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Original Research

Synergistic Antibacterial Potential of Zno-Nps with Different Antibiotics against Multidrug-Resistant *Escherichia coli* and *Pseudomonas aeruginosa*

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Abstract

Nanotechnology offers exciting prospects against infectious agents, particularly multidrug resistance bacteria (MDR), which is the roaring concern of this era. Zinc oxide nanoparticles (ZnO-Nps) efficiently deliver therapeutic agents into living systems due to their biocompatibility and bioactivity, which make them highly effective against infectious pathogens. The present investigation was designed to investigate the impact of the ZnO-Nps in combination with the Piperacillin-Tazobactam (TZP) drug against MDR. TZP are beta-lactam antibiotics highly effective against Gram-positive and Gram-negative

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bacteria such as *Pseudomonas aeruginosa*, and are recommended for the empirical treatment of Febrile neutropenia (FN) associated with chemotherapy treatment. For this study, three different-sized ZnO-Np combinations were used. The extended-spectrum beta-lactamase (ESBLs) producing *Escheria coli* and MDR *P. aeruginosa* strain sensitivity profiling towards different combinations of ZnO-Np*s* and Piperacillin-Tazobactam were measured. The synthesized Zno-Np*s* were designated as Zn-1, Zn-2, and Zn-3 based on their sizes, and were made in different combinations with the commercially available drug TZP (ZnO-Np*s*+ Drug) against MDR *E.coli* and *P. aeruginosa*. There was no synergistic effect observed against growth inhibition of *E.coli*. The combined dose of Zn-1 and TZP showed better antibacterial efficacy even as compared to the pure drug against *P. aeruginosa*. The study revealed that the dosage and biological activity of drugs used to treat fatal human diseases like cancer can be decreased while their efficacy can be boosted by using ZnO nanoparticles as powerful drug delivery systems.

Keywords: Antibacterial Potential, MDR, Piperacillin-Tazobactam, ZnO-Nps, synergistic effect

Introduction

Nano-biotechnology is a relatively young field of medicine that employs nano-sized materials for targeted cell-related, or more specifically tissue-related, therapeutic treatments [1]. In comparison to other sectors, the importance of this technology in agricultural research is relatively new [2]. NPs have greater physical properties than bulk molecules depending on their size and shape [3]. Because of their small size and high surface-to-volume ratios, nanoparticles with sizes ranging from 1 to 100 nm offer unique and exciting features [4]. The production of nanomaterials using greener and more biologically friendly approaches using safe and low-cost reactants is of tremendous interest in terms of applicability and biocompatibility [5, 6].

Antibiotic resistance is a growing issue to every healthcare system and food department, and consequently affects life expectancy. Anti-microbial resistance (AMR) or multidrug-resistant (MDR) bacteria are common problems worldwide [7]. Microbial resistance against antibiotics arises due to a systemic failure in health care research, planning, and in public health education [8].

The growth of MDR variants of numerous bacterial diseases creates the threat of a post-antibiotic world, in which previously curable illnesses are lethal, and routine surgery becomes a complicated process [9, 10]. Antibiotics become less effective, and treatment choices are restricted when a bacterium previously susceptible to an antibiotic acquires resistance in host cells [11-14]. While some bacteria have had inherent resistance for millions of years, this acquired resistance phenotype results from the microbial competition in their biological niches [15]. Moreover, the current increase in resistance that is persistent is an inevitable danger to public hygiene as it was in the pre-antibiotic era [16]. The ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* sp.) group are bacteria especially responsible for the current rise in hospital-acquired infections. The development of multiple drug resistance and negative side effects against infectious pathogens, especially *E. coli* and *P. aeruginosa*, are caused by antimicrobial drugs.

Long-term effective methods with fewer side effects will be needed using a combination methodology (such as marker technology, gene/protein sequencing, docking studies and specially nanotechnology), rather than a short-range approach and a focused conventional strategy/approach [17-19]. In this aspect, the developed "Nanotechnology-based" combination therapy decreases toxicity that is linked to a specific drug and suppresses multi-drug resistance because of different mechanisms of action [20, 21]. In comparison to other nanoparticles, inorganic, mesoporous Zinc Oxide nanoparticles (ZnO-NPs) are proven to be extraordinary [22, 23]. They are the ideal therapeutic nano-carriers because of their excellent drug loading capacity, suitability for simple functionalization, controllable particle size and shape, and biocompatibility. For example, they can effectively inhibit the growth of both gram positive and gram negative bacteria, making them potent antibacterial agents. The great sensitivity of the lipid bilayer of bacteria to the reactive oxygen species produced by these nanoparticles accounts for the antibacterial activity of zinc oxide. Despite their widespread usage in biomedicine, current investigations indicate that the effects of zinc oxide nanoparticles on various organisms are still not well known [22-25].

It is critical to develop alternatives to antibiotics for infectious diseases that are both human and animal safe [26]. Fecal microbiota transplantation (FMT), the use of bacteriophage, antimicrobial peptides (AMPs), or bacteriocins, and the competitive exclusion of pathogens using genetically modified probiotics and post biotics are a few examples of unconventional techniques that are frequently used [27-31].

Clinical diagnosis and therapy have been transformed by nanomedicine, which enables treatments and medications to target sick tissue while avoiding healthy cells [32]. To enhance delivery effectiveness and spatiotemporal accuracy, researchers have improved particle size, shape, and mesostructured regions, as

well as conjugated specific ligands and/or "gatekeepers" to increase cell selectivity and on-demand release patterns. ZnO-NPs can address a number of drawbacks of conventional therapies, such as poor bioavailability, brief half-life, and unfavorable bio-distribution, when used as nano-carriers for therapeutics delivery. Due to their small size (100 nm) and high mono dispersed nature, they are essential in numerous applications involving the controlled release of drugs [33, 34].

NPs have superior resilience to degradations caused by heat, mechanical stress, pH, and hydrolysis as compared to conventional polymer-based drug carriers. Selective functionalization with different components is possible thanks to the interior and external surfaces of NPs. The majority of drug delivery materials are made up of connected porous structures, like the branching porous structure and porous shell found in dendrimers. Additionally, NPs with the same special porosity structure that is appropriate for drug delivery [28, 35].

The existing antibiotic therapy is ineffective due to its low solubility, stability, and side effects, prompting researchers to come up with new, creative ways to combat such resistant bacterial strains. As a result, there is a considerable need for new antibiotic delivery methods. Nanotechnology has gained a lot of attention because of its favorable physicochemical features, drug targeting effectiveness, improved absorption, and bio distribution capabilities [35, 36]. The purpose of the current investigation was to examine the antibacterial effectiveness of ZnO-Nps and any possible interactions with the powerful antibiotic (*Piperacillin-Tazobactam*) when used against MDR bacteria.

Materials and Methods

The MDR strains i.e. *E. coli* as well as *P. aeruginosa* were acquired from the Laboratory of Microbiology and public Health from COMSATS University Islamabad Pakistan.

Sensitivity Profiling for Antibiotics

In accordance with the 2013 "CLSI" recommendations, the susceptibility patterns of antibiotics for the ESBL-producing *P. aeruginosa* and *E. coli* were assessed using the method of disc diffusion on Muller Hinton Agar (MHA) plates. On MHA plates, the isolates were grown using a sterile loop. These plates were inoculated with ESBL-producing *E. coli* and *P. aeruginosa*, and the surface of the plates was covered with antibiotic discs of varying concentrations. The plates were incubated at 37ºC for the entire night [37, 38].

ZnO Nanoparticles Synthesis

Using the standard Stober Method and synthetic materials, three alternative modifications were done to manufacture the ZnO nanoparticles. The ZnO-NP*s* were named as Zn-A (size 35+2), Zn-B (size 35+3) and Zn-C (size 35+2.5). Cetyl trimethyl ammonium bromide (CTAB 99%), Tetra ethyl ortho silicate (TEOS 98%), Hydrochloric acid solution (HCL 38%), Ammonium hydroxide solution (NH_3 32%) and absolute Ethanol 99%.

Zn-A Synthesis

The CTAB (about 0.3 gram) was diluted in 100 mL absolute ethanol and supplemented with about 8 mL TEOS solution. Afterwards 10µL of NH_3 was also added to the solutions. The solution was maintained for 15 min while being stirred rapidly to ensure thorough mixing.

Zn-B synthesis

Ethanol was mixed with TEOS in an 8:100 ratio. The reaction was then carried out at 60ºC with constant stirring for two hours after a few NH3 drops were also added to the solution to keep the pH = 7.5. The solution was then centrifuged for 15 minutes at maximum speed before being washed three times with 1M 0.5 mL HCL and an absolute ethanol solution subsequently. After that, the solution was agitated for two hours at 60 degrees Celsius before being dried for 24 hours at 100 degrees Celsius in a drying oven.

Zn-C Synthesis

Ethanol (8:200 ratio) was mixed with TEOS. The pH was maintained by adding NH_3, which was stirred for 30 min at 60ºC before being dried in an oven for 24 hours at 100ºC. All the samples were dried and meticulously gathered, kept, and characterized.

ZnO-NPs and Antibiotics Biological Activity

Several pathogen colonies, including *E. coli* and *P. aeruginosa*, were cultured in LB to examine the biological effects of ZnO-NP*s*/antibiotics and the combinations of both against pathogenic ESBL-producing strains. To test the synergistic action in LB, beta lactam antibiotics Piperacillin-Tazobactam (TZP) and ZnO-NP*s* were combined [39-42].

Culture Preparation

The sodium chloride (NaCl) 3 cgm, yeast (1.5 cgm), and bactotryptone (3 cgm) (OXOID, UK). To maintain the pH at 7.5, sodium hydroxide (NaOH) was added to a mixture of all the ingredients in 200 mL of distilled water.

Growth Evaluation

It was observed that the Luria Broth (LB) culture for the strains was resistant to the medication and nanoparticle combination. In order to treat 100 µL

of both the strain in a 5 mL Luria Broth (LB) culture, a serial dilution of a 20 mg per mL ZnO-NP*s* stock with 2 mg of medication per ml was used. To observe the growth kinetics of the ESBL strain, the synergism of beta lactam antibiotics with a mixture of ZnO-NP*s* was assessed. The test compound-infused Luria Broth (LB) media was used to cultivate the ESBL *E. coli* and *P. aeruginosa* cultures, which were then incubated at 37ºC. Using Nano drops, the O.Ds at 600 nm were captured for each sample of ZnO-NP*s* conjugated with the medication at intervals of 2 hours (Thermo 2000C). Readings were taken after 2, 4, 6, and 24 hours.

Results

Growth Assessment of Bacterial in LB Media

In order to ascertain the sensitivity of *P. aeruginosa* and *E. coli*, the synergism of the beta lactam antibiotics TZP with the combination of ZnO-NP*s* (Zn-A, Zn-B, and Zn-C) was studied in LB grown culture. In 5 mL L.B culture a total of 20 mgl^{-1} dilutions of ZnO-NP*s* stock solution and with 2 mgl^{-1} antibiotics was used for every 100 µL of strain.

Synergistic Activity of Both the Drugs

Zn-A was unable to demonstrate any potential ability to reduce the development of either strain of bacteria, but the combination of Ciprofloxacin and Zn-A therapy inhibits bacterial growth normally when compared to the control. While *E. coli* demonstrated only little growth inhibition, *P. aeruginosa* growth was almost completely inhibited by Zn-A and medication at 24 hours compared to pure ciprofloxacin. Fig. 1(a-d) which depict the growth patterns for the two strains, respectively.

Zn-B alone hardly affected the bacterial growth whereas in combination with the drug, growth of *P. aeruginosa* kept inhibiting the bacterial growth and showed absolute inhibition at 24 hours. In contrast, *E. coli* kept harboring the resistance against Zn-B and ciprofloxacin combination (Fig. 1a-d).

Similarly, Zn-C alone showed minimal activity. The *P. aeruginosa* growth was stunted when used with the combination therapy. Similarly, the Zn-C with the drug showed potency to inhibit the bacterial growth as compared to pure *Ciprofloxacin*. Whereas the growth of *E. coli* reduced almost two times with S3 and ciprofloxacin combined therapy in contrast with Zn-C alone (Fig. 2a-b).

Piperacillin-Tazobactam and ZnO-NPs Synergistic Activity

Zn-A In contrast to the control, TZP combined therapy hindered the typical growth pattern of *E. coli*. Zn-B and Zn-C did not demonstrate any possible efficacy to inhibit the growth of bacterial strain (Fig. 2a-d).

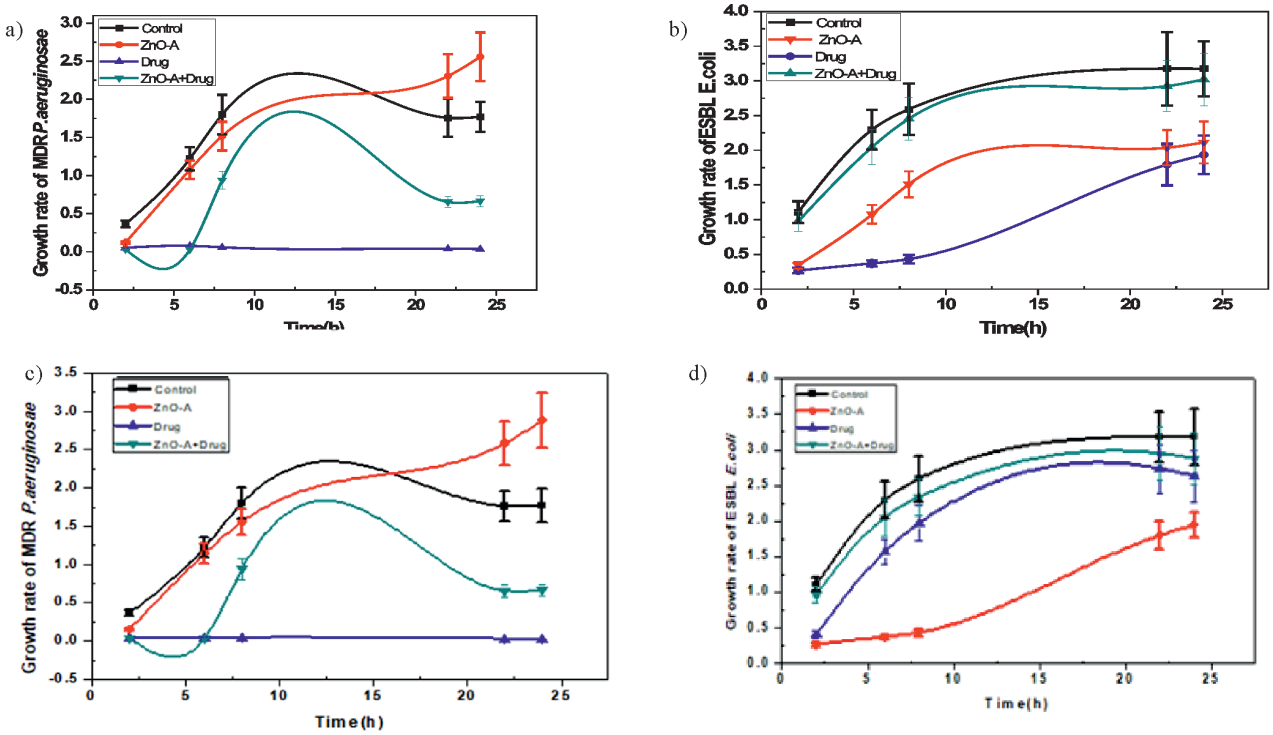

Fig. 1. a) MDR growth curve of the *P. aeruginosa* strain against combination of ciprofloxacin and Zn-A (NP) at 24 h, b) ESBL growth curve of the *E. coli* strain against various combination including ciprofloxacin and Zn-A (NP*s*) at 24 h, c) MDR growth curve of the *P. aeruginosa* strain against different combination i.e. ciprofloxacin and Zn-B over a period of 24 h, d) ESBL growth curves of the *E. coli* strain against different combination i.e. ciprofloxacin and Zn-B over a period of 24 h.

The *P. aeruginosa* growth was also slowed down by medication combination therapy. When compared to the effectiveness of Zn-A, Zn-B, and Zn- C to suppress the growth of P. aeruginosa, (Fig 3(a-d), respectively) the TZP showed more potency to limit bacterial growth.

Discussion

The treatment of severe MDR is now seriously threatened by antibiotic resistance, and the uropathogens *P. aeruginosa* and *E. coli* that produce ESBLs have differing sensitivity profiles to commercially available medications [43]. Since there is a limited selection of antibiotics, the production of these uropathogens results in the inactivation of numerous medicines and presents a significant therapeutic challenge [44, 45]. The *P. aeruginosa* infections are linked to greater rates of morbidity, mortality, and medical expenses. One of the major worries, especially in undeveloped and wealthy countries, is the transmission of these germs through human feces to streams and other natural sources. These strains are the most dangerous bacteria in caged birds, causing extra intestinal disorders such polyserositis, septicemia, and aerosacculitis in addition to human infections [46].

The *P. aeruginosa* is one of the top five pathogens responsible for infections of the lungs, circulation, urinary tract, surgical sites, and soft tissues. Unacceptably high rates of morbidity and mortality have been linked to current treatments, principally antibiotics that eradicate or prevent the growth of this bacterium. An innovative and possibly successful strategy for treating severe infections is the creation of drugs that counteract virulence factors [43, 46].

Nanotechnology-based infectious disease therapeutics have gained popularity recently as a result of the development in antibiotic resistance and the dearth of effective treatment options. ZnO-NP*s*' diversity and adaptability can be useful for infectious diseases. The antibacterial properties of zinc oxide nanoparticles (ZnO-NP*s*) have attracted a lot of attention from scientists all over the world, especially since nanotechnology has been used to create particles with dimensions in the nanometer range. ZnO-NP*s* can deliver a variety of therapeutic and diagnostic chemicals to the body because of their adaptable pore characteristics [47, 48]. Furthermore, ZnO-NP*s* are superior to other members of its family in terms of antibacterial and antifungal capabilities because they have strong photochemical and catalytic activity. The ZnO has strong optical absorption in the UVA and UVB i.e. 315-400 nm and 280-315 nm, respectively, which aids in the antibacterial

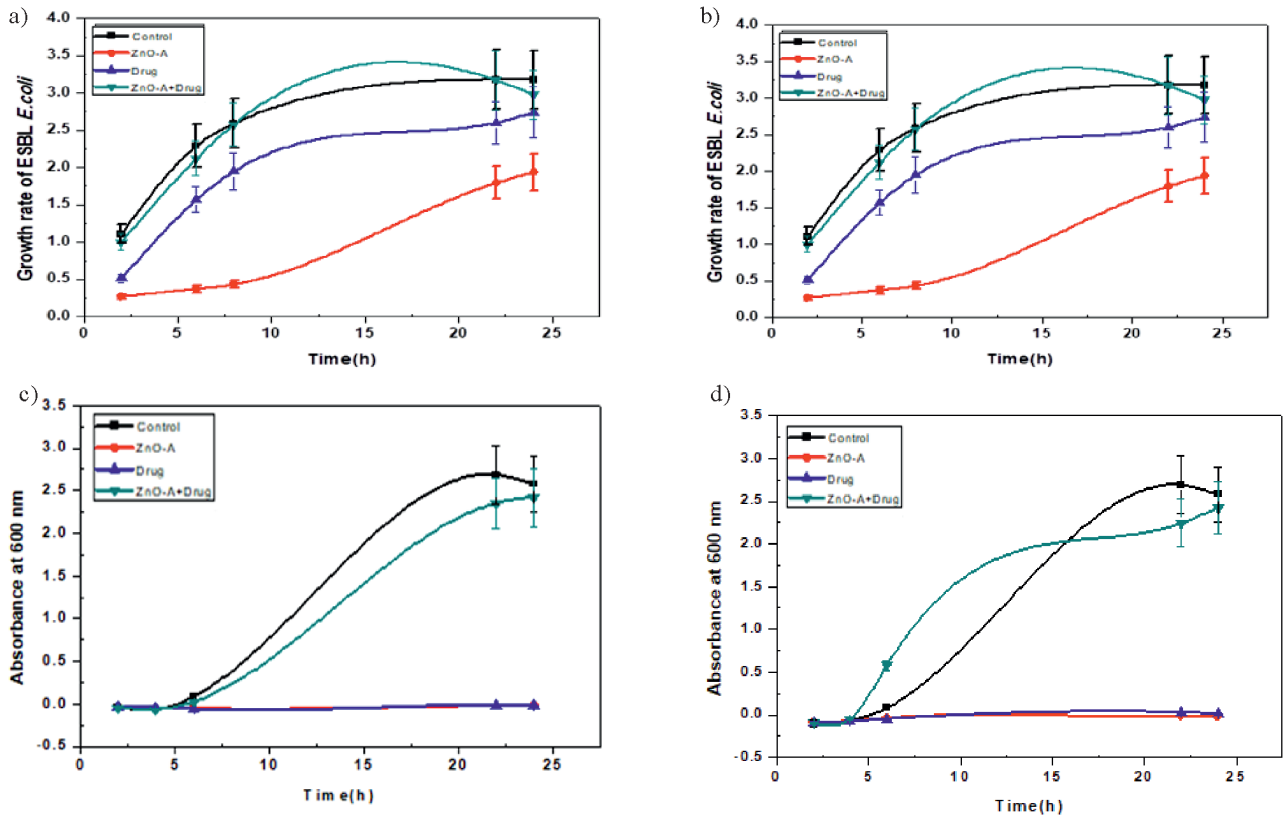

Fig. 2. a) MDR growth curve of the *P. aeruginosa* strain against combination of Zn-C and ciprofloxacin at 24 h, b) ESBL growth curves of the *E. coli* strain against different combination i.e. ciprofloxacin and Zn-C over a period of 24 h, c) ESBL growth curves of the *E. coli* strain against different combination i.e. Zn-A and TZP over a period of 24 h, d) ESBL growth curve of the *E. coli* strain with various combinations of TZP & Zn-B at 24 h.

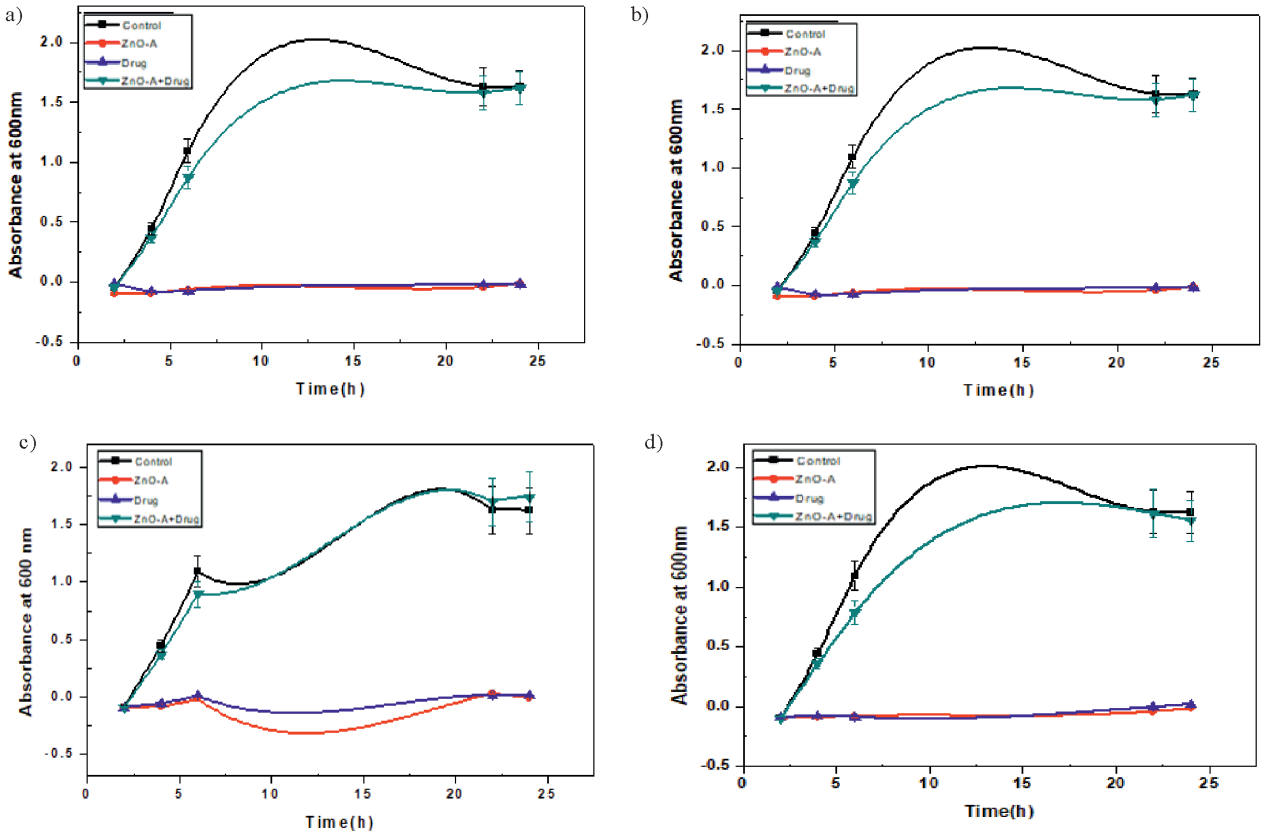

Fig. 3. a) ESBL growth curve of the *E. coli* strain against different combination of TZP and Zn-C at 24 h, b) MDR growth curve of the *P. aeruginosa* strain with various combination of TZP and Zn-A over period of 24 h, c) MDR growth curve of the *P. aeruginosa* strain with different combination of TZP and Zn-B at 24 h, d) MDR growth curve of the *P. aeruginosa* strain against various combination of TZP and Zn-C at 24 h.

response and is utilized as a UV protector in cosmetics [48-50].

ZnNP*s* can address a number of inherent treatment drawbacks, such as low bioavailability, brief circulation time, and unfavorable bio-distribution, when used as nano-carriers for therapies administration. To strengthen cell selectivity and on-demand release patterns, specific ligands and/or "gatekeepers" have been conjugated to engineered NP*s*, which have been designed to increase delivery efficiency and spatiotemporal accuracy [20, 33-34].

The present study also demonstrated the use of ZnO-NP*s* as a drug carrier to check the TZP and Ciprofloxacin with ZnO-NP*s*. The Zn-A, Zn-B and Zn-C were designed for protected delivery of TZP to the culture media. As a result of *piperacillin/antibacterial tazobactam's* action, third generation cephalosporins may not always be necessary when treating difficult illnesses. In this work, we examined the interaction between two medications and two ZnNPs for two distinct strains. The discovery that Zn-A, Zn-B, Zn-C and ciprofloxacin alone, did not reduce the *P. aeruginosa* growth, but had bactericidal efficacy when combined at 24 hours. However, no beneficial synergism between ZnNP*s* and antibiotics was found for absolute growth suppression of E. coli. Contrarily, Zn-A, Zn-B and Zn-C did not exhibit a stronger response to individual therapy of piperacillin and tazobactam, while showing significant results for the suppression of both strains when used in combination [50, 51].

Antibiotics have been discovered to have strong antibacterial properties when combined with photocatalyst ZnO-nanoparticles [51, 52]. Additionally, ZnO nanoparticles have lately been proposed as efficient photosensitizer carriers, and a lot of research has been done on their antibacterial activities [53-55], but their efficiency in terms of in-vitro cell line experiment has not been done [54, 56].

Previous research also found that increasing ZnO concentrations resulted in stronger bacterial (*E. coli*) inhibition. Similar results were found in the current research, but with the addition of antibiotics against AMR. Furthermore, the usage of Zn-based Nps is not restricted to people; it is also employed in a variety of industries, including the food business, where it is used to prevent bacterial growth due to contamination [57]. Studies have also reported the uses of antimicrobial NPs on different types of food, development of high barrier packaging materials, and use of NPs in nanosensors to track analytes related to food, such as microorganisms that cause foodborne illness [48]. Similar results were reported in previous studies

that ZnO nanoparticle inhibited bacterial growth and increased antibiotic susceptibility. They demonstrated that hydroxyl radicals produced by the coated surface played a significant part in the creation of an anti-biofilm [58]. Other investigations also demonstrated that ZnO NPs can compromise the integrity of bacterial cell membranes, decrease the hydrophobicity of cell surfaces, and suppress the transcription of genes in bacteria that are resistant to oxidative stress [59]. The results used in present study were more significant to previous studies as the Nanoparticles were used in combination with antibiotics that increases its efficiency in terms of inhibition, and it was also revealed that for AMR combine therapy can be quite useful in treating infectious diseases.

To cope with this lethal issue, the use of advanced technology by combining various fields and methodologies is of great benefit, in terms of loss reduction [17, 19, 60-64]. In this regard, specifically, it has been demonstrated that a number of classes of antimicrobial nanoparticles (NPs) and nano sized carriers for antibiotic delivery are efficient methods for the treatment of infectious or fatal diseases, including those that are resistant to antibiotics, in vitro as well as in plant or animal models [54, 65]. Antimicrobial NPs and antibiotic delivery methods have been used primarily in the visible attempts to combat infectious diseases. These are modern methods for treating infectious diseases in light of the current circumstances [54-57].

Regardless of the fact that we are living in the age of modern and advance technologies that clearly define underlying mechanisms of diseases and also help us in molecularly designing and identification of diseases, especially for new drugs/treatment [17, 19, 60-64] against lethal/infectious diseases are still considered one of the world greatest health challenges [66, 67].

Conclusions

Antimicrobial drugs are the cause of MDR and harmful side effects against infectious agents such as MRSA, *E. coli*, *P. aeruginosa*, and others. Furthermore, due to drug resistance, antibiotics must be delivered at high doses, which frequently results in severe toxicity, the need to develop new treatments, and expensive labor, material, and time expenses. Third-generation medicines for the treatment of infectious diseases have side effects such as gynecologic and vasomotor symptoms such as night sweats, hot flashes, vaginal dryness, insomnia, weight gain, and joint aches, but nanotechnology promises targeted application of anticancer drugs by minimizing the toxicity of healthy tissues. This research also indicated that nanoparticles such as ZnO-NPs can be tailored to fit the goals of current therapy and diagnosis. In addition, ZnO-NPs enable a targeted combination therapy technique for the safe transport and release of cytotoxic chemicals. This study suggests that by employing ZnO nanoparticles as potent drug vehicles in the treatment of life-threatening human diseases, dose and biological activity can be reduced while efficiency is increased.

List of Abbreviations

MDR-Multidrug resistance bacteria, ZnO-Nps-Zinc oxide nanoparticles, TZP-Piperacillin-Tazobactam, FN-Febrile neutropenia, ESBLs-Extended-spectrum beta-lactamase, AMR-Anti-microbial resistance, FMT-Fecal microbiota transplantation, AMPs-Antimicrobial peptides, pH-Power of hydrogen ion, NPs-Nanoparticles, MHA-Muller Hinton Agar, CTAB- Cetyl trimethyl ammonium bromide, TEOS-Tetraethyl orthosilicate, HCL-Hydrochloric acid solution, NH3-Ammonium hydroxide solution, mL-Milliliter, µL-Microliter, TZP-Piperacillin-Tazobactam, NaCl- Sodium chloride, NaOH- Sodium hydroxide, Zn-A-Zinc oxide A, Zn-B- Zinc oxide B, Zn-C- Zinc oxide C.

Ethics Approval and Consent to Participate

All the experiments were performed in accordance with relevant guidelines and regulations".

Author Contributions

Conceptualization: Y.Y., A.F., N.R., & A.R; Data Curation: I.K., S.H.K., S.A.K.B., & M.N.K; Formal Analysis: M.N.K., S.W., N.R., A.F., & B.A; Investigation: S.H.K., S.A.K.B., & A.K; Methodology: B.A., A.K & Y.Y; Software: R.A., S.M.A., & R.M.A; Writing-Original Draft: I.K., A.F & A.K; S.H.K; Writing - Review & Editing: M.N.K., A.K., & D.B.E.D; Funding Acquisition: R.A., S.M.A., R.M.A., & D.B.E.D.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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