Research Article

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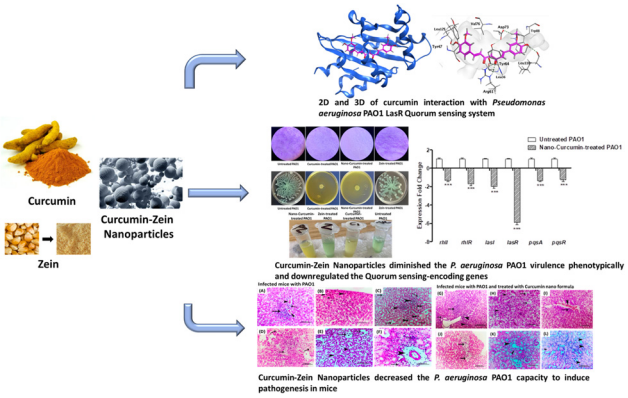
Utilization of zein nano-based system for promoting antibiofilm and anti-virulence activities of curcumin against *Pseudomonas aeruginosa*

https://doi.org/10.1515/ntrev-2023-0212
received October 22, 2023; accepted February 4, 2024

Abstract: Bacterial biofilms contribute to increased pathogenesis and bacterial resistance. Biofilms can enhance pathogenicity by shielding bacteria from the immune system and antibiotics, and they are associated with persistent infections. Additionally, the antibiotic resistance mechanisms within biofilms make them challenging to treat, emphasizing the need for strategies to be addressed. Mitigating bacterial virulence is a promising strategy that could ease their eradication by host immunity without stressing bacteria to induce resistance. The merits of this strategy are augmented when using safe anti-virulence candidates in proper formulations. The current study aimed to evaluate the antibiofilm and anti-virulence efficacy of curcumin–zein nanoparticles against *Pseudomonas aeruginosa*. *In vitro* investigations were performed to assess the effect of nanoparticles on biofilm formation, bacterial motility, and



Graphical abstract

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production of virulence factors, including proteases, hemolysins, and pyocyanin, in comparison to bulk curcumin. Furthermore, the effect on the expression of the genes that encode quorum sensing (QS) systems that regulate bacterial virulence was assessed. An *in silico* study was done to evaluate the affinity of curcumin to QS receptors. Additionally, an *in vivo* protection assay was performed to evaluate the inhibitory effect of our preparation on diminishing the *P. aeruginosa's* capacity to induce pathogenesis. The results showed significant antibiofilm and anti-virulence activities of the curcumin–zein nanoparticles compared to bulk curcumin. These anti-virulence activities were attributed to the curcumin's interfering with the *P. aeruginosa* QS systems that regulate its virulence. In conclusion, curcumin acquires significant anti-QS, anti-virulence, and antibiofilm activities that are vastly enhanced upon loading on zein nanoparticles.

Keywords: curcumin, zein nanoparticles, bacterial biofilms, antibiotic resistance, healthcare, *Pseudomonas aeruginosa*

1 Introduction

The rapid spread of antibiotic resistance in bacteria worldwide has become a significant concern for public health. Multiple studies have highlighted the alarming increase in illness and death rates linked to the growing prevalence of multidrug-resistant bacterial pathogens [1–3]. Bacterial biofilms can significantly contribute to bacterial resistance [4,5]. Biofilms are complex three-dimensional structures formed by clusters of bacterial cells surrounded by extracellular polymeric substances [6,7]. The complex structure and behavior of bacterial biofilms create a formidable challenge for antibiotic treatments [8–12]. Epidemiological research has notably identified biofilms as significant contributors to hospital-acquired infections [7,13–16]. This resistance mechanism is of particular concern in chronic infections associated with biofilm formation, such as those involving medical devices, wounds, and implant-related infections [14,17,18]. Addressing biofilm-related resistance often requires strategies that target both the biofilm matrix and the bacteria within the biofilm to achieve effective treatment [6,19,20].

Bacterial virulence is a multifactorial phenomenon, and it arises from the expression of various regulatory systems, notably quorum sensing (QS) systems. These QS systems play a pivotal role in coordinating and controlling the virulence behaviors of bacteria [21,22]. The QS systems intricately coordinate bacterial virulence through the secretion of specialized signaling molecules referred to as autoinducers [23,24]. In Gram-negative bacteria, QS systems mainly rely on *N*-acyl-homoserine lactones as signaling molecules that bind to their cognate receptors, forming autoinducer/receptor complexes that could control the expression of diverse virulence factors [21,25]. Studies have convincingly demonstrated the crucial roles of QS systems in the formation of bacterial biofilms and orchestrating the synchronized secretion of virulence factors [26,27]. *Pseudomonas aeruginosa* is a Gram-negative bacterium responsible for a broad spectrum of severe opportunistic infections [28–30]. In addition to its notable capability to develop antibiotic resistance, *P. aeruginosa* possesses an extensive array of virulence factors [3,31–33]. The QS systems of *P. aeruginosa* play the main role in controlling the expression genes that encode diverse virulence factors and are involved in biofilm formation [34–36].

In the dwindled supply of novel antibiotics against the continuous increase in bacterial resistance, there is a growing need to identify new drug targets and create innovative therapeutic strategies for addressing bacterial infections [37–39]. One of the most promising strategies involves reducing bacterial virulence without exerting pressure on bacteria to develop resistance [40–43]. This can be achieved by targeting the main virulence-controlling systems, namely QS systems [37,44–46]. Given this context, natural antimicrobial compounds extracted from plants have gained significant recognition as viable alternatives to traditional antimicrobials and antibiotics [42,47–51]. The antimicrobial, anti-biofilm, and anti-QS properties of secondary metabolites found in plants such as curcumin offer a promising avenue for combating bacterial infections [52,53]. Curcumin, a natural compound found in turmeric, has demonstrated antimicrobial activities against a wide range of microbes, including bacteria, fungi, and some viruses [54,55].

It is important to note that while curcumin shows promise as an antimicrobial, antibiofilm, and anti-virulence agent, its efficacy can vary depending on factors like concentration and formulation [56,57]. Therefore, the appropriate formulation of curcumin has the potential to ensure effective antibacterial and antibiofilm activity. The formulation process can enhance curcumin's stability, bioavailability, and ability to interact with microbial cells and biofilms, ultimately improving its antimicrobial properties for various applications. For instance, curcumin nanoparticle formulations offer a promising avenue in the development of potential antimicrobials overcoming challenges associated with traditional antibiotics delivery into the biofilm matrices [58,59].

In the current study, curcumin has been formulated as zein nanoparticles using a modified liquid–liquid phase separation technique. The prepared nanoparticles were characterized for size and zeta potential. The objective of

the current study is to evaluate the effectiveness of the prepared curcumin–zein nanoparticles in eliminating biofilms and reducing the virulence of *P. aeruginosa*.

2 Materials and methods

2.1 Materials

The chemicals were of pharmaceutical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). All microbiological media were procured from Oxoid (Hampshire, UK). *P. aeruginosa* PAO1 strain was utilized in this research.

2.2 Preparation of curcumin–zein nanoparticles

The modified liquid–liquid phase separation technique, previously described by Algandaby *et al*. [60], was adopted for preparing curcumin–zein nanoparticles in a ratio of 1:1 w/w. In brief, an ultrasonic probe (Vibra-Cell VCX 750; Sonics and Materials, Inc., Newtown, CT, USA) was utilized to dissolve accurately weighed equal amounts of curcumin (10 mg, in 15 m absolute ethanol) and Zein (10 mg, in 15 m 80% ethanol). The resulting solution was added to 30 mL deionized water, and the formed dispersion was agitated at 3,000 rpm at ambient temperature for 2 h. After complete ethanol evaporation, the aqueous dispersion was subjected to ultra-centrifugation at 20,000*g* and then lyophilized for 48 h.

2.3 Size and zeta potential measurements

After proper dilution with double distilled water, the size (z-average) and zeta potential of the prepared Curcumin-zein nanoparticles were determined using dynamic light scattering and electrophoretic techniques, respectively, using Malvern Zetasizer (Nano Z5P, Malvern Panalytical Ltd, United Kingdom). The results were expressed as the mean value of three replicates.

2.4 Detection of minimum inhibitory concentrations (MICs)

The MICs were determined using the microtiter plate broth dilution method according to Clinical Laboratory and Standards Institute guidelines [43,61]. The influence of the tested preparations on bacterial growth was assessed by measuring the optical density of *P. aeruginosa* cultures with or without the tested preparations as described [62,63].

2.5 Determination of antibiofilm activity

The inhibition of biofilm formation was evaluated using the crystal violet method as detailed [40,42]. Bacterial suspensions were prepared from an overnight culture in tryptic soya broth (TSB), and its optical density was adjusted to OD600 of 0.4 (equivalent to 1×10^8 CFU/mL). Subsequently, 10 µL aliquots of the suspension were introduced into 1 mL of fresh TSB, with and without tested preparations at sub-MIC. Portions of 100 µL of TSB, both with and without tested preparations, were dispensed into the wells of a 96-well microtiter plate and incubated at 37°C for 24 h. After incubation, planktonic cells were aspirated, and the wells were subjected to three washes with distilled water before being left to dry. The attached cells were then fixed with methanol for 15 min, followed by staining with a 1% crystal violet solution for an additional 15 min. Post-staining, the wells were washed, and the elution of the attached dye was carried out using 33% glacial acetic acid. The absorbance was measured at 590 nm.

To visualize the biofilm inhibition, bacterial biofilms were allowed to be formed on glass slides positioned in polystyrene petri plates, both in the presence and absence of tested preparations. Incubation of the plates took place for 24 h at 37°C. Post-incubation, the slides underwent a triple wash with water and were subsequently stained with crystal violet (1%) for a duration of 20 min. The stained slides were scrutinized under a light microscope at 100× magnification.

2.6 Detection of anti-virulence activity

2.6.1 Effect on bacterial motility

The *P. aeruginosa* swarming motility was evaluated on Muller Hinton (MH) agar plates (1.5% agar) provided with tested preparations, and the zones of motility were measured as demonstrated [43,49].

2.6.2 Anti-proteolytic activity

The inhibitory effect of tested preparations on protease production was evaluated using the skim milk agar

method, as previously shown [47,62]. Luria–Bertani (LB) broth tubes, both with and without tested preparations at sub-MIC, were inoculated with *P. aeruginosa* and incubated overnight at 37°C. Upon centrifugation to collect supernatants, 100 µL of these supernatants were applied to wells created in skim milk agar plates (5%). Following an overnight incubation at 37°C, the clear zones resulting from proteolytic activity were measured.

2.6.3 Effect on hemolysins

The effect of the tested preparations on the activity of *P. aeruginosa* hemolysins was assessed as detailed in previous studies [63,64]. To conduct this evaluation, 0.5 mL of the prepared supernatants, as obtained in the protease assay, were combined with 0.7 mL of a fresh erythrocyte suspension in 2% saline and incubated at 37°C for 2 h. Subsequently, the absorbance of released hemoglobin resulting from lysed erythrocytes was measured at 540 nm in the separated supernatants obtained through centrifugation at 4°C. The released hemoglobin levels were compared with a positive control (0.1% SDS in erythrocyte suspension) and a negative control (erythrocytes in LB broth).

2.6.4 Effect on pyocyanin production

The production of *P. aeruginosa* virulent pigment was assessed both in the presence and absence of curcumin preparations, as previously demonstrated [40,50]. *P. aeruginosa* was cultivated in LB broth and incubated overnight, and the resulting bacterial suspension was adjusted to an OD of 0.4 at 600 nm. Subsequently, 10 µL of the prepared bacterial suspensions were introduced into LB broth tubes (1 mL) containing tested preparations at sub-MIC, as well as into control tubes. These tubes were then incubated at 37°C for 48 h. Following centrifugation to separate the supernatants, the inhibition of pyocyanin was evaluated by measuring the absorbance at 691 nm.

2.7 Effect on the expression of QS-controlling genes

The qRT PCR was used to quantify the expression of *P. aeruginosa* QS-encoding genes. The total RNA was extracted from bacterial cells treated or untreated with the tested preparations using Gene JET RNA Purification Kit (Thermo Scientific, USA) and kept at −80°C as described [43,65]. The encoding genes of the *P. aeruginosa* QS systems lasI/R, rhlI/R, and pqsA/R were amplified using the primers listed [43,66]. The amplification was carried out using SensiFAST™ SYBR® Hi-ROX OneStep Kit (Bioline, UK) using StepOne Real-Time PCR system (Applied Biosystem, USA). The housekeeping gene *rpoD* was used to normalize the relative expressions, and the comparative threshold cycle (ΔΔCt) method was employed to calculate the relative gene expressions [65,67].

2.8 Virtual affinity of curcumin to *P. aeruginosa* QS receptors

Crystal structures of *P. aeruginosa* LasR (PDB: 6MVN) [68], QscR (PDB: 6CC0) [69], and PqsR (PDB: 4JVD) [70] were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/accessed on 7 February 2023). The protein structures were prepared utilizing the QuickPrep protocol of Molecular Operating Environment (MOE 2019.012). Curcumin structure was retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/accessed on 7 February 2023) as SMILES. Curcumin structure was energy minimized to 0.1 Kcal/mol/Å^2 gradient RMS. The docking process was performed with Alpha triangle placement through Amber10: EHT force field.

2.9 *In vivo* evaluation of the anti-virulence activity

To investigate the impact of the tested preparations on diminishing the *P. aeruginosa* pathogenesis, the mice protection assay was performed [41,43]. Four groups of 3-week-old albino mice, each containing five, were recruited. The first group was kept uninfected, and the second group was intraperitoneally injected with sterile phosphate buffer saline as negative control groups. The mice in the next group were injected with DMSO-treated *P. aeruginosa* as a positive control group. The last group was injected with *P. aeruginosa* treated with nanoparticle formulation at sub-MIC. After 5 days, the mice were humanely euthanized *via* cervical dislocation, and the kidney and liver tissues were extracted for histopathological examination as described previously [43,71].

2.10 Statistical analysis

The tests were done in triplicate, and the results were averaged. The findings are presented as mean ± SD.

Statistical significance was assessed using one-way ANOVA, and the significance is considered when $p < 0.05$.

3 Results and discussion

3.1 Particle size and zeta potential of curcumin–zein nanoparticles

The prepared curcumin–zein nanoparticles showed an average size (*z*-average) of 380.93 ± 4.21 nm. The reported size in the nano-range highlights the capability of the proposed formulation to augment the anti-virulence properties of curcumin. Furthermore, the prepared formulation showed a high degree of homogeneity, as indicated by a low polydispersity index value of less than 0.1. Regarding the zeta potential, the prepared nanoparticles exhibited an average of −1.82 ± 0.065, indicating reduced liability for immune recognition of the proposed formulation, with consequent enhanced efficacy. Representative size distribution by intensity and zeta potential plots are shown in Figure 1a and b, respectively.

Particle size plays a pivotal role in delivery systems designed for anti-virulence and antibiofilm applications. Nano-delivery systems are generally reported to have sizes in the 1–1,000 nm range [72]. More specifically, a size of less than 500 nm is considered appropriate for drug delivery applications [73]. At the nanoscale, nanoparticles exhibit an increased surface area, allowing for greater contact with microbial cells, thereby enhancing interactions for improved antimicrobial activity [74]. Thus, the increased surface area is particularly significant in anti-virulence strategies, where disrupting virulence factors is essential

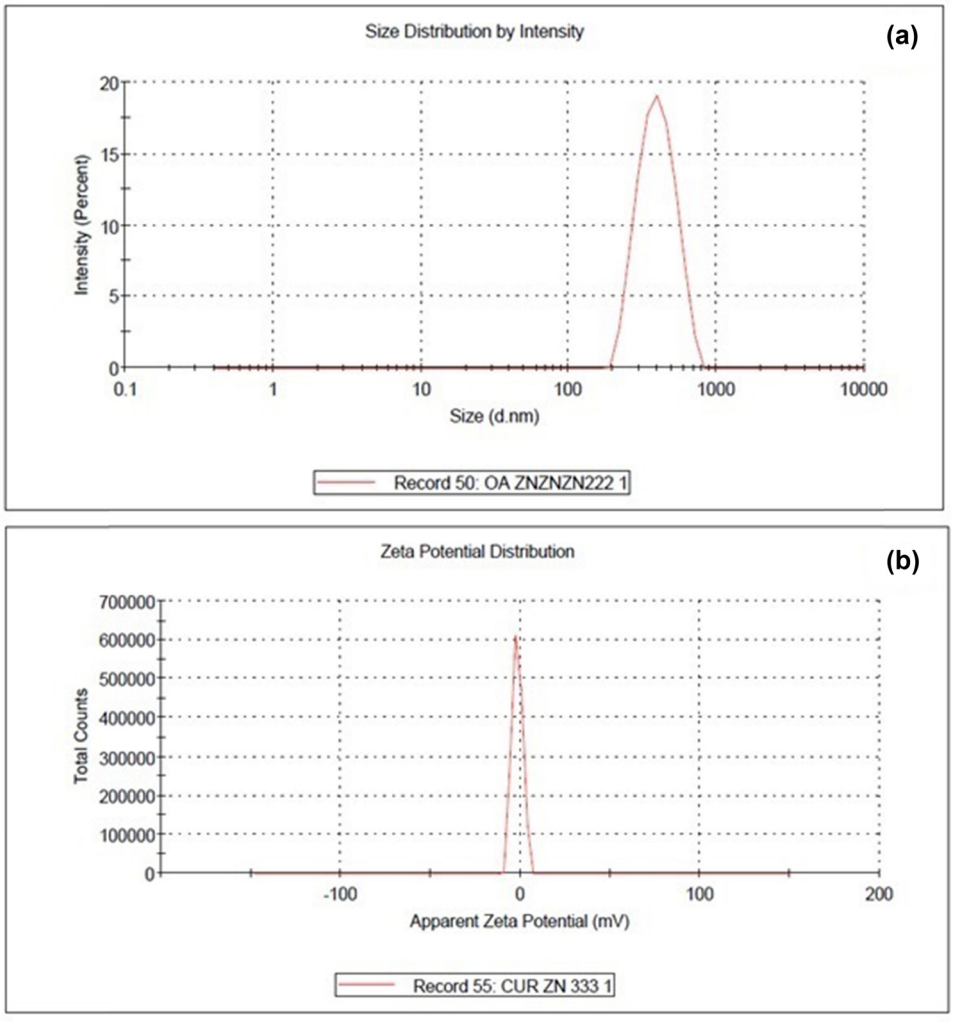

Figure 1: Particle size distribution (a) and zeta potential (b) of curcumin–zein nanoparticles.

for mitigating the severity of infections. The increased surface area facilitates the efficient binding of nanoparticles to virulence factors, impeding their function and reducing the pathogen's ability to cause harm [74]. Furthermore, a major factor is the size-dependent toxicity of nanoparticles, wherein smaller particles exhibit higher toxicity due to their enhanced ability to interact with microbial components [75]. In antibiofilm applications, particle size reduction is crucial to stop and eliminate biofilm development. Biofilms pose challenges in terms of resistance to conventional antimicrobial treatments. Nanoparticles with appropriate sizes can penetrate biofilms more effectively, disrupting the matrix and the embedded microbial cells [76]. This enhanced penetration is crucial for the successful dismantling of biofilms and preventing their recurrence. Another important hurdle that can also be addressed by nanosystems is the development of microbial resistance. The multifaceted interactions of nanoparticles with microbial cells and biofilm matrices make it challenging for pathogens to develop resistance, enhancing the overall efficacy of anti-virulence and antibiofilm strategies [77].

In conclusion, the importance of nanosize for anti-virulence and antibiofilm applications lies in its ability to influence surface interactions, penetration, and resistance prevention. Harnessing these properties through controlling size in the nano-range offers promising solutions to combat virulence and biofilm-related hurdles.

The zeta potential of nanoparticles, reflecting their surface charge, plays a pivotal role in drug delivery systems, influencing critical aspects such as stability and immune recognition. Nanoparticles with a high absolute zeta potential exhibit increased electrostatic repulsion, preventing aggregation and ensuring the stability of colloidal suspensions [78]. Nevertheless, nanoformulation can exhibit stability even when possessing a low zeta potential. This is due to the presence of additional variables, such as steric stabilization, which can also contribute to the stability of the dispersion [79].

Furthermore, the zeta potential affects the inflammatory and immune response to nanoparticles, with a neutral or slightly negative zeta potential being associated with reduced immune recognition and less inflammatory response compared to positively charged ones [80,81]. It is reported that positive surface charge leads to quick adsorption by serum proteins, including immunoglobulins that tag them for clearance by the reticuloendothelial system; on the other hand, neutral or slightly negative charges helps in avoiding removal by such phagocytic system, thereby increasing the half-life of nanoparticles in the bloodstream and enhancing their potential for drug delivery applications [81]. In summary, careful manipulation of the zeta potential is integral to optimizing the stability and immunological characteristics of nanoparticles in drug delivery systems.

3.2 Effect on bacterial growth at sub-MICs

The nanoformulation significantly reduced the MIC of curcumin as it was 1.25 or 1 mg/mL for curcumin in bulk or zein, respectively, while the MIC of the curcumin nanoformulation was 0.5 mg/mL. These results highlight the potent antibacterial activity in comparison to other nanoparticle preparations. For instance, the MIC of silver–curcumin nanoparticles inhibited *P. aeruginosa* at 10 mg/mL [82].

The strategy of mitigating the virulence of bacteria is promising to combat bacterial resistance as it is rooted in the idea of not affecting bacterial growth to avoid inducing stress in the bacteria, which can lead to the development of resistance [83,84]. To exclude any influence on the growth of bacteria, the anti-virulence and anti-QS activities of curcumin in bulk or nanoformulation, or zein, were tested at sub-MICs (1/8 MIC). There were no significant differences between the optical densities of the *P. aeruginosa* cultures with or without the tested preparations. This indicates that any *in vitro* or *in vivo* activity is due to the effect of preparation on virulence and not due to the inhibition of bacterial growth; all further experiments were conducted at sub-MICs.

3.3 Inhibition of biofilm formation

Bacterial biofilm is a complex and structured community of bacteria that adhere to surfaces and are enclosed within a protective matrix of extracellular polymeric substances [26,85]. Biofilms can form on various surfaces, such as medical devices, tissues, and natural environments. These biofilms are responsible for a wide range of persistent and chronic infections, and they exhibit increased resistance to antibiotics and the host immune system compared to planktonic (free-floating) bacteria [86,87]. Therefore, one of the most significant objectives of developing new antimicrobial agents is the disruption of biofilms that can lead to improved treatment outcomes and reduced antibiotic resistance. In this context, various studies have explored the potential of nanoparticles in disrupting and eliminating biofilms, demonstrating their promising efficacy in combating persistent microbial communities that result in the destabilization of biofilms, prevent their reformation, and enhance the efficacy of conventional antimicrobial treatments [88,89]. This is due to the unique

physicochemical properties of nanoparticles that enable them to interact with and penetrate biofilm matrices effectively. Their small size and large surface area-to-volume ratio facilitate enhanced penetration into biofilms, allowing them to directly target and disrupt the extracellular polymeric substances that encase biofilm communities [90–92].

The antibiofilm activities of the curcumin alone, curcumin in nanoformulation, and zein were performed at sub-MIC using the crystal violet method. However, zein increased the biofilm formation significantly (115%) in comparison to untreated bacteria, whereas curcumin alone or in nanoformulation significantly reduced the biofilm formation by 24 and 70%, respectively (Figure 2). Importantly, curcumin nanoformulation significantly diminished the biofilm formation compared to curcumin alone, which indicates the significant antibiofilm activity of the nanoformulation. The results of the percentages of virulence factor formation are summarized in Table 1.

These results comply with other studies that showed significant anti-biofilm activities of curcumin in bulk or formulated as nanoparticles [93–95] against both Gram-negative and Gram-positive bacteria. For instance, Loo *et al.* showed that the combination of silver nanoparticles and curcumin nanoparticles at 100 μg/mL disrupted 50% of the established *P. aeruginosa* biofilm [94], while our zein-based nanoparticles diminished 70% biofilm formation at a

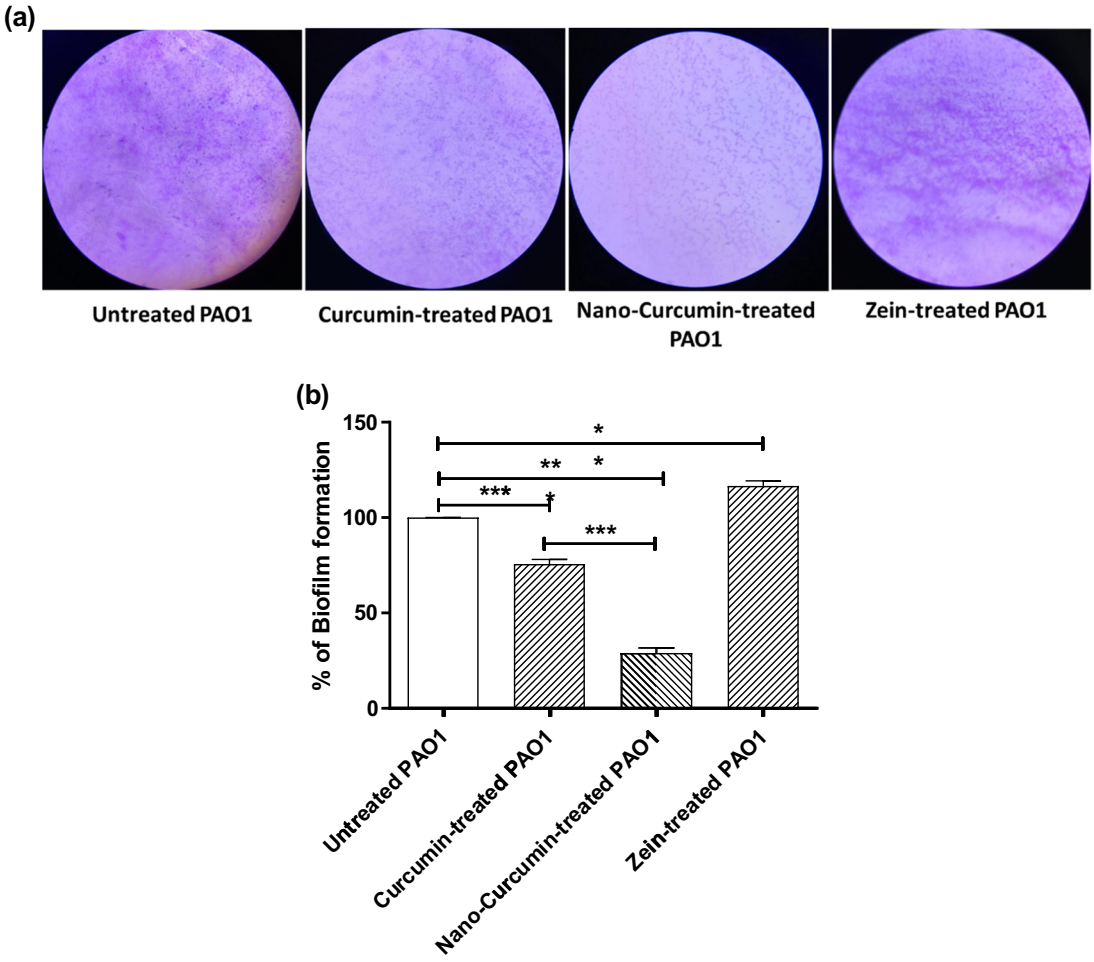

Figure 2: Curcumin in nanoformulation acquires significant antibiofilm activity. The biofilm formation in the presence of curcumin alone and nanoformulation was compared to that formed with untreated bacteria and zein-treated bacteria using the crystal violet method at sub-MIC concentrations to exclude any influence on the growth. (a) Representative light microscope images for the effect on biofilm formation. Curcumin alone or in nanoformulation (at sub-MIC) obviously decreased the biofilm formation in comparison to biofilm formed by untreated *P. aeruginosa* PAO1. On the other hand, zein increased the biofilm formation markedly in comparison to untreated bacteria. (b) Quantification of biofilm formation. However, zein increased the biofilm formation significantly compared to untreated bacteria, while curcumin diminished significantly the biofilm formation. The most significant reduction in biofilm formation was observed with curcumin in nanoformulation (about 70%) than the curcumin effect alone (about 24%). $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 1: Percentage of the production of virulence factors

Virulence factor	% of production in comparison to untreated PAO1		
	Curcumin-treated PAO1	Nano-curcumin-treated PAO1	Zein-treated PAO1
Biofilm formation	75.7 ± 4.0	29.0 ± 4.6	116 ± 4.1
Swarming motility	17.3 ± 4.2	24.3 ± 4.1	81.7 ± 4.1
Production of proteases	72.0 ± 2.6	72.3 ± 2.1	99.6 ± 4
Production of hemolysins	70.3 ± 4.5	46 ± 5.3	111 ± 4.1
Production of pyocyanin	25.7 ± 4.0	32.3 ± 2.5	99.6 ± 4.0

concentration of 150 µg/mL. In another example, Jaiswal *et al.* showed that curcumin–silver nanoparticles inhibited 85% of *P. aeruginosa* biofilm at a concentration of 10 mg/mL [82], which is a very high concentration in comparison to our preparation. These findings underscore the considered antibiofilm efficacy of our zein-based nanoparticles at low concentrations (150 µg/mL) in comparison to other nanoparticle preparations.

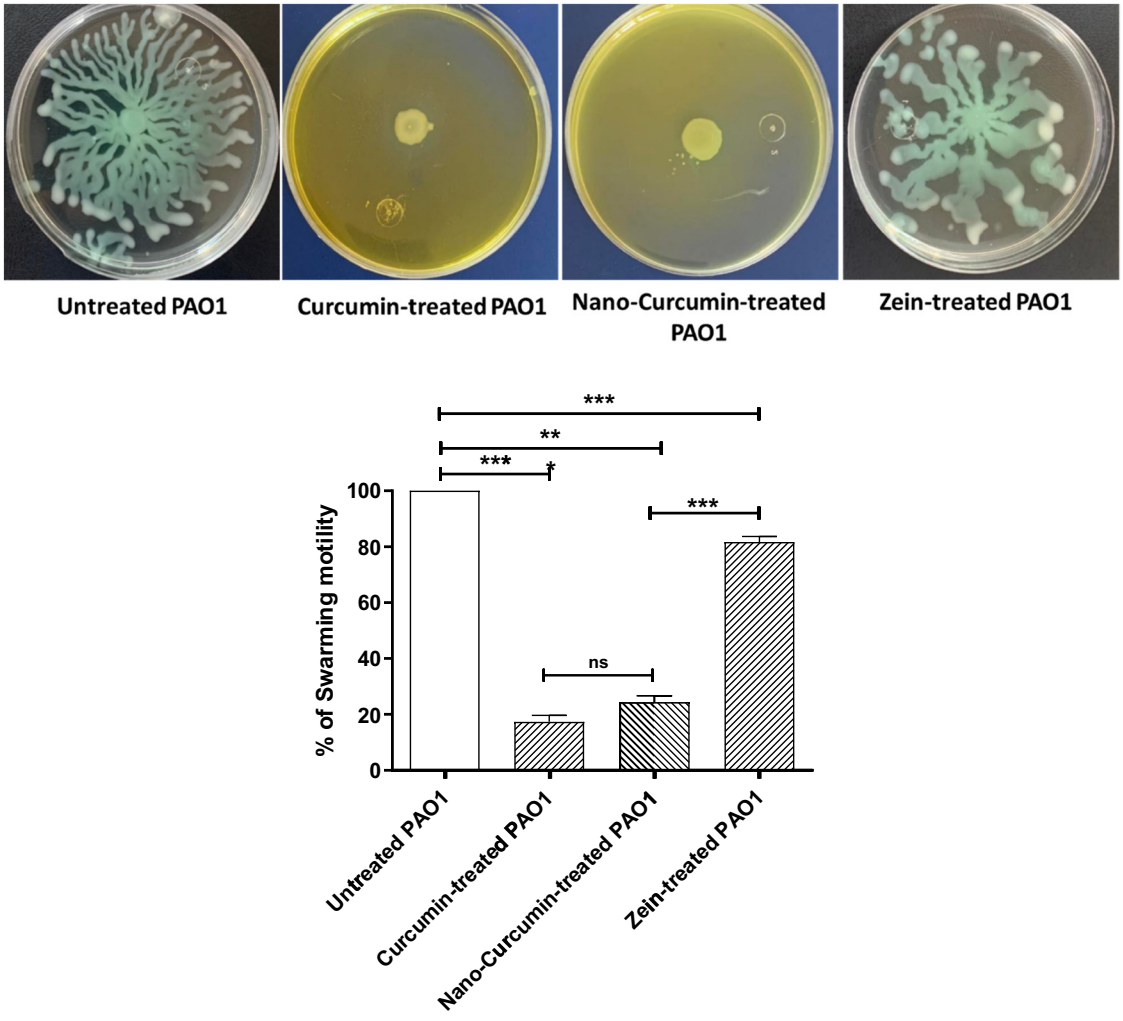

Figure 3: Curcumin reduces bacterial motility. The swarming zone of *P. aeruginosa* PAO1 was measured on plates provided with or without curcumin, curcumin nanoformulated, and zein at sub-MIC. However, zein significantly reduced the motility as compared to control untreated bacteria, curcumin alone, or curcumin nanoformulated and significantly reduced the motility as compared to zein-treated bacteria or untreated bacteria. There was no significant difference between the inhibitory effect of curcumin or curcumin in nanoformula on motility. Non-significant (ns): $p > 0.05$; ***$p < 0.001$.

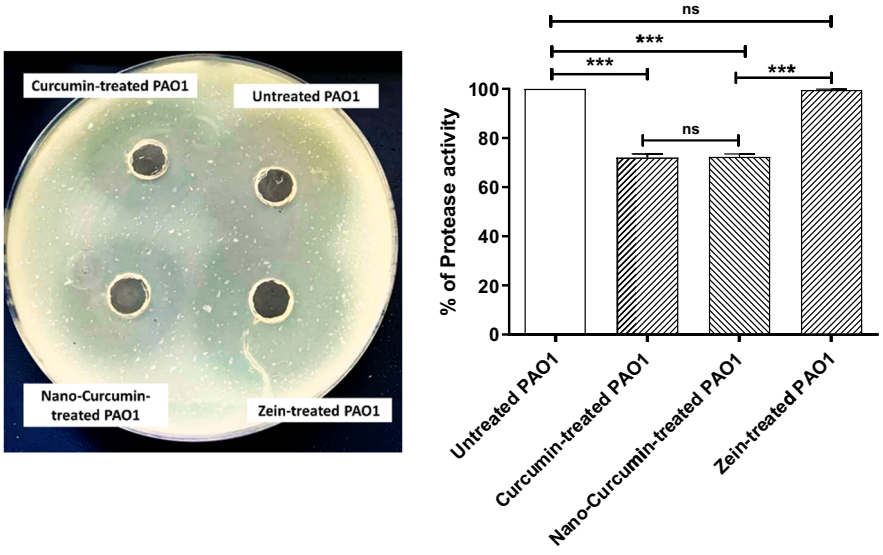

Figure 4: Curcumin decreased the production of protease. The developed hydrolysis zones around wells containing equivalent amounts of *P. aeruginosa* PAO1 treated with or without curcumin alone or nanoformulated, or zein at sub-MIC were measured on skim milk containing agar plates. Zein has no effect on protease production compared to control untreated bacteria, curcumin alone, or curcumin nanoformulated, and significantly reduced the protease production as compared to zein-treated bacteria or untreated bacteria. There was no significant difference between the inhibitory effect of curcumin or curcumin in nanoformula on protease production. ns: $p > 0.05$; ***: $p < 0.001$.

3.4 Curtailing of bacterial motility

Bacterial motility is required for infection spread and establishment into the host and its inhibition could reflect effective anti-virulence activities [96,97]. Furthermore, the nonmotile mutants were unable to adhere to host tissues and form biofilms [98,99], which resulted in diminishing the bacterial capacity to induce pathogenesis [98,100]. The swarming motility of *P. aeruginosa* was assessed in MH agar plates provided with curcumin alone, curcumin nanoformulated, or zein at sub-MIC in comparison to untreated bacteria (Figure 3). The curcumin alone, curcumin nanoformulated, and zein significantly reduced the bacterial motility by 85, 75, and 20%, respectively. However, there was no significant difference between the effect of curcumin alone or curcumin in nanoformulation on motility; the motility was significantly reduced in plates containing curcumin as compared to plates containing zein. These findings indicate the significant effect of curcumin in inhibiting bacterial motility.

3.5 Reduction of production of proteases

The virulent bacteria employ diverse extracellular enzymes to establish their accommodation in the host tissue. Protease is one of the important enzymes that enables bacteria to spread and establish infections in the host tissues [101,102]. The skim milk method was used to assess the inhibitory effects on the production of protease at sub-MIC concentrations. The hydrolysis zone on the skim milk agar was measured and compared. Curcumin alone or curcumin

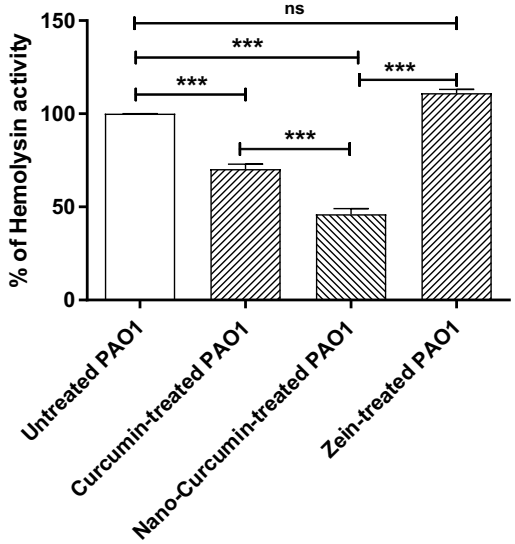

Figure 5: Curcumin decreased the hemolysin activity. The hemolytic activity of *P. aeruginosa* PAO1 was assessed in the presence of curcumin alone, curcumin nanoformulated, or zein at sub-MICs. The curcumin alone or curcumin nanoformulated significantly decreased the bacterial hemolysis activity as compared to untreated or zein-treated bacteria; however, curcumin nanoformulated significantly decreased hemolysin activity in comparison to curcumin alone. In contrast, zein increased the blood hemolysis but nonsignificantly. ns: $p > 0.05$; ***: $p < 0.001$.

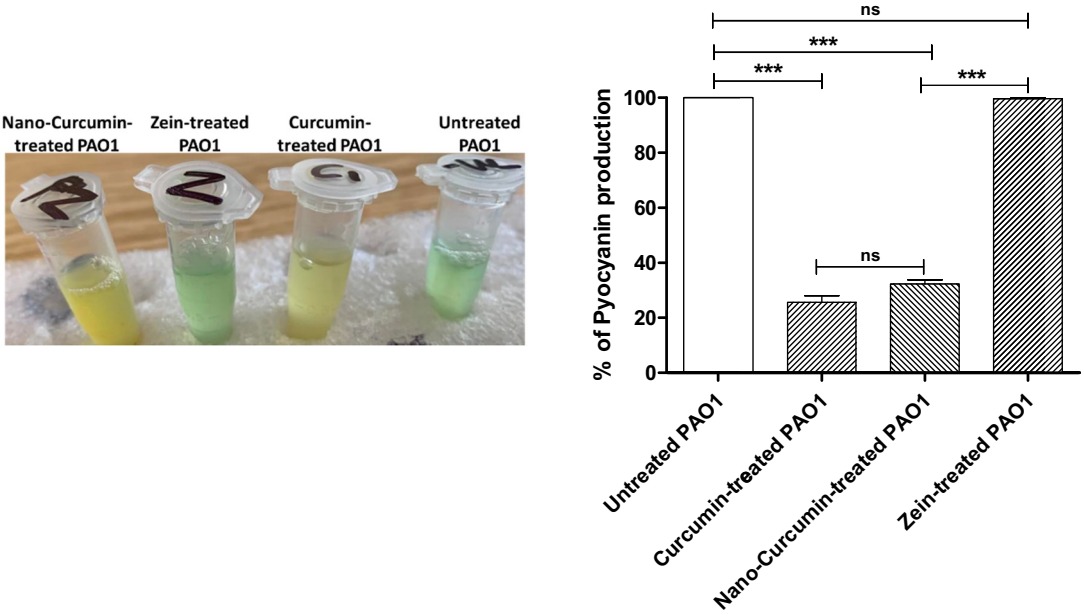

Figure 6: Curcumin decreased pyocyanin production. The absorbances of the pyocyanin pigment were measured in the presence of curcumin alone, curcumin nanoformulated, or zein at sub-MICs in comparison to untreated bacteria. The curcumin alone or curcumin nanoformulated significantly decreased the production of pyocyanin as compared to untreated or zein-treated bacteria. There was no significant difference between curcumin alone or curcumin nanoformulated on the pyocyanin production. ns: $p > 0.05$; ***$p < 0.001$.

nanoformulated significantly decreased the production of protease to about 25%. It is worth mentioning that zein does not affect the production of protease (Figure 4).

3.6 Diminishing of hemolysin activity

Among *P. aeruginosa's* huge arsenal of virulence factors, hemolysins play roles in the establishment of its infection and escape from the immune systems [103,104]. The effect of curcumin on the production of hemolysins was evaluated at sub-MIC. However, zein did not increase the hemolysis significantly in comparison to untreated bacteria, curcumin alone, or curcumin nanoformulated, and significantly decreased the bacterial hemolysin activity to about 30 and 50%, respectively. The nano-formulated curcumin showed a significant ability to reduce hemolysin activity in comparison to curcumin alone (Figure 5).

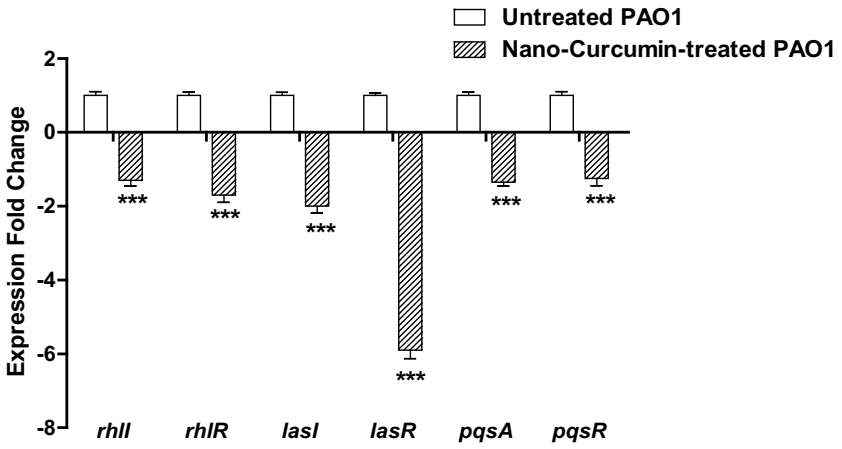

Figure 7: Curcumin downregulated the expression of QS-encoding genes. RT-PCR was employed to quantify the expression of genes that encode QS receptors and inducer synthetases in the presence or absence of curcumin nanoformulation at sub-MIC. Curcumin in nanoformula significantly downregulated the expression of all the QS-controlling genes. ***: $p < 0.001$.

Table 2: Docking details of curcumin and reference ligands with *P. aeruginosa* LasR, QscR, and PqsR

Compound	*S* score (kcal/mol)	H-bond interaction	Pi interaction
LasR (PDB: 6MVN)			
Curcumin	−7.9979	Arg61, Asp73, Leu110, Leu125	Trp88
Co-crystallized ligand: 3M5	−8.7019	Tyr56, Trp60, Asp73, Ser129	Trp88
QscR (PDB: 6CC0)			
Curcumin	−7.5263	Ile125	Tyr58
Co-crystallized ligand: EWM	−9.8344	Tyr58, Trp62, Asp75	—
PqsR (PDB: 4JVD)			
Curcumin	−7.2839	—	Leu208, Ile236, Tyr258
Co-crystallized ligand: NNQ	−6.6745	Leu197	Leu208

3.7 Reduction of pyocyanin production

P. aeruginosa employs a wide diverse array of virulence factors, including its greenish pigment pyocyanin, which plays an important role in its virulence [105,106]. Pyocyanin plays a role in the oxidative stress response, biofilm formation, and the virulence of *P. aeruginosa* in various infections [106,107]. It can also have toxic effects on host tissues and cells, making it an important factor in the study of *P. aeruginosa* infections and their treatment [108]. In the current study, zein did not show any effect on the pyocyanin production. Curcumin alone or curcumin nanoformulated significantly decreased the production of pyocyanin in comparison to untreated bacteria. There was no significant difference between the effect of curcumin alone or nanoformulated curcumin on pyocyanin production (Figure 6).

3.8 Anti-QS activities of curcumin

P. aeruginosa mainly employs three QS systems to orchestrate virulence during the different stages of infections [109]. The QS systems include two Lux-type systems, Las and Rhl and one non-Lux-type Pqs QS system [34,110]. Furthermore, there is a fourth QS system Qsc that senses the Lux-type autoinducers [111].

3.8.1 Downregulation of QS encoding genes

To investigate the anti-QS activity, the expressions of QS encoding genes were quantified in the presence of curcumin in nanoformulation (Figure 7). The results showed significant downregulation of the QS encoding genes in the presence of curcumin, which could explain the diminishing of other virulence factors that are controlled by the QS system phenotypically.

3.8.2 Virtual affinity of curcumin to QS receptors

To explore molecular interactions of curcumin with *P. aeruginosa* QS receptors, *in silico* docking studies were performed. The docking procedures were first validated by re-docking the co-crystallized ligand for each studied receptor. 3M5 (*N*-(3-oxodecanoyl)-L-homoserine lactone), EWM (*N*-[(3S)-2-oxooxolan-3-yl] dodecanamide), and NNQ (2-nonylquinolin-4(1*H*)-one) are the co-crystallized ligands for *P. aeruginosa* LasR, QscR, and PqsR, respectively. The root mean standard deviation (RMSD) values of re-docked 3M5, EWM, and NNQ are 1.2689, 1.4791, and 1.4520 Å, respectively, which indicate the validity of the docking procedures. Molecular docking of curcumin and the co-crystallized ligands (3M5, EWM, and NNQ) with *P. aeruginosa* QS receptors revealed comparable results within the active site (Table 2).

Curcumin showed good binding affinity within the active site of *P. aeruginosa* LasR (PDB: 6MVN), where the binding energy score is −7.9979 kcal/mol. Notably, one phenolic OH group showed an H-bond interaction with Leu 110, and the second one showed an H-bond interaction with Leu 125 on the other side of the active site. One carbonyl group exhibited an H-bond interaction with the basic Arg 61. The methoxy group formed an H-bond interaction with the acidic Asp 73. Additionally, the curcumin structure was stabilized through pi–pi bond interaction with Trp 88. The curcumin structure showed hydrophobic contacts with Leu 36, Tyr 47, Tyr 64, Val 76, and Trp 88 residues (Figure 8a and b).

Curcumin was able to fill the active pocket space and showed a good binding affinity with *P. aeruginosa* QscR (PDB: 6CC0) with an S score of −7.5263 kcal/mol. The phenolic OH group formed an H-bond interaction with Ile 125. The phenyl ring participated in the curcumin stability through the formation of pi–H bond interaction with Tyr 58. Moreover, the curcumin structure revealed hydrophobic contacts with the following residues: Phe54, Tyr 58, Tyr 66, and Met 127 (Figure 8c and d).

Curcumin structure could fill the active pocket of *P. aeruginosa* PqsR (PDB: 4JVD) where the binding energy of curcumin is lower than that of the co-crystallized ligand (NNQ) (Table 1). On one side of the active pocket, the phenyl ring exhibited two pi–H bond interactions with Leu 208 and Ile 236 residues. The second phenyl ring, on the other side of the pocket, showed pi–pi bond interactions with Tyr 258. Besides, the curcumin structure stabilized through hydrophobic interactions with Leu 207, Leu 208, Ile 236, Tyr 258, and Ile 263 residues (Figure 8e and f).

Overall, these molecular docking studies present the potential inhibition effect of curcumin into *P. aeruginosa* QS receptors. The molecular interactions of curcumin with *P. aeruginosa* LasR (PDB: 6MVN), QscR (PDB: 6CC0), and PqsR (PDB: 4JVD) are depicted in Figure 8.

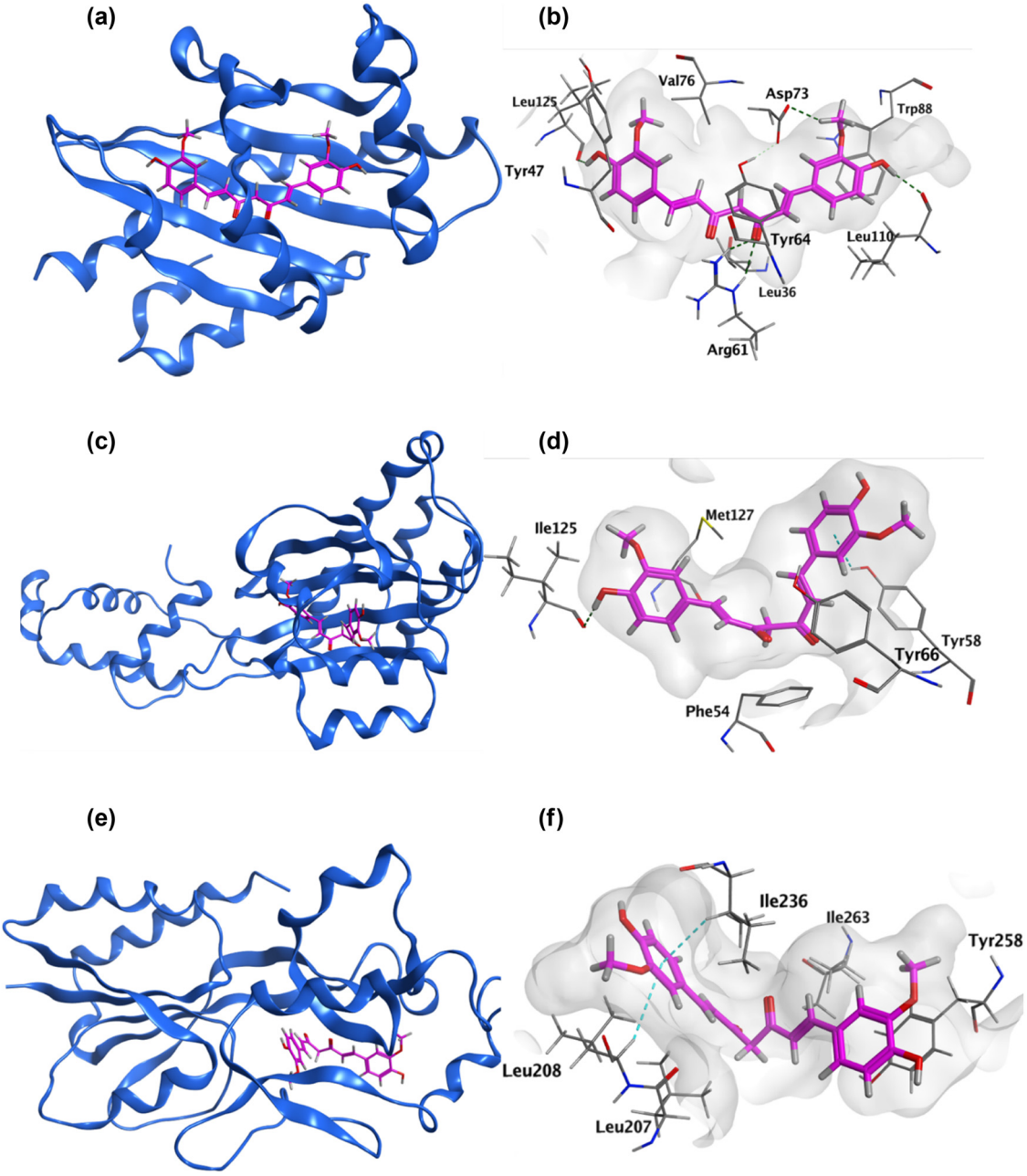

Figure 8: (a) 3D curcumin–*P. aeruginosa* LasR (PDB: 6MVN) interaction diagram. (b) Curcumin in the molecular surface of the LasR active site. (c) 3D curcumin – *P. aeruginosa* QscR (PDB: 6CC0) interaction diagram. (d) Curcumin in the molecular surface of the QscR active site. (e) 3D Curcumin–*P. aeruginosa* pqsR (PDB: 4JVD) interaction diagram. (f) Curcumin in the molecular surface of the PqsR active site.

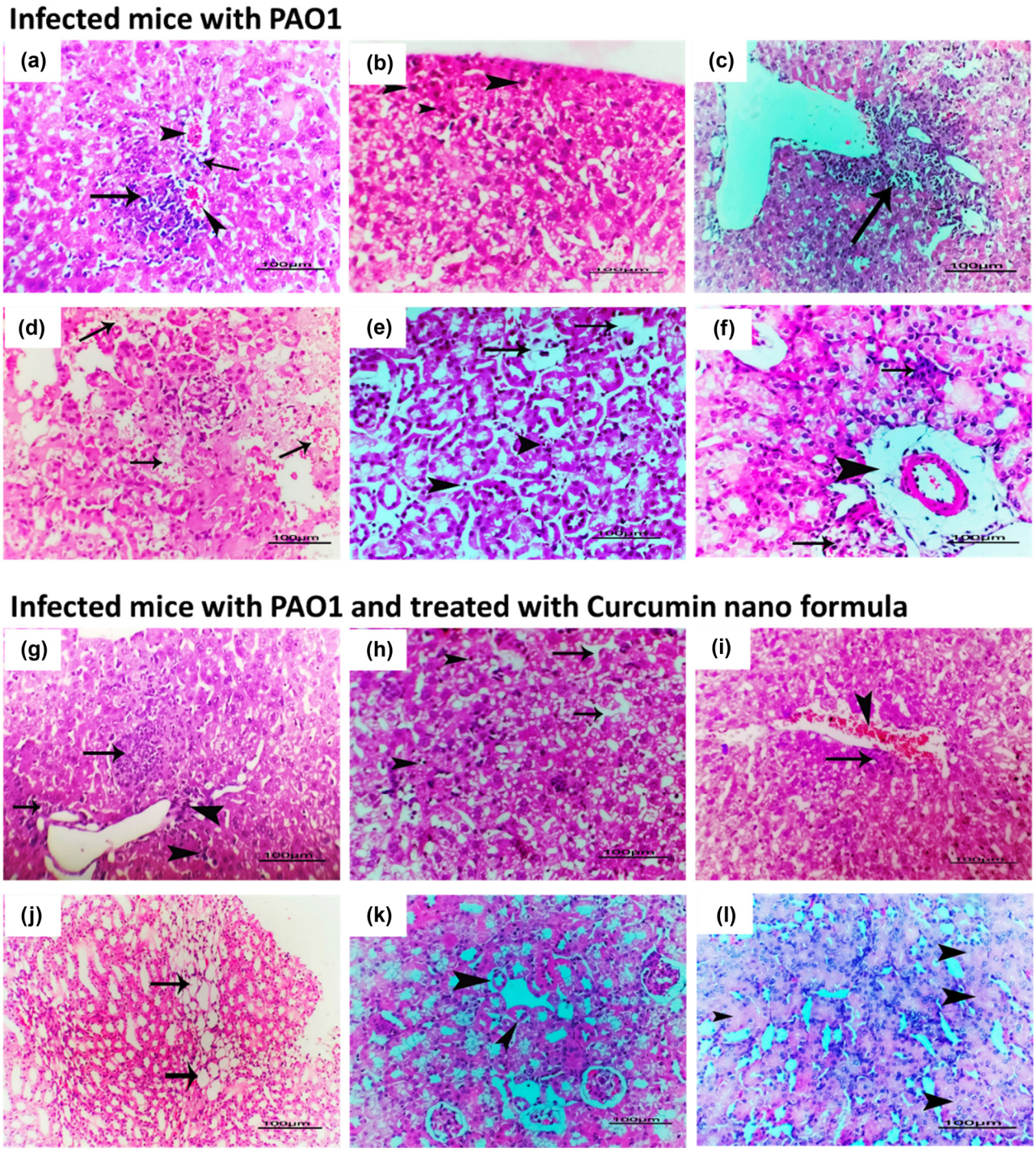

Figure 9: Histopathological examination of the isolated renal and hepatic tissues from mice groups that were infected with *P. aeruginosa* PAO1 or injected with *P. aeruginosa* PAO1 treated with curcumin nanoformula at 1/8 MIC (0.15 mg/mL). Photomicrograph of the H&E-stained liver section of the infected group showing (a) severe congestion of hepatic blood vessels (arrows head) with perivascular leucocyte cell infiltration (arrows), (b) subcapsular coagulative necrosis of hepatocytes (arrowhead) represented by nuclear pyknosis, and (c) severe focal perivascular and periductal leucocytic cell infiltration (arrow). Photomicrograph of the H&E-stained kidney section of the infected group showing (d) diffuse interstitial extravasated erythrocytes (hemorrhage) (arrows) with interstitial edema (arrowhead) and degenerated renal tubules within renal cortex, (e) severe hypotrophy of renal glomeruli in the renal cortex (arrows) with diffuse inflammatory cell infiltration (arrows head), and (f) perivascular edema (arrowhead) with endothelins and interstitial leucocytic cell infiltration cloudy swelling (arrows). Photomicrographs of the H&E-stained liver section of the infected and treated group showing (g) focal moderate perivascular areas of cellular infiltration (arrows) with mildly dilated sinusoids and macrophage infiltration (arrows head), (h) diffuse infiltration of von Kupffer cells (arrows head) with mild sinusoidal dilation (arrows), and (i) mild congestion of hepatic blood vessels (arrowhead) with mild perivascular inflammatory cell infiltration (arrow). Photomicrographs of the H&E-stained kidney section of infected and treated group showing (j) focal area of cystic dilation of some renal tubules (arrows) with normal medulla, (k) focal splitting of some renal tubules (arrows head), which appeared atrophied within the normal renal cortex, and (l) mild diffuse degeneration of renal tubules within renal medulla represented by cloudy swelling (arrows head) (scale bar = 100 µm).

3.9 Nanoformulated curcumin-protected mice

To appraise the protective effect of curcumin nanoformula on alleviating the *P. aeruginosa*-induced pathogenesis, demonstrative renal and hepatic photomicrographs were taken for tissues isolated from mice intraperitoneally injected with treated or untreated bacteria (Figure 9). The liver tissues of mice infected with untreated *P. aeruginosa* showed severe hepatic blood vessel congestions accompanied by severe perivascular and periductal infiltration of leucocytes (Figure 9a–c). In the same way, the kidney tissues isolated from the group that was infected with untreated *P. aeruginosa* showed hemorrhage with interstitial edema and severe hypotrophy of renal glomeruli in the renal cortex with diffuse inflammatory cell infiltrations (Figure 9d–f). On the other hand, the liver (Figure 9g–i) and kidney (Figure 9j–l) tissues that were isolated from the group injected with treated *P. aeruginosa* showed no to mild congestions of blood vessels with minimal infiltrations of lymphocytes. These data show obviously the *in vivo* protecting effect of curcumin nanoformula against *P. aeruginosa* pathogenesis.

In a nutshell, targeting bacterial virulence is a promising strategy for controlling bacterial infections, and it offers several advantages when natural compounds are used. The effectiveness of this approach can be significantly enhanced by utilizing nanoparticle formulations. The anti-virulence and anti-QS activities of curcumin–zein nanoparticles have been evaluated in comparison to bulk curcumin. In bulk, curcumin showed a significant ability to diminish *P. aeruginosa's* ability to form biofilms and produce virulence factors, such as hemolysins, proteases, and pyocyanin. However, nanoparticle formulation significantly decreased the biofilm formation and reduced the production of virulence factors at lower MIC than curcumin in bulk. Curcumin acquires significant anti-QS activities that could explain its anti-virulence activity. These anti-QS activities are attributed to the curcumin's interference with the QS receptors besides its ability to downregulate the QS-encoding genes. Furthermore, the *in vivo* results emphasized the nanoparticle's significant effects in relieving the *P. aeruginosa*-associated pathogenesis. Collectively, the curcumin zein nano-based formulation demonstrated effective antibacterial, anti-virulence, and antibiofilm activities against *P. aeruginosa* in both *in vitro* and *in vivo* studies.

Acknowledgments: This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Saudi Arabia, Jeddah, under grant no. (RG-17-166-43). The authors, therefore, acknowledge and thank DSR for technical and financial support.

Funding information: The Deanship of Scientific Research (DSR) at King Abdulaziz University (KAU), Jeddah, Saudi Arabia, has funded this project.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflict of interest: The authors state no conflict of interest.

Ethical approval: All *in vivo* procedures were carried out in strict accordance with the relevant ethical guidelines for animal welfare, as approved by the Institutional Animal Care and Use Committee at Zagazig University (ZU-IACUC), Egypt (Approval number: ZU-IACUC/3/F/154/2022).

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