ISSN 2664-4169 (Print) & ISSN 2664-7923 (Online)
South Asian Research Journal of Biology and Applied Biosciences
Abbreviated Key Title: South Asian Res J Bio Appl Biosci

| Volume-7 | Issue-3 | May-Jun -2025 | **DOI: https://doi.org/10.36346/sarjbab.2025.v07i03.002**

Original Research Article

Targeting Klebsiella Pneumoniae Pathogenicity Impending Biofilm and Virulence Gene Activity Using Zn Nano Particles and Ultrasound

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Article History
Received: 27.03.2025
Accepted: 02.05.2025
Published: 12.05.2025

Abstract: ***Background:*** Biofilm formation is a major challenge in healthcare, as bacteria encased in a protective matrix become highly resistant to antibiotics and immune defenses. Which urgently required a highly attention ***Objectives:*** This study investigates the antibiofilm activity of zinc oxide nanoparticles (ZnO NPs) and low frequency sonication against multidrug- resistant (MDR) bacterial isolates. ***Material and methods:*** Bacterial isolates were identified, including Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli, and Staphylococcus aureus, which were isolated form different clinical samples. *E. coli* strains were nominated to synthesize the ZnO NPs. Production of the Zn nanoparticles were confirmed using various professional analysis applications using UV-Visible spectroscopy, FTIR, XRD, and SEM. ***Results:*** treatment using 40 kHz ultrasound bath alone showed a 20% reduction in the ability of the *K.pneumoniae* of biofilm production after 30 min. ZnO nanoparticles (ZnO NPs) at MIC (12.5 mg/ml) exhibit clear antibiofilm activity 48.68% reduction, while the subMIC concentration revealed only 20.3%., but their efficacy is significantly enhanced when combined with 40 kHz ultrasound, leading to up to 18% biofilm inhibition against Klebsiella pneumoniae after 24 hours. Notably, sub-MIC below (12.5mg/ ml) of ZnO NPs exhibit a fluctuating effect on gene expression, for several biofilm-associated genes (FimH, K2A) as analyzed by real-time PCR (RT-PCR). ***Conclusion:*** These findings underscore the potential of combined therapy of ultrasound and ZnO NPs as a cost-effective, eco-friendly alternative for managing (MDR) bacteria and biofilm-related diseases, with implications for future therapeutic applications.

Keywords: Low frequency ultrasound; Gene expression; nanoparticles: Iraqi bacterial isolates.

INTRODUCTION

Klebsiella pneumoniae is a major nosocomial pathogen responsible for a wide range of infections, including pneumonia, urinary tract infections, and bloodstream infections, particularly in immunocompromised patients, the elderly, and those with underlying conditions such as diabetes [1] One of the key factors that contribute to its pathogenicity is its ability to form biofilms, which are complex, multicellular structures composed of bacterial cells embedded in an extracellular matrix. Biofilms provide bacteria with a protective niche, making them more resistant to both host immune defenses and antibiotic treatments [2] This biofilm-associated resistance is a significant challenge in treating infections, as biofilm-associated bacteria are up to 1,000 times more resistant to antimicrobial agents than their planktonic counterparts [3].

Biofilm formation in *Klebsiella pneumoniae* is a multifactorial process regulated by both environmental and genetic factors. The production of polysaccharide capsules, Fimbriae, and other surface-associated proteins plays a central role in this process, promoting adherence to surfaces and inter cellular aggregation [4]. Critical to the formation and maintenance of biofilms are specific virulence genes, such as those involved in capsule biosynthesis (*wcaJ*), iron uptake (*kfu*), and quorum sensing [5]. Additionally, the transition from planktonic to biofilm growth is regulated by stress responses, including oxidative stress, nutrient limitation, and the presence of host immune components, which trigger changes in gene expression and facilitate biofilm development [1].

The ability of *Klebsiella pneumoniae* to form robust biofilms and its associated resistance to conventional antibiotic therapy underscore the need for novel therapeutic strategies to combat these infections. In this context, nanotechnology offers promising alternatives, particularly the use of nanoparticles for antimicrobial applications. Zinc oxide nanoparticles (ZnO- NPs) have emerged as a potent tool for tackling bacterial infections due to their antimicrobial, anti-biofilm, and anti-virulence properties. ZnO- NPs exert their antimicrobial activity through several mechanisms, including disruption of bacterial cell membranes, generation of reactive oxygen species (ROS), and inhibition of cellular functions, all of which contribute to bacterial death [6].

The ZnO- NPs have been shown to interfere with the integrity of biofilms by penetrating the extracellular matrix and disrupting the structural cohesion of biofilm cells [7]. By interacting with the biofilm matrix, ZnO- NPs can promote the detachment of bacterial cells and decrease the formation of new biofilm layers. Additionally, ZnO- NPs can inhibit key virulence factors in *Klebsiella pneumoniae*, such as capsule production and biofilm-specific gene expression, by modulating bacterial gene regulatory networks. For example, studies have demonstrated that ZnO- NPs can down regulate the expression of the *kfu* gene involved in iron acquisition, a critical factor for biofilm growth [8]. By targeting these biofilm-specific genes and virulence factors, ZnO- NPs not only reduce the bacterial load but also decrease the pathogenicity of the organism.

Furthermore, the combination of ZnO- NPs with physical treatments such as ultrasound has shown to enhance the antimicrobial efficacy of nanoparticles. Ultrasound, particularly in the form of high-intensity focused ultrasound (HIFU), can improve nanoparticle penetration into biofilm structures, increase the localized temperature, and promote cavitation effects that disrupt bacterial cell membranes and biofilm matrices [9]. The synergistic effects of ZnO- NPs and ultrasound could lead to enhanced bacterial uptake of nanoparticles, improved biofilm disruption, and a reduction in the expression of virulent genes, making this combined approach a promising strategy for combating biofilm-associated infections caused by antibiotic-resistant *Klebsiella pneumoniae*.

The present study aims to investigate the efficacy of ZnO- NPs, both alone and in combination with ultrasound, in targeting biofilm formation and virulent gene expression in *Klebsiella pneumoniae after treatment*. This research could contribute to the development of novel therapeutic strategies that overcome biofilm-mediated antibiotic resistance and provide an alternative to conventional treatments in the fight against multidrug- resistant pathogens.

MATERIALS AND METHODS

Pathogenic Microorganisms

A total of thirty pathogenic bacterial isolates (Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Escherichia coli, and Staphylococcus aureus) were collected from various clinical sources (urine, wound, sputum, and nasal swabs) from patients of different ages and genders at several hospitals in Baghdad City. All samples were identified by the Vitec 2 system and transported and stored in the laboratory under standard septic conditions.

Detection of biofilm formation: A microtiter plate method was used to assess bacterial susceptibility to biofilm formation. Experiment done according to [10].

Biosynthesis of Zinc Oxide (ZnO) Nanoparticles

Fifty milliliters of sterilized nutrient stock were prepared and inoculated with an 18-hour-old culture of Escherichia coli, then incubated in a rotary shaker at 37°C for 24 hours. Afterward, the supernatant was collected via centrifugation at 10,000 rpm for 10 minutes. This supernatant was used to synthesize Zinc Oxide nanoparticles [11].

Characterization of Biosynthesized ZnO Nanoparticles UV-Visible Spectroscopy

The primary characterization of the biosynthesized ZnO nanoparticles was performed using UV-Vis spectroscopy (CARY 100 CONC and UV-Vis−NIR, Split−beam Optics, Dual detectors), scanning in the wavelength range of 200-900 nm after dispersing the ZnO nanoparticles in deionized water [12].

X-Ray Diffraction (XRD)

The crystalline structure of ZnO was determined using X-ray diffraction (XRD, Shimadzu 6100) [8].

Fourier Transform Infrared (FTIR) Spectroscopy

Employing the FTIR analysis is to detect the functional groups involved in the synthesis of ZnO nanoparticles the analysis followed [13]

Scanning Electron Microscopy (SEM): The shape and structure synthesized ZnO NPs were observed using (SEM, Carl Zeiss Ultra 55).

Determination Minimum Inhibitory Concentration (MIC) and sub-MIC of the synthesized ZnO nanoparticles

MIC and sub-MIC of the synthesized ZnO nanoparticles was determined. By adding a 1:1 concentration of 0.1 ml ZnO nps to 5 ml BHA, 4 different dilutions were prepared to get (100, 50, 25, 12.5 mg/ml) concentrations. Afterward, 100 µl of an overnight bacterial culture (1.510^8 CFU/ml) was added to each tubes and incubated at 37°C for 16-24 h growth assessed by measuring the O.D 450nm [14].

Effect of ZnO Nanoparticles on Biofilm Formation

The antibiofilm activity of the biosynthesized ZnO nanoparticles was evaluated using the method described by [15] with modification.

Sonication of bacterial suspensions

100 ml of bacterial suspension were sonicated using 40 kHz sonication bath. The temperature was maintained at 25 ºC by employing a ice bath. About one ml were drawn every 0, 5, 10, 15, 30 min after sonication. The effect of the antimicrobial has been analyzed using the spectrophotometer [16].

Antibiofilm effect of 40 kHz Sonication

Bacterial suspension, displayed to sonication 40 kHz for 30 minutes, following 1 ml of each interval sonication treatment time (0, 5, 10, 15, 30 min) were withdrawn and add to microtiter plate to ensure the antibiofilm effect of sonication alone on treated isolates followed same antibiofilm procedure has been described above [11].

RNA extraction

Transom Up Plus Kit (TRANS, China) was used to extract RNA. Bacterial cultures were centrifuged at 8,000 × g for 2 min at 4°C. Then, chloroform was added to the mixture, after that centrifugation at 10,000 × g for 15 min at 4°C to separate phases. The supernatant was collected and added into RNase-free tube. The final RNA precipitation was done using an equal volume of ethanol (96%-100%), and the solution was passed through a spin column. Flashed with buffers (CB9 and WB9), then centrifuge to remove excessive ethanol. The final products (RNA) were eluted with RNase-free water kept in at -80°C until the day of experiments [17].

Removing of Genomic-DNA

EasyScript® One-Step gDNA Removal and cDNA Synthesis Super-Mix (TRANS/China) kit, genomic DNA was removed. First-Strand cDNA Synthesis: Primers used in this study illustrated in (Table 1)

Table1 : Primers used in this study

Primer Name	DNA sequence (5 to 3)	Size (bp)
K2A	**F-** CAACCATGGTGGTCGATTAG **R-** TGGTAGCCATATCCCTTTGG	**543**
FimH	**F-** ATGAACGCCTGGTCCTTTGC **R-** GCT GAACGCCTATCCCCTGC	**688**

The reaction component of illustrated in (table 2):

Table 2: Components employed for analysis of cDNA

Component		**Volume**
Total RNA/mRNA		0.1 ng-5 /10 pg-500 ng
Anchored Oligo $(dT)_{18}$ Primer (0.5 µg/µl)		1 µl
Or Random Primer (0.1 µg/µl)		1 µl
Or GSP		2 pmol
2 × ES Reaction Mix		10 µl
EasyScript® RT/RI Enzyme Mix		1 µl
gDNA Remover		1 µl
RNase-free Water		Complete to 20 µl
Reaction Components for RT-PCR		
2×EasyTaq® PCR SuperMix		10
cDNA		2
Primers		2
Nuclease-free Water		6
Cycle conditions		
Steps	**Temperature**	**Time**
Denaturation	95°C	10 sec
Annealing	64°C	15 sec
Extension	72°C	20 sec

Multi experimental steps such as incubation at 25°C for 10 minutes and 42°C for 15 minutes were done to achieve the extraction at 85°C for 5 sec which employed to assure enzyme inactivation. RT-PCR was performed for RNA sample.

RESULTS

Thirty various bacterial isolates were collected from patients suffering from a multiple clinical disorder. all bacterial isolates were identified by the Vitek 2 system (Table 3).

Table 3: Distribution of Pathogenic Bacterial Isolates from Clinical Sources

Source	No. of Isolates	Percentage (%)	Isolate(s)
Wounds	5	16.66	*Pseudomonas aeruginosa*
Urine (3) / Sputum (3)	6	20.00	*Acinetobacter baumannii*
Wounds 8	8	26.66	*Klebsiella pneumoniae*
Urine (5) / Wounds (3)	8	26.66	*Escherichia coli*
Urine (1) / Wound (1)	2	6.6	*Staphylococcus aureus*
Nasal	1	3.3	*Klebsiella pneumoniae*
Total	**30**	**100**	

Then the following experiment carried out on K. pneumoniae only to assess the effect of Zno nanoparticles alone and in combination with 40 kHz sonication on the biofilm production.

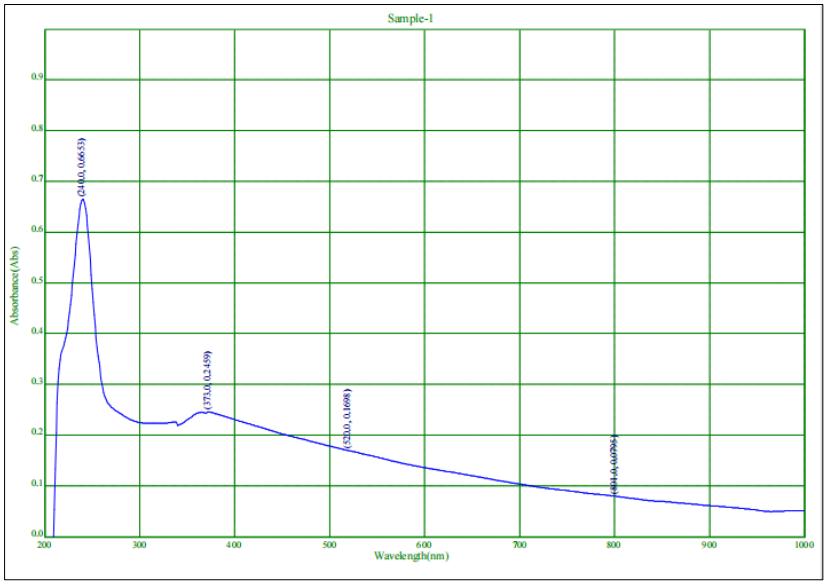
Biofilm Formation Using the Microtiter Plate Method

The ability of pathogenic bacterial isolates to form biofilms was assessed using the microtiter plate method [18], where biofilm formation was quantified by comparing the optical density (O.D.) values of stained bacterial cells adhered to the wells. The results indicated the following Klebsiella pneumoniae: 4 isolates produced strong biofilms, 2 isolates formed moderate biofilms, and 2 isolates displayed weak biofilm formation.

Biosynthesis of ZnO Nanoparticles (ZnO Nps): Three nonpathogenic Escherichia coli isolates (E. coli 1, E. coli 2, E. coli 3) were selected for the biosynthesis of zinc oxide nanoparticles (ZnO NPs) (Figure 1 A,B,C,D) [14].

Characterization of ZnO Nanoparticles

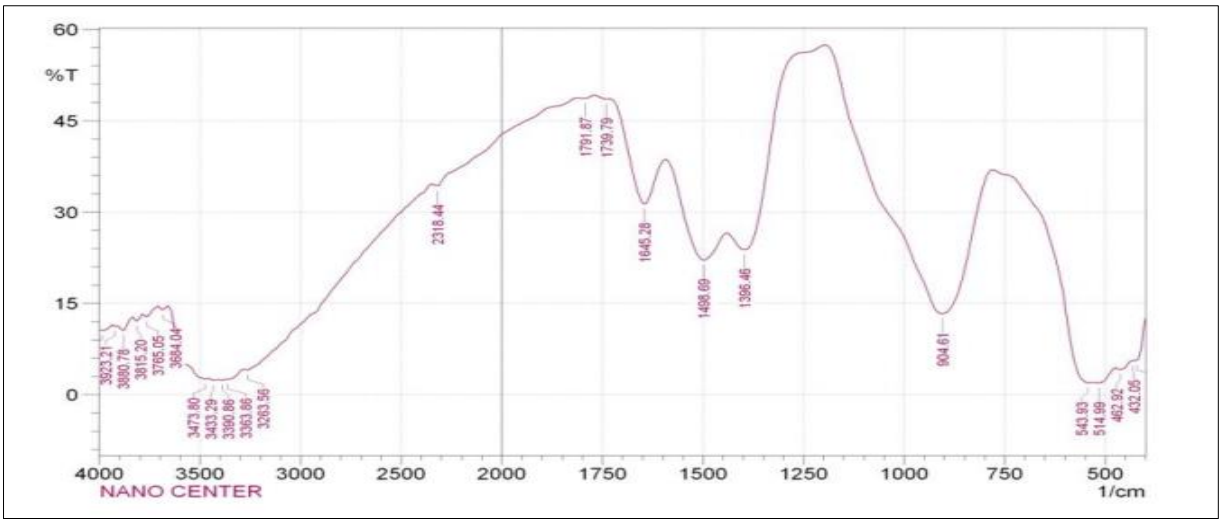
A. UV-Visible Spectroscopy: UV-Visible spectroscopy was used to examine the optical properties of the ZnO NPs, with an absorption peak at 240 nm confirming successful biosynthesis. This peak corresponds to the surface Plasmon resonance (SPR) of the nanoparticles, a characteristic feature [19] (Ifeanyichukwu et al., 2020; [20] Thamer, 2016). A similar absorption peak at 270 nm was observed by [21] Balogun et al. (2020), while [22] Huda (2018) noted a peak at 207 nm for ZnO NPs.



A

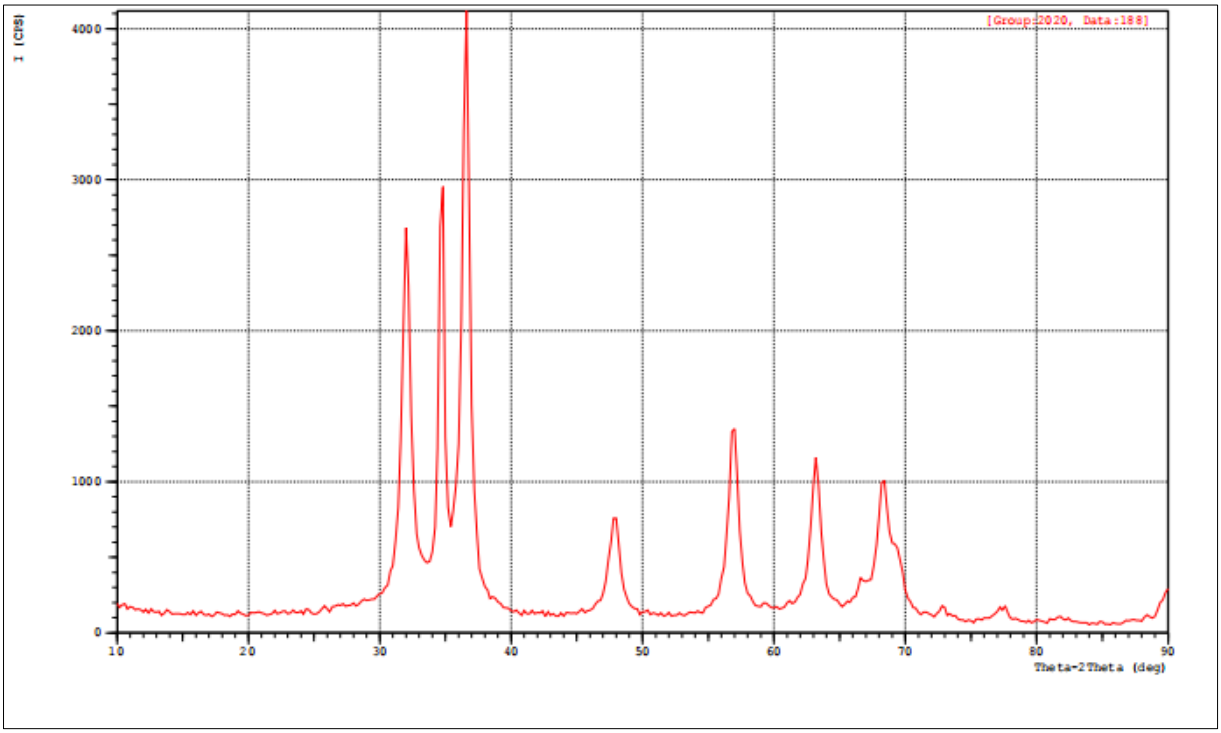
B. Fourier Transform Infrared (FTIR) Spectroscopy: FTIR analysis revealed key functional groups, including O-H stretching at 3684.04 and 3263.56 cm−1, C−H groups at 2315.44 cm−1, C−N stretching at 1396.46 cm−1, N−H

bending at 904.61 cm−1, and ZnO stretching at 432.05 cm−1. These findings are consistent with previous studies [22]; [23]; [24]; [25].



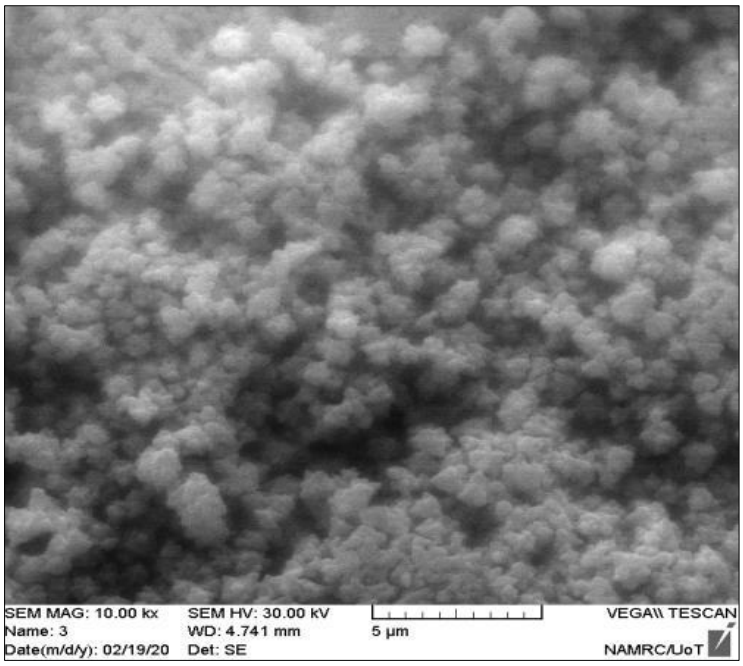
B

C. X-ray Diffraction (XRD): XRD analysis confirmed the hexagonal phase of ZnO NPs, with diffraction peaks at 32.48°, 34.73°, 36.57°, and others corresponding to the (100), (002), (101), (102), (110), (103), and (112) planes (JCPDS card No. 89-0510). The average crystalline size, calculated using Scherer's equation, was found to be 10 nm.



C

D. Scanning Electron Microscopy (SEM): SEM imaging showed that the ZnO NPs had a flower-like morphology, with particle sizes ranging from 23.87–26.57 nm. This flower shape was produced through a solution process at 90°C using zinc acetate dehydrate and NaOH [26].



D

Figure 1: Characterization of ZnO Nanoparticles A. UV-Visible Spectroscopy B. Fourier Transform Infrared (FTIR) Spectroscopy C. X-ray Diffraction (XRD) D. Scanning Electron Microscopy (SEM)

E. Shelf Life: The synthesized ZnO NPs were stable for 7 months when stored at temperatures of 4°C, 25°C, and 37°C, with no significant changes in color or properties. UV-Visible spectroscopy and FTIR reanalysis showed that the NPs maintained good transparency across the range of 200–900 nm (Figure 3.19). FTIR analysis further confirmed the stability, with characteristic peaks at 3401 cm−1 (O-H), 1471 cm−1 (C−O−H bending), 915 cm−1 (N-H), and 434 cm−1 (ZnO stretching), indicating the continued presence of functional groups associated with the ZnO NPs.

Antibiofilm Activity on Microtiter Plate Method: Data reveled ZnO NPs has a moderate effect on inhibiting biofilm formation by multidrug-resistant bacterial isolates with 48.68% reduction in biofilm formation using 12.5% mg/ml of ZnONps (MIC) concentration, while the subMIC concentration revealed only 20.3% reduction on ability of biofilm formation for *K. pneumoniae* isolates.

Calibration of ultrasonic equipment: Calorimetry is the most common and simple method employed to calibrate ultrasonic equipment in terms of power input. Calorimetry method done according to [16].

Table 4: Calculation of ultrasonic power using 40kHz sonication bath

Frequency kHz	Polynomial equations	Power = dT/dt x Cp x m (Watt)
40	**y = 0.0286x + 14.46** **R^2 = 0.9803**	0.0286 ˚C x 4.19 J / (g x ˚C) x100g **P = 11.983 W**

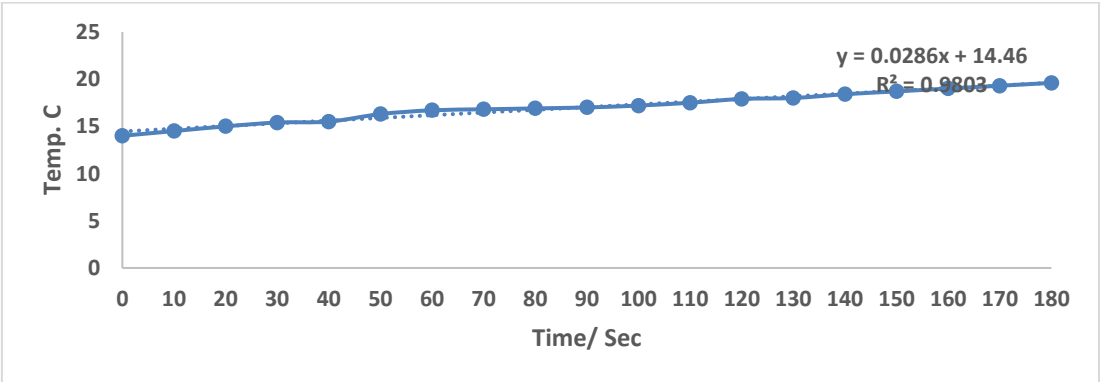


Figure 2: Calibration of ultrasonic equipment 40 kHz ultrasonic bath, power setting 100%, using 100 ml of distilled water

Anti-biofilm effect of K. pneumonia treated using 40 kHz ultrasonic bath for 30 min.

The effect of ultrasound frequency at 40 kHz bath on 100 ml *K. pneumonia* bacterial suspension biofilm forming analyzed using a spectrophotometer with a wavelength of 630 nm. In this experiment, three readings were averaged at different intervales times (0, 5, 10, 15, 30 mins).

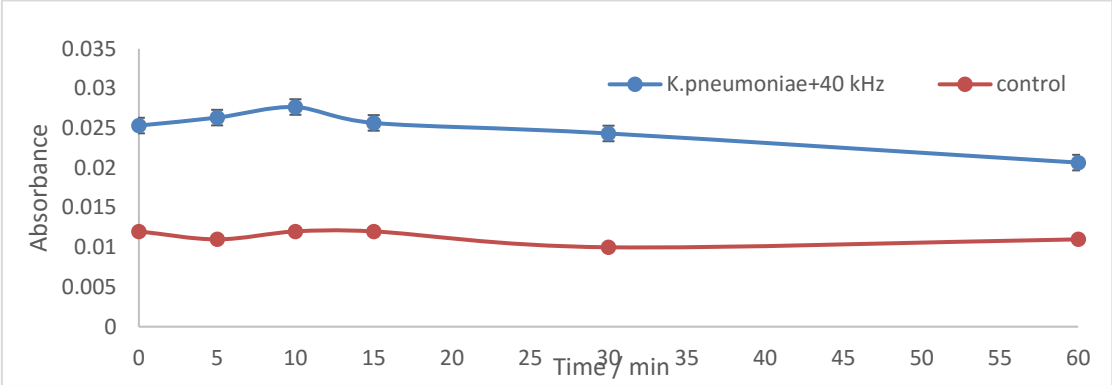


Figure 3: Anti biofilm effect of Klebsiella pneumonia treated with 40 kHz ultrasonic bathe for 30 min

Result revealed a moderate effect for the 40 kHz frequency on the biofilm forming ability, of As well as, sonication Cavitation process has the potential to clean biofilms from a substrate at the microscopic level with minimal damage to the underlying surface.

Impact of combined treatment (ZnO NPs and 40 kHz ultrasound) on Biofilm-Related Genes in K. pneumoniae

This research examined, the expression of key biofilm-associated genes (fimH, magA). Real-time PCR (RT-PCR) traditionally employs reference or housekeeping genes to account for variability between samples [30]. However, recent findings suggest that some housekeeping genes may exhibit significant variation in certain biological samples. The RT-PCR technique was used to assess the genotypic expression of these genes in Klebsiella pneumoniae.

The expressions of the (Fim H, mag A genes) which are considered as important biofilm formation genes were examined. RNA expressions using real-time PCR has traditionally used reference or housekeeping genes to control for error between samples [30]. Undouble some housekeeping genes might considerably vary in their expression in different biological samples effect of combined treatment on Fim H gene showed in (figure 4).

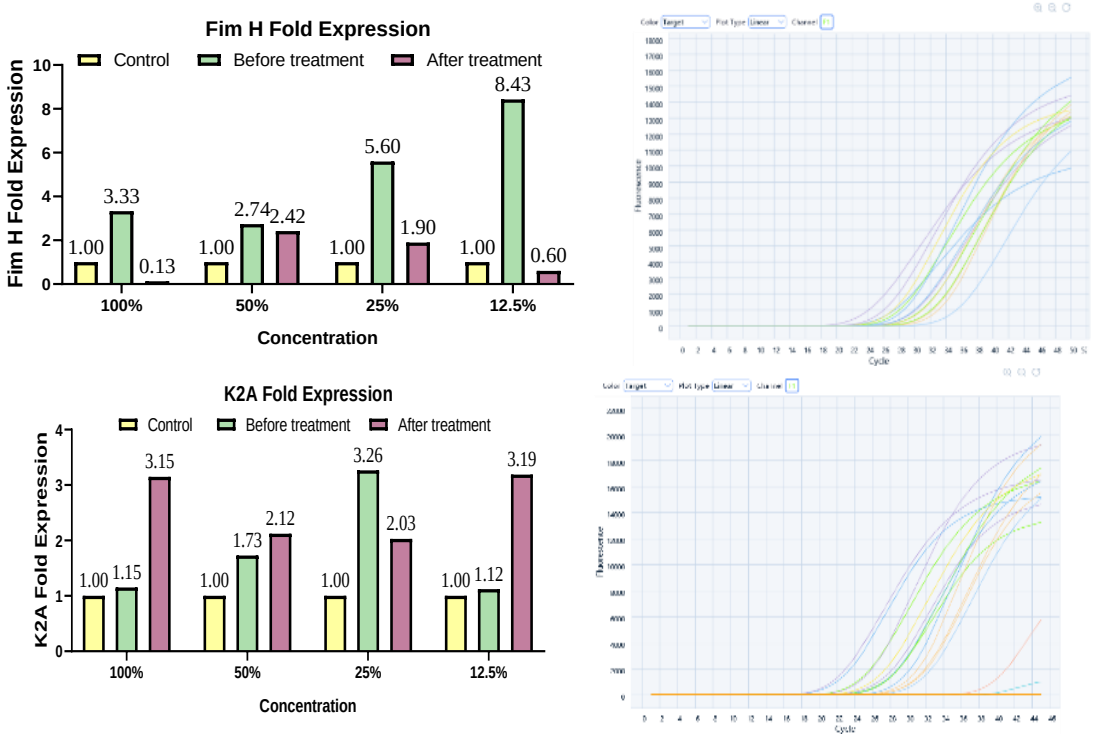


Figure 4: Fold Expression of key biofilm-associated genes (FimH, K2A) analyzed using Real-time PCR (RT-PCR)

DISCUSSION

The large surface area of nanoparticles and the presence of functional groups, such as amino and hydroxyl groups, enable nanoparticles to interact with bacterial cell wall [27]. Now adays Biofilm-related diseases consider a major issue due to resistance to wide range of conventional antibiotic choice [28]. ZnO nanoparticles exhibit bioactivity to control biofilm, thus the significant depend on the many factors such as concentration. To sum up, ZnO nanoparticles represents a hopeful antibacterial agent, offering advantages over conventional antibiotics [29]. Current studies are exploring the potential of nanoparticles' penetrating power for biofilm eliminating [30]

The range of high energy, low frequency high intensity ultrasound is between 20 and 100 kHz. Ultrasound with a low frequency produces significant mechanical and shear stresses [26]. Studies showed that wave pressure variations cause bubbles to form and collapse during a process known as acoustic cavitation. When ultrasonic waves are shed on water and other liquids, this process occurs. Both the hydrodynamic and thermal boundary layers are disturbed by this appearance. The small gas bubbles suspended in the liquid expand quickly and break up into numerous smaller bubbles when the local pressure in the vacuum period is less than the liquid's vapor pressure, which raises the temperature and intensifies the projected frequencies [27]. The extraordinarily high temperatures produced when the cavitation bubble collapsed, bacterial cells was unable to perform the regular activity, thus ultrasound has been used to control pathogens biologically [28].

The mechanical properties of the ultrasound, such as frequency and acoustic pressure, and the properties of the fluid such as surface tension and temperature, can be tailored for more effective disruption of biofilms. The impact of such parameters on ultrasonic biofilm removal in particular requires more research and has been addressed in the past study [26]. As a result, various effects are created. The chemical effect is summarized in the primary radicals generated by ultrasound are H and OH On the other hand, when an ultrasonic wave passes through a solid medium, it produces a series of alternating contractions and expansions, a phenomenon known as the “sponge effect,” which facilitates the transfer of matter with the medium surrounding the solid. Moreover, this mechanical stress can cause the formation of micro channels in the interior of the solid, also favoring mass transfer processes [27].

Data The combined treatment of sonication and ZnO nanoparticles significantly influenced the fold expression of the studies genes, which plays a crucial role in biofilm formation and adhesion in Klebsiella pneumoniae. The synergistic effect of these two treatments resulted in a flatulated regulation of Fim H gene expression, as observed through real-time PCR analysis [30]. In contract the combined treatment of sonication and ZnO nanoparticles significantly downregulated K2A gene expression, reducing biofilm stability in Klebsiella pneumoniae. Sonication disrupted biofilm structure through acoustic cavitation, while ZnO nanoparticles enhanced this effect via reactive oxygen species (ROS) production and membrane disruption. The synergy of both treatments led to a greater reduction in K2A expression than individual treatments, weakening biofilm integrity and bacterial virulence which comes compatible with [30]. These findings highlight the potential of this combined approach for controlling biofilm-associated infections, warranting further optimization for enhanced therapeutic efficacy. This study highlights that sub-MIC ZnO nanoparticles (ZnO NPs) can induce bacterial stress, potentially upregulating biofilm and virulent gene expression in Klebsiella pneumoniae. Low ZnO NP concentrations may trigger a bacterial survival response, enhancing biofilm formation through increased EPS production and quorum sensing activation [31].

However, when combined with 40 kHz sonication, the antibiofilm efficacy of ZnO NPs is significantly enhanced, counteracting this adaptive response. Sonication disrupts biofilm structure, enhances ZnO NP penetration, and increases ROS production, leading to oxidative stress and bacterial cell damage [32]. Clinically, these findings suggest that while sub-MIC ZnO NPs alone may promote biofilm formation, their effectiveness can be maximized through sonication, preventing bacterial adaptation. Further research should explore the specific gene expression changes induced and their impact on bacterial resistance.

CONCLUSION

This study shows that sub-MIC levels of ZnO nanoparticles (ZnO NPs) can influence biofilm formation in Klebsiella pneumoniae, with some biofilm-related genes becoming more active as a bacterial stress response. However, when combined with 40 kHz ultrasound, the antibiofilm effect was significantly stronger, likely because ultrasound helps ZnO NPs penetrate the biofilm, increases ROS exposure, and physically disrupts the biofilm structure. These findings suggest that using ZnO NPs with ultrasound could be a promising approach to tackling MDR biofilm infections. More research is needed to fine-tune this combination and better understand how it affects bacterial gene expression, making it a potential solution for medical applications like antimicrobial treatments and device coatings**.**

Acknowledgements: Authors in this study would like to thank Mustansiriyah University (http://uomustansiriyah.edu.iq/) in Baghdad, Iraq for its support to finish the present work.

Author Contributions: All authors read and approved the manuscript.

Data Availability: All data and strains in this study are available from the corresponding author with a reasonable request.

Conflict of Interest: The author declares no competing interests.

Ethical Approval: This article does not contain any studies with human participants or animals that were performed by any of the authors.

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