogens (2.1) after the Kirby−Bauer disc diffusion protocol[39] by using the following antibiotic discs: gentamicin, ciprofloxacin, chloramphenicol, ceftazidime, piperacillin and tazobactam, amikacin, cefepime, trimethoprim/sulfamethoxazole, cefotaxime, meropenem, tetracycline, imipenem, ampicillin, norfloxacin, nitrofurantoin, vancomycin, levofloxacin, rifampicin, linezolid, and erythromycin. The distances of the inhibition zones were established, and the results were recorded as resistant (R), intermediate (I), or susceptible (S), according to CLSI.[39]

2.6. Nanoparticle's Formulation and Determination of the Efficacy of Nanoparticles Using Agar Well Diffusion Method. The stock solution of the NPs was prepared by adding 100 mg of ZnONPs and AgNPs into 10 mL of distilled water, followed by vigorous shaking for 5 min to ensure the complete dispersion of the NPs; to obtain a homogeneous solution, this solution was sterilized by autoclaving at 121 °C for 15 min and then left at room temperature. The final concentration was 10 mg/mL.[40] The agar well diffusion was conducted as described by Valgas et al.[41]

2.7. Determination of MIC of ZnONPs and AgNPs. The MIC was established by using the broth microdilution technique as stated in the CLSI[39] using 96-well microtiter plates. AgNPs and ZnONPs were prepared and serially diluted 2-fold between 10 mg/mL to 0.0195 mg/mL with 10^6 standardized microbial concentration cfu/mL of 0.5 McFarland's standard. Briefly, "100 μL of the synthesized AgNPs and ZnONPs stock solution (10 mg/mL) was transferred and diluted 2-fold with the bacterial inoculums in 100 μL of Muller Hinton Broth starting from column 1 to 10. The microtiter plate column 1 contains the maximum concentration of NPs, while column 10 contains the minimum concentration. Column 11 was used as negative control (containing medium only), and column 12 served as a positive control (bacterial inoculums" coupled with ciprofloxacin) and incubated at 37 °C for 24 h. Resazurin (0.015% w/v) was used as an indicator. Thirty microliters (30 μL) of the resazurin solution was mixed into each well of the microtiter plate after incubation and further incubated for 2−4 h. The assay was conducted in triplicate, and the lowest concentration that inhibits visible color change was recorded as the MIC.

2.8. MIC of Antibiotics. The MIC of the antibiotics was determined by employing five different antibiotics (vancomycin, ciprofloxacin, ceftazidime, meropenem, and ampicillin). This assay was done by using the microdilution method according to CLSI[39] while ATCC 29213 (*Staphylococcus aureus*) served as the control.

2.9. Synergy Testing. Antibiotic interaction in pairs was carried out using a checkerboard assay according to Petersen et al.[42] The stock solutions of vancomycin and ampicillin varied between 0.005 and 0.512 mg/mL and 0.0003 to 0.032 mg/mL, accordingly. The Ag and ZnO nanoparticles stock solutions that were used varied between 0.0024 and 0.156 mg/mL and 0.009 to 10 mg/mL, respectively. Analysis was done with 2-fold dilutions of both the drug and the nanoparticles. A total of 50 μL of the drug was sequentially diluted along its horizontal axis, as the dilution of the nanoparticle was conducted vertically. An inoculum of 5×10^5 cfu/mL was prepared in Mueller Hinton broth, of which 100 μL was mixed with each well. The plate incubation was carried out at 37 °C for 24 h, and the test was done twice per isolate. "The ∑FICs (fractional inhibitory concentrations) were calculated" using eq 1:

$$\sum \text{FIC} = \text{FIC A} + \text{FIC B} \quad (1)$$

where "FIC A is the MIC of drug A (antibiotic) in the combination/MIC of drug A" (antibiotic) "alone, and FIC B is the MIC of drug B (nanoparticle) in the combination/MIC of drug B [...] (nanoparticle) alone. The combination is considered synergistic when the ∑FIC is ≤0.5, [...] indifferent when the ∑FIC is >0.5 and <2, and antagonistic when the ∑FIC is ≥2."[43]

2.10. Time-Dependent Killing Assay. The time−kill assay was conducted using the broth macro dilution procedure by Pankey et al.[44] Vancomycin and ampicillin were used in the time-dependent killing assay. The concentration values for nanoparticles and antibiotics were 1/2 MIC, MIC, and 2 MIC. Summarily, tubes having LB with antibiotics and nanoparticles were inoculated with 5×10^5 cfu/mL of *A. baumannii* and *Enterococcus* species and incubated at 35 °C. Serial 10-fold dilutions were carried out at the time 0, 2, 4, 6, 8, and 24 h after inoculation, and 100 μL aliquots from each dilution factor were plated onto Muller Hinton Agar (MHA). The experiment was carried out in triplicate. The findings were evaluated using average colony count values from the triplicate plates per isolate (Rodriguez et al., 2019).[45] The bactericidal effect of drugs alone or the combination has been identified as the ≥3 log 10 cfu/mL reduction in feasible count related to the initial inoculum. "Synergism and antagonism were respectively defined as ≥2 log 10 cfu/mL reduction or increase in the viable count with the combination compared with the most active agent alone at different time points."[46]

2.11. Cytotoxicity Study. The toxic effect of nanoparticles was evaluated on the monkey kidney cell line (Vero cell lines), which were

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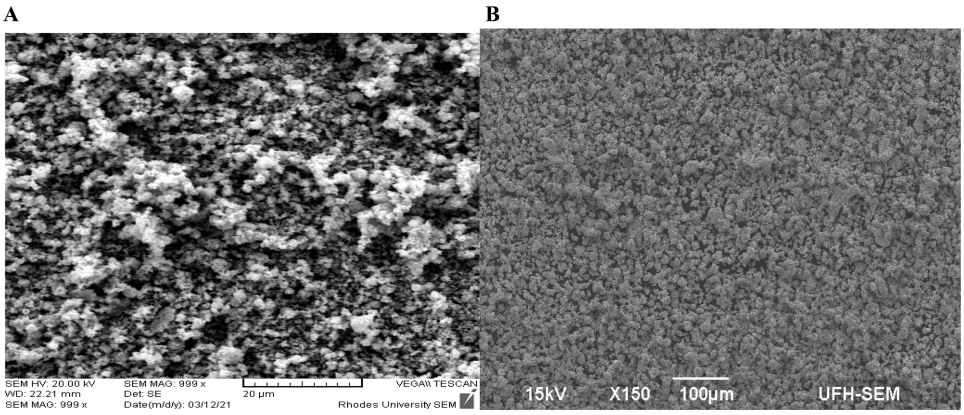

Figure 2. SEM micrograph of (A) ZnONPs and (B) AgNPs.

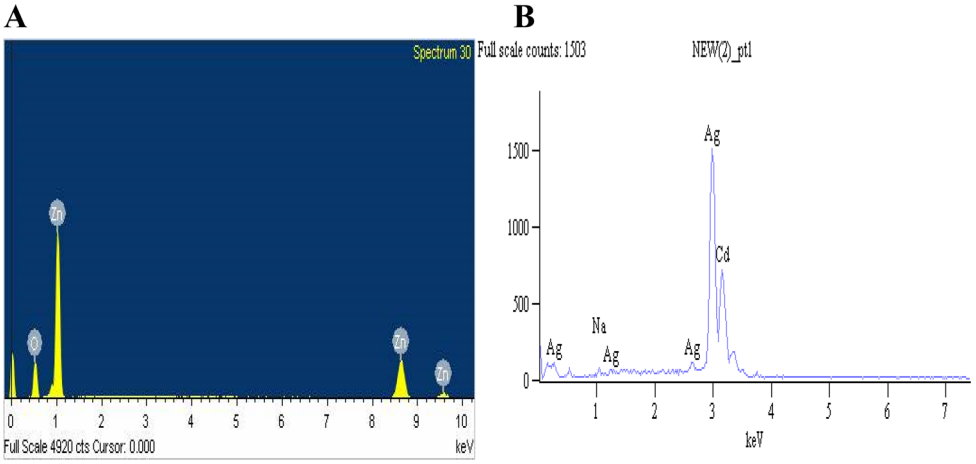

Figure 3. Energy dispersive X-ray spectra of (A) ZnONPs and (B) AgNPs.

procured from Highveld Biological, Johannesburg, South Africa. The 3,4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was carried out to evaluate the cell survival rate of the cells. The seeding of the Vero cells was achieved at a density of 5000 cells/100 μL/well and incubated overnight at 37 °C for attachment. A cytotoxicity assay treatment for the nanoparticles was done by adding an additional 100 μL of samples to achieve definitive concentrations of 0.02, 0.04, 0.08, 0.16, and 0.32 mg/mL for silver nanoparticles and 0.625, 1.25, 2.5, 5, and 10 mg/mL for zinc oxide nanoparticles. The cells were incubated at 37 °C for 48 h. Melphalan at 12.5, 25, 50, 100, and 200 μM served as the positive control. After 48 h, the treatment medium was removed. MTT was diluted in DMEM low glucose cell culture medium/FBS to the last concentration (0.5 mg/mL) and added to the wells using 100 μL aliquots. The cells were made to undergo incubation at 37 °C for 2 h, after which, the medium was aspirated and washed twice with PBS. Dissolution of the purple insoluble formazan product was followed with the addition of 100 μL of DMSO. The absorbance was measured with a spectrophotometer at 540 nm. The amount of product generated is proportionate to the number of living cells.[47]

2.12. Statistical Analysis. In this study, data are presented as mean ± standard deviation. The analyses were performed using Microsoft Excel, Origin Pro 7, and ImageJ software. Assessments between and within multiple groups were made using one-way analysis of variance (ANOVA) using SPSS 21.0 statistical software. The threshold for statistical importance was set at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of Nanoparticles. In this study, two different metallic nanoparticles comprising AgNPs and ZnONPs were synthesized. These metallic nanoparticles were characterized using a range of techniques to ascertain their successful synthesis.

3.1.1. TEM Analysis. A transmission electron microscope micrograph helps to appraise the size and shape of a material. The TEM image of the synthesized ZnONPs is presented in Figure 1. The result revealed that the ZnONPs showed some agglomeration and aggregated; the particulates were found to have an irregular spherical shape, joined together by overlapping. Conversely, the TEM image of AgNPs shows that the particles are spherically shaped and well dispersed with little or no agglomeration. The particle size of the nanoparticles was assessed by ImageJ software. ZnONPs were found to have a mean particle size of 43.37 ± 11.52 nm, while the size of AgNPs was 21.03 ± 5.10 nm.

3.1.2. SEM Analysis. SEM analysis was carried out to examine the morphology of the prepared nanoparticles. The current investigation showed that the ZnONPs were spherical and agglomerated (Figure 2A). At the same time, the AgNPs had a spherical shape with a low-density dispersion, as displayed in Figure 2B. These results from the SEM micrographs for these metallic nanoparticles complement the result obtained from the TEM images for the materials (Figure 1). Similar results have been published for metallic nanoparticles by Ojemaye and Okoh.[48]

3.1.3. EDX Analysis. Figure 3 shows the results from the EDX analysis of the synthesized materials. The prominent atoms present were Zn and O with 77.89% and 18.24%

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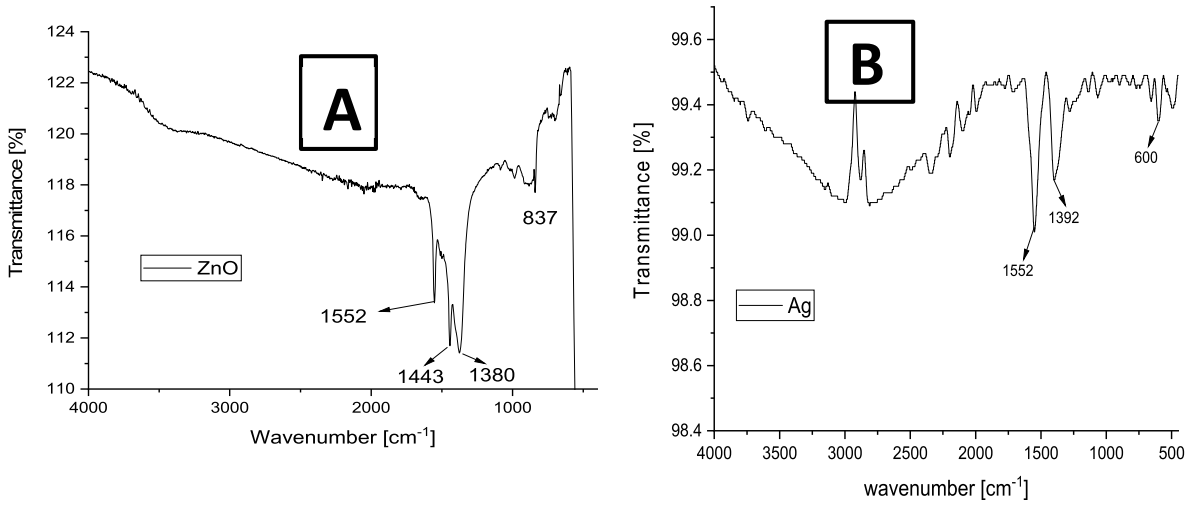

Figure 4. Fourier transform infrared (FTIR) spectra of (A) ZnONPs and (B) AgNPs.

abundance, respectively, in Figure 3A. The abundance of Ag was 95.65% in Figure 3B. The presence of these elements in these spectra is a signal of the purity of the synthesized nanoparticles. Wang et al.[49] reported that EDX can be used to determine the purity of nanomaterials.

3.1.4. FTIR Analysis. FTIR spectroscopy is a method used for characterization: it helps to detect functional groups in compounds. The result of the FTIR analysis is shown in Figure 4; the spectra were observed between 4000−400 cm^{-1}. The peaks corresponding to Zn were obtained at 480 and 837 cm^{-1}. The broad band is an indication that the Zn^{2+} may be present as ZnO. The peaks at 1552, 1443, and 1380 cm^{-1} are assigned to N═O stretching, C−H bending, and O−H bending, respectively (Figure 4A). FTIR indicates that the zinc oxide nanoparticles were very pure. Likewise, the FTIR spectrum of the synthesized AgNPs show prominent peaks at 475 and 600 cm^{-1}, corresponding to the vibration frequency of the Ag−O band (Figure 4B). These peaks are characteristic of all metallic nanoparticles.[50] The peaks at 1392 and 1552 cm^{-1} correspond to the C−H bending of aldehyde and N−O stretching, respectively. In a preceding research study, the FTIR bands at 3405, 2100, 1788, 1635, 1048, and 832 cm^{-1} showed the vibrational frequencies of typical AgNPs.[51] These reported vibrational bands were also observed in the FTIR spectra of these materials in this study.

3.1.5. UV−vis Spectroscopy. The main method and easiest approach to verify the formation of these nanoparticles is through UV−vis spectroscopy. Absorption spectra of the sample were obtained between 200−800 nm by using the UV−vis spectrometer with purified water as the blank. A strong UV−visible spectra broad peak was detected at 400 nm (Figure 5). Sastry et al.[52] reported that "AgNPs are known to exhibit a UV−visible absorption maximum in the range of 400−500 nm because of surface plasmon resonance."

3.2. Antibacterial Efficiency of AgNPs and ZnONPs against MDR *Enterococcus feacium*, *Enterococcus faecalis*, and *Acinetobacter baumanni* Using Agar Well Diffusion. The antibacterial effectiveness of the characterized nanoparticles was assessed against studied pathogens at different concentrations (Figure 6). Following the results generated, it was discovered that all the strains were susceptible to AgNPs with the inhibition zone between 14 mm to 17 mm, as shown in Figure 6, which revealed that the synthesized

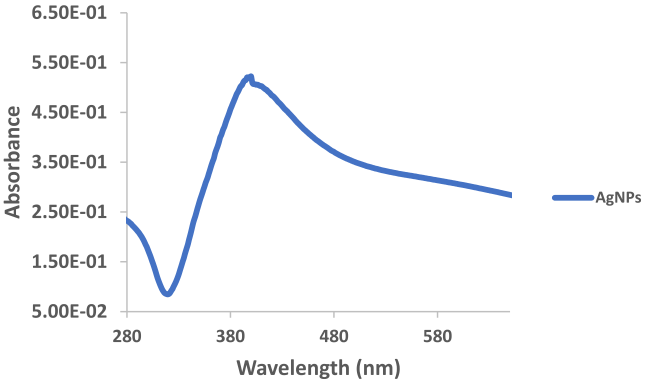

Figure 5. UV−visible absorption spectrum of silver nanoparticles.

AgNPs have high antibacterial activity. Oei et al.[53] reported silver nanoparticles as the most common antimicrobial substances used to prevent and treat various diseases because of their solid biocidal effect. All the isolates tested against ZnONPs did not show any inhibition zone. This is per a report by Singh et al.[54] that zinc oxide nanoparticle suspension prepared with simple laboratory equipment and vortexing was not the best method of forming the suspension since it does not guarantee consistent interference of nanopowder aggregates. Likewise, it was said that a better zinc nanoparticle suspension was to be obtained by using a sonicator to allow even dispersion of the zinc oxide nanopowder in the distilled water.[54]

3.3. Antibiotic Sensitivity Testing. The antibiotic sensitivity test was performed against two of the studied pathogens (*Acinetobacter baumannii* and *Enterococcus* species). Overall, we observed that all the studied pathogens exhibited resistance to more than three different classes of antibiotics, which indicated that they are MDR organisms, as shown in Table 1. The isolated "bacteria were characterized as MDR according to the definition of MDR as the resistance of bacteria to at least one agent in three or more antibiotics classes."[55] This agrees with a recent report by Adeniji et al.[56]

Acinetobacter baumannii isolates were resistant to the 10 antibiotics tested, including tazobactam, piperacillin, amikacin, meropenem, ceftazidime, cefepime, cefotaxime, gentamicin, tetracycline, trimethoprim−sulfamethoxazole (SXT), and ciprofloxacin. This is in agreement with the results of Kareem

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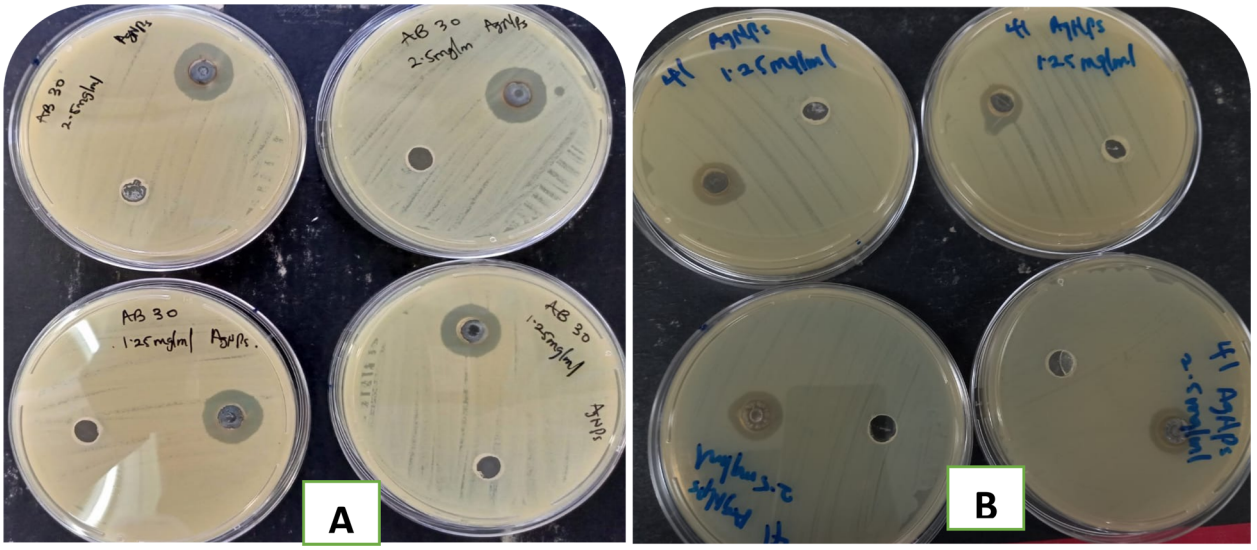

Figure 6. Antibacterial activity of AgNPs against (A) *Acinetobacter baumanni* and (B) *Enterococcus* species.

Table 1. Antibiotic Sensitivity Testing of *E. faecalis, A. baumannii, and E. faecium*[a]

antimicrobial class	antimicrobial agents	disc content μg	*Acinetobacter baumanni* (30)	*Acinetobacter baumanni* (44)	*Enterococcus feacium* (26)	*Enterococcus feacium* (44)	*Enterococcus faecalis* (41)	*Enterococcus faecalis* (35)
β-lactam/β-lactamase inhibitor combination	piperacillin tozobactam	110	R	R	NE	NE	ND	ND
cephems	ceftazidime	30	R	R	NE	NE	ND	ND
	cefotaxime	30	R	R	NE	NE	ND	ND
	cefepime	30	R	R	NE	NE	ND	ND
carbapenems	imipenem	10	S	R	NE	NE	ND	ND
	meropenem	10	R	R	NE	NE	ND	ND
aminoglycosides	amikacin	30	R	R	NE	NE	ND	ND
	gentamicin	10	R	R	R	R	S	S
lipopeptide	Polymyxin B	300	S	S	NE	NE	ND	ND
tetracyclines	tetracycline	30	R	R	R	R	R	R
quinolones	ciprofloxacin	5	R	R	R	R	R	S
	levofloxacin	5	NE	NE	R	R	R	S
	norfloxacin	10	NE	NE	R	R	R	I
foliate-pathway inhibitor	trimethoprim-sulfamethoxazole (SXT)	25	R	R	NE	NE	ND	ND
penicillin	ampicillin	10	NE	NE	S	R	R	R
glycopeptide	vancomycin	30	NE	NE	R	R	R	R
macrolides	erythromycin	15	NE	NE	R	R	R	R
phenicol	chloramphenicol	30	NE	NE	S	S	S	R
nitrofurantoin	nitrofurantoin	300	NE	NE	S	R	R	R
oxazolidinones	linezolid	30	NE	NE	R	R	R	R
ansamycins	rifampicin	5	NE	NE	R	R	R	R

[a]NE = not evaluated; S = susceptible; I = intermediate.

et al.[57] Nevertheless, all were sensitive to Polymyxin B. The *Enterococcus* species showed resistance to nine antibiotics belonging to different classes, including vancomycin, the last resort glycopeptide, and linezolid and oxazolidinones (Table 1). Adeniji et al.[56] reported that "*Enterococcus* species are among pathogens known to be intrinsically resistant to a wide range of antibiotics such as beta-lactams, aminoglycosides, and ampicillin." It is also good to know that "the unrestrained use of antibiotics in the treatment of animals and their integration in animal feed has also been presumed to account considerably for the upsurge in antimicrobial resistance in pathogenic bacterial isolates."[58]

3.4. MIC Determination of Zinc Oxide and Silver Nanoparticles. The antimicrobial effect of the nanoparticles was evaluated up to a concentration of 10 mg/mL of NPs, which is nearly twice the level of toxicity in normal cells. For therapeutic concern as a bactericidal for synthesized nanoparticles, activity acquired at 5 mg/ml or lower only were considered according to Punjabi et al.[59] In this research, the use of both AgNPs and ZnONPs as an antibacterial agent was evaluated against selected Gram-positive and Gram-negative microbes. The results of the MIC of both NPs against MDR infectious agents are shown in Table 2. The excellent lowest concentration of chemically synthesized AgNPs that inhibited

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Table 2. Antimicrobial Implications of the Examined Metallic Nanoparticles Minimum Inhibitory Concentration (MIC) against Studied Bacteria

organisms	AgNPs (mg/mL)	ZnONPs (mg/mL)
MDR *Acinetobacter baumanni* (30)	0.039	>10
MDR *A. baumanni* (44)	0.039	>10
MDR *Enterococcus feacium* (26)	0.078	5
MDR *Enterococcus feacium* (44)	0.039	>10
MDR *Enterococcus faecalis* (35)	0.078	2.5
MDR *Enterococcus faecalis* (41)	0.039	>10

bacteria growth was 0.039 mg/mL for MDR-*A. baumannii*, 0.078 mg/mL for *E. faecalis* and *E. faecium*, respectively. The MIC value for ZnONPs was ≥10 mg/mL for MDR-*A. baumannii*, 2.5 mg/mL for *E. faecalis* and 5 mg/mLfor *E. faecium*. The MICs of AgNPs against both Gram-negative and Gram-positive organisms were significantly lower than the ZnONPs. Gurunathan et al.[60] reported that the small-sized AgNPs with large surface areas are more effective than the larger ones. The antimicrobial effectiveness of AgNPs has been reported by many scholars.[25,28] Nevertheless, the values obtained for the MIC in prior studies demonstrated a large degree of variance. For this reason, a comparison of outcomes is hard because there is no reference spectrometry of the antibacterial activities of AgNPs, and various approaches have been implemented by the researchers.[61] In the current study, AgNPs annex a good bactericidal effect against the examined multidrug-resistant microbes compared with ZnONPs. This was not in agreement with Kareem et al.,[57] whose findings showed that the efficiency of ZnONPs against multidrug-resistant bacteria is higher compared with that of AgNPs. Mohammad et al.[62] reported that ZnONPs were effective against *Klebsiella pneumonia*, *Listeria monocytogenes*, and *Salmonella enteritidis* pathogens. The chemically synthesized silver nanoparticles have proven to inhibit the studied pathogenic organisms used for the study, though zinc oxide nanoparticles only inhibited Gram-positive organisms.

3.5. MIC of Antibiotics. The MICs of the selected antibiotics used in treating the studied pathogens were carried out. Table 3 displays the result of the MIC for each test strain. The results showed that tested bacteria were resistant to vancomycin, ampicillin, ciprofloxacin, and ceftazidime according to CLSI[39] guidelines. However, chemically synthesized AgNPs showed much higher antibacterial activity than ciprofloxacin, vancomycin (≤0.256 mg/mL), and ceftazidime (0.512 mg/mL) against *Enterococcus* species and *Acinetobacter baumanni*, respectively.

3.6. Checkerboard Assay. For the verification of the synergy of prepared nanoparticles with selected antibiotics against test organisms, checkerboard testing was conducted. Two isolates of *A. baumannii*, *E. faecalis*, and *E. faecium* were chosen for the checkerboard assay. The study revealed that both the nano-AgNPs and nano-ZnO particles, in combination with commercial antibiotics such as meropenem, ciprofloxacin, and ceftazidime, had an additive indifference but not synergistic effect against *A. baumannii* (FIC ≤ 2). At the same time, *Enterococcus* species isolates, in combination with vancomycin and ampicillin antibiotics, showed synergistic effects (FIC ≤ 0.5) (Table 4). Different authors have shown different effects of silver nanoparticles combined with antibiotics from synergistic to antagonistic.[57,59] Furthermore, in the current study, the synergistic and nonsynergistic impacts of the blend of chemically synthesized silver nanoparticles with antibiotics were evaluated. Remarkably, the synergistic interaction was detected for ampicillin and vancomycin against MDR *E. feacium* and MDR *E. faecalis*. The FIC value and explanation of drug interactions are as shown in Table 4.

Table 4. Effect of the Synthesized Nanomaterials Blended with Different Antibiotics on the Studied Pathogens

pathogens Gram-positive	nanoparticles with antibiotics(mg/mL)	FIC types of interaction
MDR *E. feacium* (44)	AgNPs + vancomycin	0.7 additive
	AgNPs + ampicillin	2.3 indifference
MDR *E. feacium* (35)	AgNPs + vancomycin	0.36 synergistic
	AgNPs + ampicillin	0.28 synergistic
	ZnONPs + vancomycin	0.38 synergistic
	ZnONPs + ampicillin	0.28 synergistic
MDR *E. faecalis* (41)	AgNPS + vancomycin	0.7 additive
	AgNPs + ampicillin	1.45 indifference
MDR *E. faecalis* (26)	ZnONPs + vancomycin	0.5 synergistic
	ZnONPs + ampicillin	1 additive
	AgNPs + vancomycin	0.36 synergistic
Gram-negative	AgNPs + ampicillin	0.75 additive
MDR *Acinetobacter baumanni* (30)	AgNPs + ciprofloxacin	1 additive
	AgNPS + ceftazidime	1.5 indifference
	AgNPS + meropenem	2 indifference
MDR *Acinetobacter baumanni* (44)	AgNPS + ciprofloxacin	0.98 additive
	AgNPS + ceftazidime	2 indifference
	AgNPS + meropenem	1 additive

$$\text{FIC} = (\text{MIC of AgNPs in combination with antibiotic})/(\text{MIC AgNPs alone}) + (\text{MIC of antibiotic in combination with AgNPs})/(\text{MIC of antibiotic alone})$$

Nanoparticles are regarded as a worthwhile alternative to drugs and are revealed to have great capability to combat the muddle of the increase of MDR bacteria.[63] In this research,

Table 3. MIC Results for the Selected Conventional Antibiotics[a]

pathogens	vancomycin (mg/mL)	ampicillin (mg/mL)	ciprofloxacin (mg/mL)	meropenem (mg/mL)	ceftazidime (mg/mL)
MDR *Acinetobacter baumanni* (30)	ND	ND	0.256	0.001	0.128
MDR *A. baumanni* (44)	ND	ND	0.128	0.008	0.512
MDR *Enterococcus feacium* (26)	0.128	0.008	NE	NE	NE
MDR *Enterococcus feacium* (44)	0.128	0.016	NE	NE	NE
MDR *Enterococcus faecalis* (35)	0.256	0.016	NE	NE	NE
MDR *Enterococcus faecalis* (41)	0.256	0.008	NE	NE	NE

[a]NE: not evaluated.

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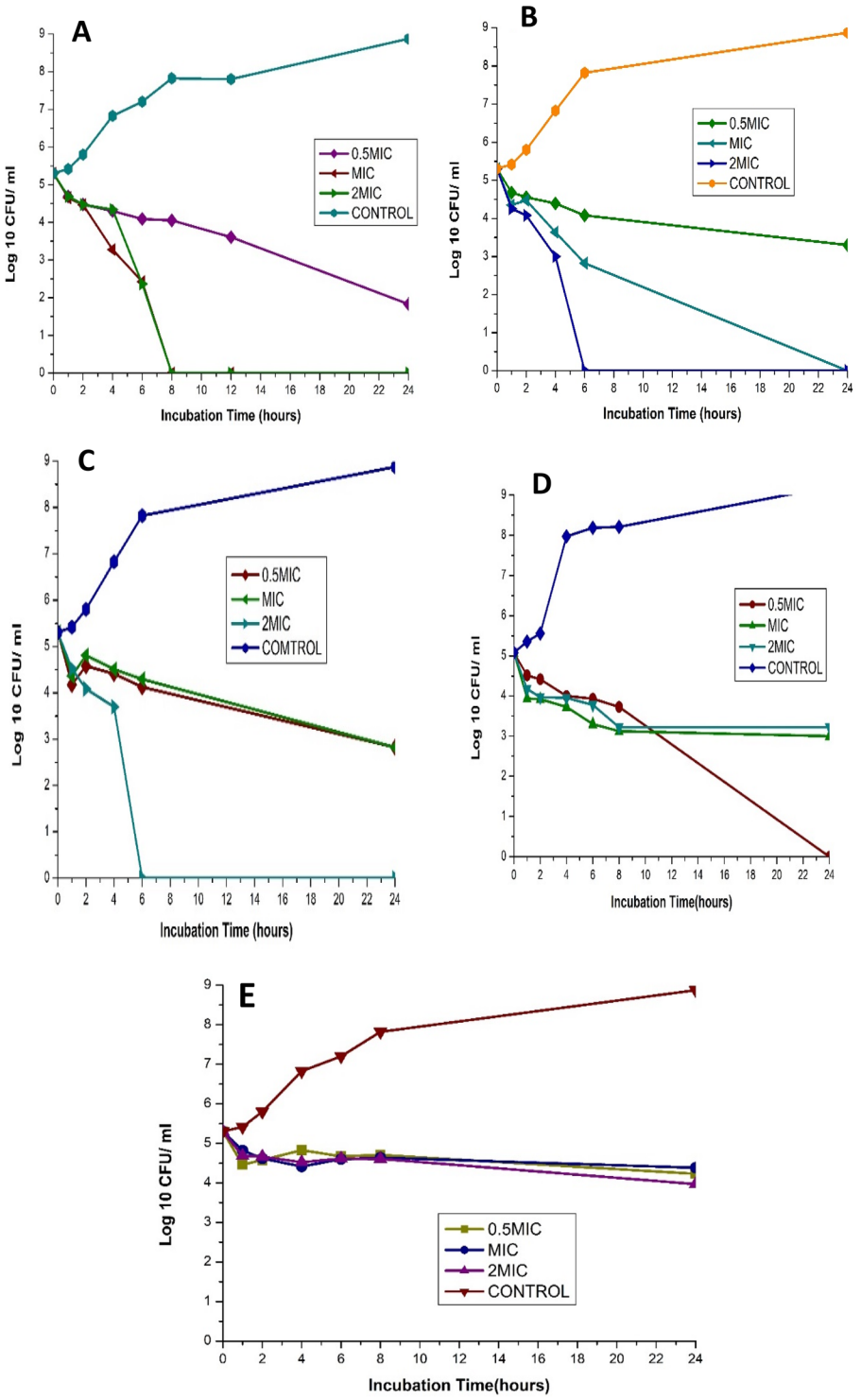

Figure 7. Plots for (A) time−kill assay of silver nanoparticles (0.078 mg/mL) with vancomycin (0.512 mg/mL) against MDR *Enterococcus faecalis*, (B) time−kill assay of silver nanoparticles (0.078 mg/mL) with vancomycin (0.128 mg/mL) against MDR *Enterococcus feacium*, (C) time−kill assay of silver nanoparticles (0.078 mg/mL) with ampicillin (0.008 mg/mL) against MDR *Enterococcus feacium*, (D) time−kill assay of zinc oxide nanoparticles (5 mg/mL) with vancomycin (0.256 mg/mL) against MDR *Enterococcus feacium*, and (E) time−kill assay of zinc oxide nanoparticles (2.5 mg/mL) with vancomycin (0.256 mg/mL) against MDR *Enterococcus faecalis*.

chemically synthesized AgNPs and ZnONPs revealed antimicrobial activity against some of the studied pathogens. It is known that the antimicrobial efficacy of metal nanoparticles is dependent upon their shape and size.[64] Smaller NPs present a greater level of harmfulness to bacterial pathogens because these nanoparticles perhaps disperse more quickly compared with the bigger ones.[65] In addition, Ebrahimi et al.[66] also confirmed synergy between AgNPs and vancomycin using a microdilution assay. Positive results were evaluated for the synergistic activity of ZnONPs with vancomycin and ampicillin in the case of MDR *Enterococcus faecium* and *E. faecalis*. In large part, the results of the antibiotic−AgNPs combination studies

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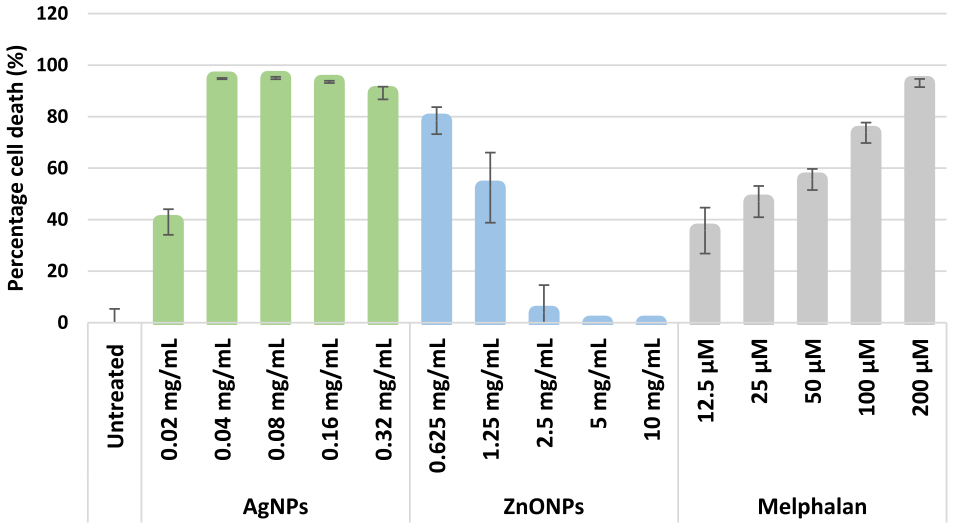

Figure 8. Percentage cell death results for samples and positive control.

showed that AgNPs could boost the antimicrobial activity of many antibiotics.

3.7. Time-Dependent Killing. The time-dependent killing assay is determined to elucidate bacterial growth and death and to observe the bactericidal effects with time.[67] All synergistic interaction results obtained from checkerboard determination were re-examined using a time−kill kinetic assay, as shown in Table 4. The time−kill diagrams for effective blends are shown in Figure 7. None of the additive and indifferent interactions obtained from the checkerboard assay were established by the time−kill assays. As stated by Scheetz et al.: "The bactericidal activity was defined as being equal to 3log10 cfu/mL or greater reduction in the viable colony count relative to the initial bacteria inoculum."[68]

The bactericidal effect of AgNPs combined with vancomycin is effective against vancomycin-resistant *E. faecalis* and *E. faecium* microbes. The decrease in the cfu/mL number was ≥3 Log units (99%). The biocidal end point of silver nanoparticles combined with vancomycin for MDR *E. faecalis* achieved between 4 and 6 h of incubation at 1 MIC (0.078 mg/mL; 0.256 mg/mL) and 8 h at 2 MIC (0.156 mg/mL; 0.512 mg/mL) (Figure 7a), while for *E. faecium*, the bacteria were eliminated after being incubated for 6 h at 2 MIC (0.156 mg/mL; 0.256 mg/mL) (Figure 7b). Also, the biocidal effect of AgNPs blended with ampicillin against MDR *E. faecium* (ampicillin-resistant) is effective after 4 h of incubation at 1 MIC (0.078 mg/mL; 0.008 mg/mL) and 2 MIC (0.156 mg/mL; 0.016 mg/mL) (Figure 7c). Hemeg[69] documented that the effectiveness of drugs in combination with nanoparticles was indistinguishable in both Gram-positive and Gram-negative bacteria, unlike the complexity of killing MDROs with drugs alone.

The bactericidal activity of ZnONPs blended with vancomycin antibiotic was appraised against MDR *E. faecalis* and *E. feacium* organisms. Figure 7d shows that 1 MIC and 2 MIC combination of ZnONPs with vancomycin achieved bactericidal effects against MDR *E. feacium* at 4 h by reducing the bacterial count >3 log 10 cfu/mL compared with the initial inoculum, while *E. faecalis* showed a bactericidal effect at 6 h for 0.5 MIC, 1 MIC, and 2 MIC (Figure 7e). This result agrees with the study of Jasim et al.[70] where they reported that some ZnONPs were effective against vancomycin-resistant enterococci (VRE) and *S. aureus*. In addition, Baëtz et al.[71] documented promising results of zinc oxide NPs on enterococci used in combination with vancomycin conventional antibiotics, which is similar to our findings. It has been discovered that zinc oxide nanoparticles with insignificant particle size demonstrated improved efficiency of biological effect because of the increase in the specific surface area to volume ratio and surface reaction of zinc oxide NPs.[72]

3.8. Toxicity of Nanoparticles. Cytotoxicity was estimated from 0.02 to 10 mg/mL of NPs. The proportion of cytotoxicity is drawn against the level of nanoparticles, as shown in Figure 8. Untreated cells were used as a negative control. The toxic effect of a compound is evaluated by specifying the IC_{50} value, that is, the amount in which cells' growth is inhibited by 50%. AgNPs were shown to cause cell death at concentrations ≥ 0.04 mg/mL. On the basis of the toxicity of nanoparticles to the Vero cell line, the IC_{50} values applicable to silver were found to be 0.02 mg/mL. Devi and Bhimba[73] reported less cytotoxicity of synthesized AgNPs against normal Vero cell line with IC_{50} = 95 µg/mL, while ZnONPs caused cell death at all concentrations tested (absorbance values were affected because of the precipitate of nanoparticles, which is why the dose−response curve shows the opposite trend to what would be expected; the data point for the lowest level of 0.625 mg/mL is the only one that is reliable). These results show high cytotoxicity values, similar to that reported by Brunner et al.[74] for ZnONPs prepared chemically using the same cell line, lower concentrations, and similar particle size. However, other authors reported significantly lower cytotoxicity using similar mergers and cell lines. ZnONPs cytotoxicity is still questionable, and the mechanism has not been well detected.[75]

4. DISCUSSION

Antibiotic resistance in Gram-negative organisms has turned out to be threatening because of the rapid growth of drug resistance to even a higher standard of drugs, such as third-generation cephalosporins and carbapenems.[76] Amidst the Gram-positive bacteria, *Enterococcus* spp. have been ever more reported.[56] The usage of nanotechnology in human health research has brought about an enormous development in drug and gene delivery and diagnostics.[77] The current study

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evaluated the chemical synthesis of ZnONPs and AgNPs. EDX and FTIR analysis confirmed the successful synthesis of these nanoparticles (Figures 3 and 4). The peak corresponding to Zn was obtained at 837 cm^{-1} in the EDX spectra of ZnONPs, while silver showed prominent peaks at 600 cm^{-1}, equivalent to the vibrational frequency of the Ag−O ionic bond. TEM and SEM analysis identified spherical shapes for both ZnONPs and AgNPs with a mean particle size of 43.34 and 21.03 nm, respectively (Figures 1 and 2). Previous research carried out by Agnihotri et al.[78] established that antibacterial effectiveness was increased for the silver nanoparticles of less than 10 nm in size. They also found that silver nanoparticles with the size of 5 nm have the most rapid bactericidal effect in comparison with other sizes of silver nanoparticles.

MIC values for the chemically synthesized nanoparticles ranged from 0.078−0.039 mg/mL against studied Gram-positive and Gram-negative MDR organisms for AgNPs, while ZnONPs ranged between 2.5−5 mg/mL against Gram-positive strains. This is an indication that both nanoparticles are more sensitive to the groups of bacteria strains tested and that both inhibit them at a low concentration (0.078- 5 mg/mL). ZnONPs have no effect on *Acinetobacter baumanni* from our findings, which negates the study by Jaafar et al.[79] stating that ZnONPs were lethal both for the Gram-negative and Gram-positive organisms. The antibacterial implications of silver are generally attributed to the silver ions.[24] The larger surface area of AgNPs makes them demonstrate a better and stronger bactericidal activity, as described by Rudramurthy et al.[9] It was documented that AgNPs exhibit bactericidal effect by impeding the structural integrity of the bacterial cell by attaching to the essential cellular structure and predominantly to their Sulfhydryl Groups in Activating Enzymes (SH-groups).[80] The bactericidal effects of zinc oxide NPs were equally linked to the nanoparticles nanosizes and large surface area to volume ratio, which makes their interaction with the cell membranes of microorganisms direct, in addition to metal ions that exit from the cell in solution.[9]

AgNPs and ZnONPs that were combined with ciprofloxacin, vancomycin, ampicillin, ceftazidime, and meropenem were investigated in relation to FICs on the basis of the time−kill dependent analysis and checkerboard test. The combination of AgNPs with vancomycin and ampicillin for the Gram-positive bacteria showed a more rapid killing, and no antagonism was detected about any of the studied isolates in the time−kill dependent assay or checkerboard tests. The combination of silver nanoparticles with vancomycin and ampicillin might be beneficial because both AgNPs and ZnONPs act as bactericidal on both vancomycin and ampicillin-resistant strains. This is in agreement with the report of Mohammed et al.[81] It was also established by Esmaeillou et al.[82] that the vancomycin-bound AgNPs had improved bactericidal effect against Gram-positive organisms, but not Gram-negative bacteria compared with pure vancomycin.

However, ZnONPs exhibited narrow-spectrum antibacterial activities against the Gram-positive organisms tested in the present study. This contradicts the report of Sharma et al.[83] who stated that the MIC of ZnONPs against test strains has an insignificant effect on the growth of Gram-positive bacteria compared with the Gram-negative bacteria because of the structural disparity in cell wall composition. Orooji et al.[84] and Karami et al.[85] have shown that metallic oxides work in a consistent manner by enhancing the antibacterial properties of both Gram-positive and Gram-negative pathogens.

The toxicity of the materials is the maximum vital parameter determining its therapeutic feasibility.[59] The cell survival rate assay is a significant method for toxicology study that explains the cellular response to a lethal substance. This can offer evidence on cell death, survival, or metabolic activities.[86] Thus, in vitro cellular toxicity of the two nanoparticles prepared were evaluated on a noncancerous epithelial cell line (Vero). The method employed was the MTT assay, a quantitative colorimetric assay based on the metabolic activity of cells set by the absorbance values after being treated with the drug for a specific period. The result from the present study showed half-maximal inhibitory concentration (IC_{50}) of silver nanoparticles against Vero cells to be 0.02 mg/mL, while zinc oxide nanoparticles indicated greater cytotoxicity. Previous results indicated that the cytotoxicity of ZnO nanopowder depends on the cell type, concentration, exposure time, and growth,[87] irrespective of the type of synthesis. Also, Kao et al.[88] revealed that the solubility of ZnONPs is the central factor in producing in vitro cytotoxicity. Zn^{2+} in low amounts is crucial to maintaining cellular processes and metabolism; however, Zn^{2+} in high amounts can generate toxic effects.

5. CONCLUSION

In this research, the ZnONPs and AgNPs were prepared by the precipitation and chemical reduction methods, respectively. The characterization results have demonstrated success in the synthesis of these nanomaterials, and the EDX pattern confirms the purity of the compounds. Findings from this research investigated the potential of chemically synthesized silver nanoparticles and zinc oxide nanoparticles as bactericidal agents against MDR *Acinetobacter baumanni*, *Enterococcus faecium*, and *E. faecalis* organisms. We quantified the antibacterial activity of these nanomaterials on the basis of MIC, checkerboard assay, and time-dependent killing assay. The results highlighted that silver nanoparticles showed improved bactericidal activity compared with ZnONPs. The synergistic activity of AgNPs blended with drugs brought about the increased antibacterial activity. Likewise, the concurrent effect of drugs and AgNPs will render it challenging for the disease-causing organism to exhibit resistance. Therefore, this combinational therapy could be studied more in developing new formulations of AgNPs coupled with antibiotics. In vitro bactericidal examinations showed AgNPs exhibited substantial activity against VRE.

In addition, the cytotoxicity study by MTT assay of AgNPs has demonstrated less cytotoxicity than ZnONPs when tested against noncancerous cells. The results pointed out that the toxicity of ZnONPs was concentration-dependent and significantly more toxic at lower concentrations. This exploratory cytotoxicity study of silver nanoparticles could contribute to the understanding of this substance for use in in vivo studies such as in drug delivery systems. However, AgNPs showed less toxicity on Vero cells at lower concentration and demonstrated serious toxicity at higher concentrations. The current study puts in place AgNPs as potent antimicrobial agents; however, their use dosage should be carefully selected on the basis of their cytotoxic effects. The present results showed zinc oxide nanoparticles exerted toxic effects in vitro. In conclusion, there is a need to develop standard protocols to thoroughly evaluate nanoparticle toxicity to minimize possible discrepancies that are associated with their final use.

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A.I. Okoh: Conception and design of the study, proofreading the manuscript. O.O. Adeniji: Conception and design of the study, collection of data, writing of the manuscript. O.M. Ojemaye: Characterization of metallic nanoparticles and proofreading.

Notes

The authors declare no competing financial interest.

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