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Antimicrobial and Anti-biofilm Activity of Zinc Oxide and Copper Oxide Nanoparticles Against Multidrug-Resistant *Klebsiella pneumoniae* Clinical Isolates: Synergistic Effects and Virulence Gene Expression

النشاط المضاد للميكروبات والأغشية الحيوية لأكسيد الزنك وأكسيد النحاس النانوية ضد العزلات السريرية لبكتيريا كليبسيلا نيومونيا المقاومة للأدوية المتعددة التأثيرات التآزرية والتعبير الجيني الفوعة

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Abstract

Klebsiella pneumoniae represents a significant nosocomial pathogen causing urinary tract infections, wound infections, and burns with increasing multidrug resistance. This study investigated the antimicrobial efficacy of zinc oxide (ZnO) and copper oxide (CuO) nanoparticles against clinical *K. pneumoniae* isolates. From 100 clinical specimens, 50 *K. pneumoniae* isolates were identified, with 64% from urine, 22% from wounds, and 14% from burns. Antibiotic susceptibility testing revealed high resistance to ampicillin (96%) and amoxicillin-clavulanate (72%), while imipenem and ciprofloxacin showed lower resistance (10% each). CuO nanoparticles demonstrated superior antimicrobial activity with minimum inhibitory concentration of 12.5 µg/mL compared to ZnO at 25 µg/mL. Both nanoparticles exhibited synergistic effects with antibiotics, converting resistant isolates to sensitive/intermediate for ciprofloxacin, meropenem, and imipenem. ZnO nanoparticles significantly reduced biofilm formation by 85-90%, while CuO stimulated biofilm production. Gene expression analysis revealed that both nanoparticles downregulated virulence genes (uge and mrkA), with ZnO showing greater anti-virulence effects than CuO nanoparticles.

Keywords: *Klebsiella pneumoniae*, Metal oxide NPs, Multidrug Resistance, Biofilm Inhibition, ZnO/CuO NPs.

Introduction

Klebsiella pneumoniae is recognized as an opportunistic pathogen with a high capability to produce a wide range of infections in man [1]. *K. pneumoniae*

represents one of the most significant nosocomial pathogens in modern healthcare settings belonging to the Enterobacteriaceae family of Gram-negative bacteria [2]. This encapsulated, non-motile bacterium has emerged as a leading cause of healthcare associated infections, particularly in immunocompromised patients and those with prolonged hospital stays [3]. The pathogenicity of *K. pneumoniae* stems from its ability to produce a thick polysaccharide capsule that enhances virulence and provides protection against host immune response [4].

K. pneumoniae exhibited predilection for causing infections in multiple anatomical sites, with urinary tract infection (UTI) [5], wound infections and burn related sepsis representing the most common clinical presentations [6]. In UTI, *K. pneumoniae* demonstrated particular affinity to catheter associated infections, where biofilm formation on catheter surface provides a protected niche for bacterial persistence and growth [7]. In wound and burn infections, *K. pneumoniae* take advantage of compromised tissue integrity and impaired local immune response, often leading to deep tissue invasion and systematic dissemination [8].

The emergence of multidrug resistant *K. pneumoniae* stains has transformed this pathogen into a formidable therapeutic challenge [9]. *K. pneumoniae* demonstrated high capacity for acquiring resistance mechanisms via horizontal gene transfer, including extended spectrum beta-lactamases (ESBLs), carbapenemases, and metallo-beta-lactamases [10]. The use of Nanoparticles or Nanomaterials has lately been a scientific major aspect, since it is a promising technology utilized in many sectors of life to mitigate or remove various forms of environmental pollutants, hence maintaining a healthy and safe environment free of diseases [11, 12]. The limitation of conventional antibiotic treatments has driven research toward alternative antimicrobial approaches, with metal nanoparticles (NPs) emerging as promising candidates [13]. These nanoscale materials possess antimicrobial action including membrane disruption, reactive oxygen species (ROS) generation, and interference with cellular processes [14]. The high surface to volume ratio of NPs enhances their antimicrobial efficacy while potentially reducing the likelihood of resistance development due to their multi-target approach [15]. Among metal NPs, zinc oxide (ZnO NPs) and copper-based NPs have demonstrated exceptional antimicrobial potential against *K. pneumoniae* [16, 17]. ZnO NPs excert antimicrobial effects through multiple mechanisms including membrane destabilization, intracellular zinc accumulation leading to enzyme dysfunction, and generation of ROS that damage cellular

components [18]. Copper NPs similarly demonstrate broad-spectrum antimicrobial activity via copper ion release, membrane distribution and oxidative stress induction [19]. Both metals possess inherent antimicrobial properties that have been recognized for many years, and their NPs forms offer enhanced activity with controlled release characteristics positioning them as viable alternatives for combating MDR *K. pneumoniae* infections.

Accordingly, this research involved the isolation and identification of *K. pneumoniae* isolated from clinical specimens, investigating *K. pneumoniae* multidrug resistance pattern and the impact of ZnO and CuO NPs as stressors on key virulence factors (biofilm formation and gene expression of capsule production) in clinical *K. pneumoniae*.

Materials and Methods

Study Area and Specimens Collection

One hundred clinical specimens collected from patients admitted to the emergency room of Ramadi Teaching Hospital for Maternity and Children and Ramadi General Hospital, Ramadi – Al-Anbar province of both sexes and different age groups. Specimens were subdivided into 23 specimens from wounds, 27 specimens from burns and 50 specimens from urine. Clinical specimens were collected from patients following established microbiological protocols [20]. All specimens were collected using sterile disposable cotton swabs immersed in transport medium. The collected specimens were promptly transported to the microbiology laboratory at Al-Anbar University, College of Science, for the purposes of culturing, identification, and characterization.

Isolation and Identification of *K. pneumoniae*

Clinical samples were spread on nutrient agar plates and incubated at 37°C for 18 hours. After incubation, growing colonies were streaked directly on MacConkey agar and incubated for 18 hours at 37°C. The lactose fermenting colonies on MacConkey agar were selected and subculture on another MacConkey agar plate and incubated for another 18 hours at 37ºC for obtaining pure culture. The suspected isolates were further identified using VITEK 2 system (Biomerieux, France).

Antibiotic Sensitivity Test

Antibiotic sensitivity test was performed using the Kirby-Bauer method [21]. In brief, a sterile cotton swab was dipped into the bacterial suspension (0.5

MacFarland standard). The swab was then used to inoculate Mueller-Hinton agar plates by streaking across the dried surface in three directions. Using sterile forceps, antibiotic discs were carefully placed on the agar surface, with a maximum of five discs per plate. The plates were then incubated at 37°C overnight. Post-incubation, the inhibition zones were measured in millimeters using a ruler, with these zones identified as areas surrounding the discs where no visible growth occurred. The measurements were compared with standard values to classify each drug's effectiveness as susceptible (S), intermediate (I), or resistant (R), according to Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines.

ZnO and CuO Nanoparticles (NPs) Stock Solution Preparation

A stock solution of both ZnO and CuO NPs (US Research Nanomaterials, Inc, USA) was prepared by dissolving 1 g of each nanopowder in 10 mL normal saline to obtain stock solution of 100,000 µg/mL. The solution was sterilized by filtration (0.45 µm filter). The sterilized solution was stored at 4°C until used.

Determination of Minimum Inhibitory Concentration (MIC) of ZnO and CuO NPs

The inhibitory activity of ZnO and CuO NPs against *K. pneumoniae* isolates was performed using resazurin-based 96-well plate method [22]. Aliquot of 100 µL of Brain Heart Infusion broth containing serial dilution (1.56, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) of ZnO or CuO NPs was added in each well. A volume of 50 µL of *K. pneumoniae* inoculum was added to each well. The 96-well plate was incubated for 24 hours at 37°C. Inoculated wells containing Brain Heart Infusion broth without treatment and wells containing only Brain Heart Infusion broth were set as control. The experiment was run in triplicate. After incubation, 30 µL of resazurin (0.015% in D.W.) was added to each well and allowed to stand for 2-4 hours at 37°C. After the final incubation, the plate was observed for color change. Treated wells with no color change (the blue color of resazurin unchanged) indicating the MIC concentration, which means all bacterial cells were killed due to treatment, while transitions from blue and non-fluorescent to pink and highly fluorescent upon chemical reduction via aerobic respiration resulting from cellular division, signifying cell viability.

Synergistic of ZnO and CuO NPs with Antibiotic Disk

The susceptibility of *K. pneumoniae* clinical isolates toward selected antibiotics was determined in the presence of ZnO and CuO NPs at sub-MIC concentration using Kirby-Bauer. The Mueller-Hinton agar plates were supplemented with ZnO or CuO NPs at sub-MIC concentration before applying bacteria and antibiotic disks.

Antimicrobial Activity of ZnO and CuO NPs using Well-Diffusion Method

The agar based well-diffusion method was used to estimate the antimicrobial activity of ZnO and CuO NPs against selected *K. pneumoniae* isolates at MIC and sub-MIC concentrations [23]. A specified volume of the bacterial suspension (0.1 – 0.2 mL, 0.5 MacFarland standard) was meticulously transferred and uniformly spread on Mueller Hinton agar medium using a sterile cotton swab, followed by a 10 min incubation period. Five millimeter-diameter wells were created in the agar layer, with 3 - 5 wells per plate. Aliquots of 0.1 mL of various treatments were dispensed into each well using micropipette. The diameter of the inhibitory zones was quantified following overnight incubation at 37°C on the plates. Normal saline served as the negative control in the experiment.

Biofilm Formation Assay

The *K. pneumoniae* isolates were examined for biofilm production applying a previously established microtiter plate method [24]. Each *K. pneumoniae* isolate was inoculated in 5 mL of Brain Heart Infusion broth and incubated at 37°C for 24 hours. Aliquot of 20 µL of microbial suspension (equivalent to 0.5 McFarland) was used to inoculate 96-microliter wells containing 180 µL of Brain Heart Infusion broth supplemented with 2% sucrose to each well plate and incubated at 37°C for 24 hours. After incubation, the plate was washed three times with a normal saline solution in order to eliminate non-adherent cells. The adherent cells forming biofilm were fixed with 200 µL of 99% methanol for 15 minutes. The methanol was decanted, and the plate was allowed to dry at room temperature for 30 min. Subsequently, 200 µL of crystal violet (0.5%) was introduced to each well for a duration of 15 min. The staining step was stopped by washing the plate with distilled water and the attached dye was solubilized with ethanol (96%) and the optical density was appointed in a micro-titer plate reader at 630 nm and the biofilm formation was evaluated as described in Table (1).

Table 1. Evaluation of biofilm formation by microtiter plate method.

Optical Density	Adherence
OD ≤ ODc	Non-Biofilm Former
2ODc > OD > ODc	Weak Biofilm Former
4 ODc > OD > 2 ODc	Moderate Biofilm Former
OD > 4 ODc	Strong Biofilm Former
Cut-off value (ODc) = Average OD of negative control + (3 x Standard deviation)	

OD: Optical density.

Expression of *mrkA* and *uge* Biofilm Associated Genes

RNA was isolated from *K. pneumoniae* isolates using TransZol Plus RNA isolation kit (TransGen Biotech, USA) according to the protocol described by the manufacturer. The conversion of total RNA into first strand cDND was preformed using GoScript™ Reverse Transcription kit (Promega, USA). *K. pneumoniae* isolates were selected prior to and following treatment with CuO and ZnO NPs to assess the gene expression of virulence biofilm associated genes (*mrkA* and *uge*) in relation to a housekeeping gene (*recA*) using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Cycle of threshold (Ct) means were employed, and the $2^{-\Delta\Delta Ct}$ technique was utilized to compute fold expression differences across groups. Primers for *mrkA*, *uge* and recA were designed using Primer 3 Plus software (Table 2).

Table 2. Reverse transcriptase - polymerase chain reaction primers.

Primer	Sequences	bp	Product Size(bp)	Annealing Temp.
uge	Forward: GCGCACACCTATTCTCACCT	20	114	58
	Reverse: CATCGCTTTCGTGAACTTGA	20		58
mrkA	Forward: GTCAGGGTTACCGGAGACAG	20	128	58
	Reverse: GCAGCTGATACCACTGTTGG	20		58
recA	Forward: GGGTAACCTGAAGCAGTCCA	20	130	58
	Reverse: ACGCACAGAGGCGTAGAACT	20		58
Reference			**Designed in this study**	

Statistical Analysis

GraphPad Prism version 8.0 was used to analyze the data statistically. Chi-square was used to analyze differences in counts. A one-way analysis of variance ANOVA (Tukey Test) was used to determine whether group variance was significant or not, statistical significance was defined as * $p < 0.05$ or ** $p < 0.01$. Experiments were repeated thrice, and data were expressed as mean ± standard deviation.

Results and Discussion

Identification of *K. pneumoniae* isolated from Clinical Samples

All the 100 clinical specimens showed successful growth on nutrient agar and MacConkey agar after 24 h growth. The isolates that exhibited morphological characteristics of *K. pneumoniae* on MacConkey agar were further identified using VITEK-2 system. Results showed that 50% of clinical specimens were identified as *K. pneumoniae*, while the remaining comprised other types of bacteria, including *Staphylococcus* sp. (18%) and *Escherichia* coli (32%). Table (3) shows significant (p <0.0001) variation among the different types of specimen collection, in which the majority of *K. pneumoniae* isolates (32 isolates) were obtained from urine specimens representing 64% of the total clinical specimens, followed by 11 isolates (22%) from wounds, while burn specimens accounted for the lowest population (14%).

Table 3. The frequency of *K. pneumoniae* isolation from clinical specimens

Type	Specimen No.	Sig.	*p* Value
Urine	32 (64%)		
Wound	11 (22%)	**	<0.0001
Burn	7 (14%)		
Total	50 (100%)		

**** $p < 0.01$.**

K. pneumoniae demonstrates distinct prevalence pattern across different clinical specimen types, with notable variation between UTIs and wound/burn infections. The higher isolation rate of *K. pneumoniae* from urine samples compared to wound/burn specimens reflecting the propensity for urogenital tract colonization and infection [25]. *K. pneumoniae* enhanced survival in urinary environments, coupled with the ability to form biofilms on urinary catheters, contributes to the predominance of *K. pneumoniae* in nosocomial UTIs [26]. Conversely,

wound/burn isolates typically showed a lower frequency rate of *K. pneumoniae* which may be attributed to anatomical site-specific factors, including local immune response, tissue oxygenation and microbial competition [27].

Antibiotic susceptibility of *K. pneumoniae*

Susceptibility and resistance profiles of *K. pneumoniae* isolates were evaluated using Kirby-Bauer method by employing 8 different ready-to-use antibiotic disks. Antimicrobial sensitivity testing protocols and interpretive criteria were established in accordance with Clinical Laboratory Standards Institutes (CLSI) guidelines and standardized breakpoint values for recommended antibiotic categories. Results in Table (4) revealed that *K. pneumoniae* isolated from clinical specimens exhibited a varying level of sensitivity and resistance patterns to these antibiotics.

Table 4. Susceptibility test of *K. pneumoniae* clinical isolates to 8 different antibiotics

Antibiotic	S Isolate, No.(%)	I Isolate, No.(%)	R Isolate, No.(%)	Chi-Square	*p* Value
Azithromycin (AZM)	28 (56%)	6 (12%)	16 (32%)	14.56	0.0007 **
Ciprofloxacin (CIP)	40 (80%)	5 (10%)	5 (10%)	49.00	<0.0001 **
Levofloxacin (LEV)	29 (58%)	11 (22%)	10 (20%)	13.72	0.0010 **
Meropenem (MEM)	33 (66%)	11 (22%)	6 (12%)	24.76	<0.0001 **
Imipenem (IPM)	43 (86%)	2 (4%)	5 (10%)	62.68	<0.0001 **
Ampicillin (AM)	1 (2%)	1 (2%)	48 (96%)	88.36	<0.0001 **
Amikacin (AK)	15 (30%)	20 (40%)	15 (30%)	1.000	0.6065 NS
Amoxicillin-Clavulanate (AMC)	13 (26%)	1 (2%)	36 (72%)	37.96	<0.0001 **
Chi-square	57.72	42.51	101.1		
p Value	<0.0001**	<0.0001**	<0.0001**		

S: Sensitive, I: Intermediate, R: Resistance. NS: Non-significant, ** *p* < 0.01.

The β-lactam antibiotic Ampicillin exhibited the highest percentage of antibiotic resistance (96%), followed by Amoxicillin-Clavulanate (72%). Clinical *K.*

pneumoniae isolates showed considerable resistance toward Azithromycin (32%), Amikacin (30%) and Levofloxacin (20%), while Meropenem, Imipenem and Ciprofloxacin exhibited the lowest resistance percentages (12%, 10% and 10%, respectively).

The high resistance against ampicillin is attributed to the intrinsic resistance caused by chromosomally SHV-1 β-lactamase production, which hydrolyze SHV-1 β-lactam antibiotics by cleaving β-lactam ring structure. The presence of SHV-1 penicillinase on the chromosome confirms the intrinsic resistance to ampicillin, amoxicillin and ticarcillin [28]. Results showed significant resistance toward amoxicillin-clavulanate despite the β-lactamase inhibitor of clavulanate. The result was in agreement with Bayatinejad et al., which indicated that almost all *K. pneumoniae* isolated from patients with ventilator-associated pneumonia were resistant to amoxicillin-clavulanate [29]. Moderate to low resistance was observed against azithromycin, amikacin and levofloxacin, ciprofloxacin, imipenem and meropenem. In this investigation, *K. pneumoniae* exhibited moderate to minimal resistance to ciprofloxacin (10%) and levofloxacin (20%). *K. pneumoniae* isolates showed moderate susceptibility to aminoglycosides antibiotics, especially amikacin. Although resistance genes encoding for aminoglycoside-modifying enzymes are often reported in pathogenic *K. pneumoniae* and confirm resistance to amikacin, studies showed that K. pneumoniae exhibited moderate susceptibility against amikacin [30]. The results revealed that carbapenem antibiotics, imipenem and meropenem, demonstrated significant antimicrobial activity against clinical *K. pneumoniae*. These results correspond closely with local study which documented a 90.7% imipenem sensitivity among *K. pneumoniae* isolates [31].

Antimicrobial activity of ZnO and CuO NPs

The antimicrobial activity of ZnO and CuO NPs against *K. pneumoniae* isolates was investigated using MIC (microtiter plate method) and agar well-diffusion methods. The results of MIC experiment indicated that the lowest inhibitory concentration of CuO NPs against *K. pneumoniae* isolates was 12.5 µg/mL. On the other hand, the lowest inhibitory concentrations of ZnO NPs against clinical *K. pneumoniae* isolates were 25 µg/mL. In agar-well diffusion experiments, results indicated that ZnO NPs treatment at MIC concentrations showed an inhibitory inhibition zone of 13 mm (Fig. 1). Regarding CuO NPs, the agar-well diffusion experiment was unable to provide clear results due to a reaction between Cu and the media components.

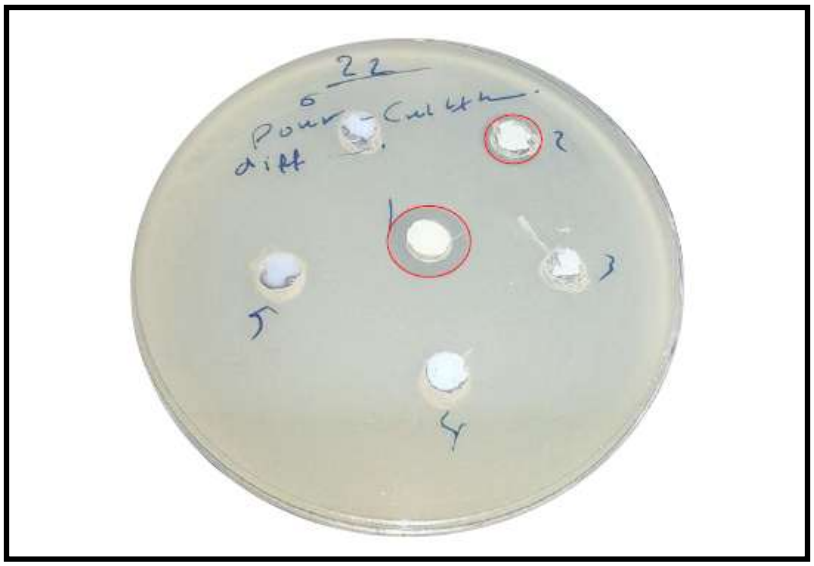

Figure 1: Antimicrobial activity of ZnO NPs at MIC concentration against *K. pneumoniae* clinical isolates using agar-well diffusion method.

ZnO NPs demonstrated significant antimicrobial efficacy against clinical and environmental *K. pneumoniae* isolates. Antimicrobial mechanisms involve multiple pathways, including reactive oxygen species (ROS) generation, direct membrane interaction, and intracellular damage [32]. ZnO NPs generate hydroxyl radicals, superoxide ions and H_2O_2 upon contact with bacterial cells, leading to oxidative stress and membrane lipid peroxidation [33]. The bactericidal activity of CuO NPs was confirmed against K. pneumoniae isolates. CuO NPs inhibition mechanism against bacterial cells comprises the release of Cu ions and subsequent cellular damage. CuO NPs generates Cu^{2+} which interact with bacterial cell components, leading to protein denaturation, enzyme inactivation and DNA damage. The generation of ROS, including hydroxyl radicals and superoxide ions, induce sever oxidative stress within bacterial cells [34].

Synergistic Effect of ZnO and CuO NPs with Different Antibiotics

The synergistic effect of ZnO and CuO NPs in combination with antibiotics was investigated in clinical *K. pneumoniae* isolates using Kirby-Bauer method on Mueller-Hinton agar plates supplemented with sub-MIC concentration of ZnO NPs or CuO NPs. Results in Table (5) illustrate the antibiotic susceptibility before and after CuO/ZnO NPs treatments against *K. pneumoniae* clinical isolates. CuO NPs and ZnO NPs convert the susceptivity of all isolates from resistance to sensitive/intermediate for ciprofloxacin, meropenem and imipenem. However, the

treatment of CuO NPs and ZnO NPs did not change the resistance capabilities of all isolates toward azithromycin, ampicillin, amikacin and amoxicillin-clavulanate except for isolate 4 in which CuO/ZnO NPs treatment convert the amikacin resistance characteristic into intermediate resistance. In addition, CuO NPs were more effective in some cases than ZnO NPs, with samples showing a greater increase in sensitivity to some antibiotics after treatment with CuO/ZnO NPs.

Table 5. Synergistic effect of CuO/ZnO NPs with different antibiotics against *K. pneumoniae* clinical isolates.

Isolate 1	Antibiotics						
	AZM	CIP	MEM	IPM	AM	AK	AMC
Before	R	R	R	R	R	R	R
CuO NPs	R	S	S	S	R	R	R
ZnO NPs	R	I	S	I	R	R	R
Isolate 2							
Before	R	R	R	R	R	R	R
CuO NPs	R	S	S	S	R	R	R
ZnO NPs	R	I	S	I	R	R	R
Isolate 3							
Before	S	R	R	R	R	R	R
CuO NPs	S	I	S	I	R	R	R
ZnO NPs	S	S	S	S	R	R	R
Isolate 4							
Before	S	R	R	S	R	R	R
CuO NPs	S	S	S	S	R	I	R
ZnO NPs	S	S	S	S	R	I	R

S: Sensitive, R: Resistant, I: Intermediate.

ZnO/CuO NPs demonstrated synergistic interaction with conventional antibiotics, effectively reversing multidrug resistance in *K. pneumoniae* clinical isolates and restoring the susceptibility of some antibiotics (ciprofloxacin, meropenem and imipenem). The synergistic mechanism involves multiple complementary pathways that collectively overwhelm bacterial defense systems. ZnO/CuO NPs disturb efflux pump functionally, a primary resistance mechanism in MDR resistant K. pneumoniae, by interfering with energy dependent transport systems and membrane integrity [35]. Simultaneously, metal NPs induced oxidative stress depleting cellular antioxidant reserve, compromising the bacteria to neutralize antibiotic mediated damage [36]. The generation of ROS generates membrane perforations that enhance antibiotic penetration, effectively increasing intracellular drug concentrations beyond resistance thresholds.

The Effect of ZnO/CuO NPs on Biofilm Formation

The antibiofilm formation of *K. pneumoniae* isolates by the effect of sub-MIC ZnO/CuO NPs was determined using a pre-sterilized 96-well polystyrene microtiter plate test. In Fig. (2), ZnO NPs treatment exhibited significant ($p < 0.01$) biofilm inhibition efficacy compared to control across all tested *K. pneumoniae* clinical isolates (except for isolate No. 1, no change in biofilm formation). The antibiofilm measurement revealed that ZnO NPs reduced biofilm formation to approximately 0.06-0.09 OD units, representing an 85-90% reduction compared to untreated controls. In contrast, CuO NPs treatment stimulated the significant ($p < 0.01$) production of biofilm formation compared to untreated controls, suggesting that resistant *K. pneumoniae* clinical isolates promote biofilm formation when exposed to CuO NPs.

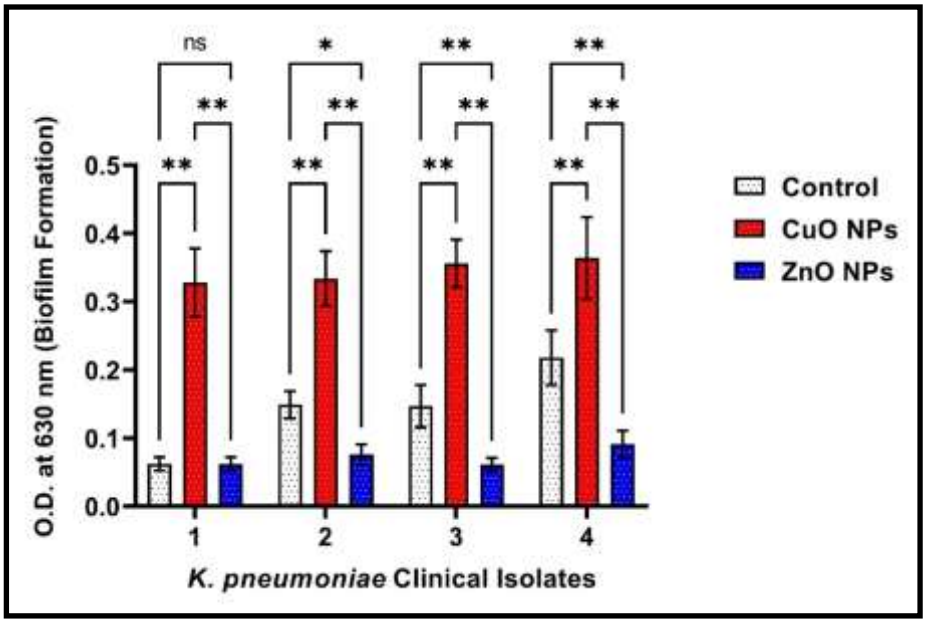

Figure (2): Biofilm formation of *K. pneumoniae* clinical isolates. Results represent the mean ± SD optical density (at 630 nm) of three independent experiments. NS: Non-significant, * $p < 0.05$, ** $p < 0.01$.

The results highly confirmed the antibiofilm activity of ZnO NPs against *K. pneumoniae* clinical isolates. Pourmehdiabadi et al., reported the efficacy of ZnO NPs in reducing the formation of *K. pneumoniae* ATCC 1383 biofilm by investigating the level of biofilm associated genes (*mrkH* and *fimH*) with notable decrease in mRNA level after treatment with ZnO NPs [37]. ZnO NPs at concentration 200 µg/mL showed effective biofilm reduction up to 88% in pathogenic *K. pneumoniae* isolated from clinical samples [38]. However, CuO NPs treatment exhibited unexpected pro-biofilm effect on *K. pneumoniae* isolates, representing paradoxical response to antimicrobial stress.

The Expression of *mrkA* and *uge* Biofilm Associated Genes

The expression of *uge* was significantly reduced upon NPs treatment. With CuO NPs, the mean *uge* transcript level significantly ($p < 0.01$) dropped to roughly 0.8 ± 0.03-fold compared to control (untreated) level. With ZnO NPs, *uge* was even further suppressed, down to about 0.4 ± 0.05-fold of control (indicating a 60% reduction, $p < 0.01$). Similarly, the *mrkA* gene showed significant ($p < 0.01$) downregulation to 0.6 ± 0.04-fold with CuO NPs and as low as 0.4 ± 0.04-fold with ZnO NPs. These changes were highly significant, underscoring that both nanoparticle types can dampen the expression of major virulence factors in clinical strains. Notably, ZnO NPs consistently caused a greater reduction in *uge* and *mrkA* expression than CuO NPs, suggesting that ZnO NPs exerts a stronger anti-virulence effect at the molecular level than CuO NPs (Fig. 3).

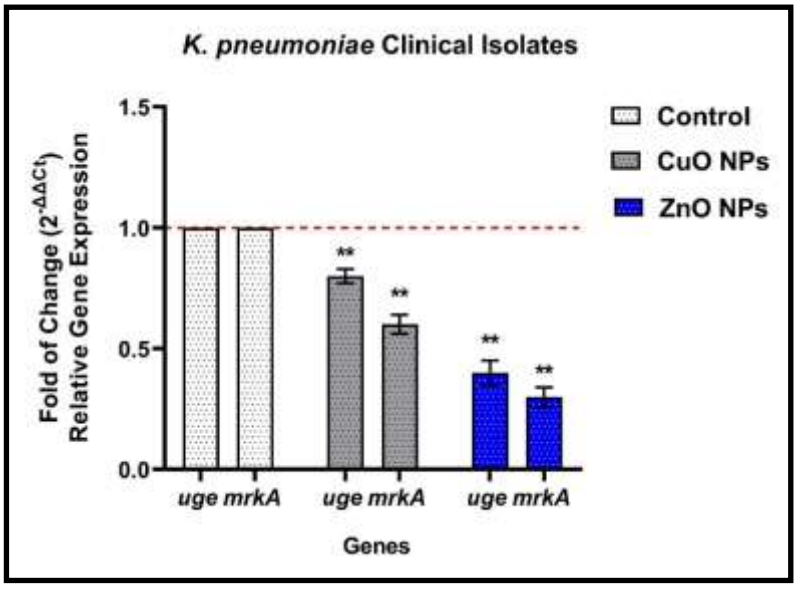

Figure (3): Relative expression ($2^{-\Delta\Delta Ct}$) of virulence capsule *uge* and fimbrial *mrkA* genes in clinical *K. pneumoniae* isolates following sub-MIC ZnO and CuO NPs exposure. ** $p < 0.01$.

A consistent pattern was observed: ZnO NPs had a stronger impact than CuO NPs in suppressing virulence gene expression. Statistically, the difference between CuO NP and ZnO NP treatments was significant for both *uge* and *mrkA*. ZnO NPs have been reported to generate ROS and cause cell envelope stress to a greater extent than CuO NPs [19, 39]. The greater stress imposed by ZnO NPs could lead to more extensive disruption of normal virulence factor production. The results corroborate and extend previous findings; for example, a recent study by Shivaee et al., also found that ZnO nanoparticles drastically reduced the expression of *mrkA* (by ~8.5-fold) in drug-resistant *K. pneumoniae* [40], which is in line with the downregulation we observed in clinical *K. pneumoniae* isolates treated with ZnO NPs. Likewise, capsule gene expressions have been shown to be attenuated by ZnO NPs in *K. pneumoniae*. This result was in concordance with other reports supporting the observation of *uge* suppression [41, 42].

Conclusion

ZnO and CuO nanoparticles demonstrate potent antimicrobial activity against multidrug-resistant K. pneumoniae clinical isolates, offering promising therapeutic alternatives through synergistic antibiotic enhancement and biofilm inhibition. ZnO nanoparticles showed superior anti-virulence effects, significantly downregulating key virulence genes while reducing biofilm formation by 85-90%, presenting innovative solutions for combating nosocomial infections.

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