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Evaluation of vitamins d3 and k1 as anti-virulence against clinical *Pseudomonas aeruginosa* isolates from burn infections

Haneen Haitham Al Saraj, Rasmia Omar Al Jobory
Department of Biology, College of Education for Women, University of Mosul, Mosul, Iraq
Corresponding Author: Haneen Haitham Al-Saraj

Abstract
Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen commonly associated with burn infections.
Aim: This study aimed to evaluate the effect of cholecalciferol (vitamin D3), phylloquinone (vitamin K1) on selected virulence factors, antibiotic activity, and phagocytic activity.
Materials and Methods: A total of 120 burn swab samples were collected and examined for the presence of *P. aeruginosa*, *identified using* VITEK 2 system. One *P. aeruginosa* isolate exhibiting the highest virulence-associated activity was selected. The sub-minimum inhibitory concentrations of vitamin D3 and vitamin K1 were determined. The effect of these treatments was evaluated virulence factors, phagocytic activity, and antibiotic activity. Experiments were performed in triplicates, and the data were expressed as mean ± standard deviation and analyzed using one-way ANOVA followed by Duncan's multiple range test, with statistical significance set at $p < 0.05$.
Results: the isolated rate of *P. aeruginosa* was 53.33%. Vitamin D3 produced the greatest reduction in protease activity (67.94%) and increased phagocytic activity by 16.56%. Vitamin K1 produced the greatest reduction in pyocyanin production (94.58%) and increased the tigecycline inhibition-zone diameter from 9mm to 16 mm. The combined treatment produced the greatest inhibition of biofilm formation (98.32%).
Conclusion: At sub-MIC concentration, Vitamin D3 showed the greatest effect on protease activity and phagocytic activity, vitamin K1 markedly reduced pyocyanin production and also increased the *in vitro* activity of tigecycline under the tested conditions, the combined treatment strongly inhibited biofilm formation. These findings support further investigation of these vitamins as antibiotic adjuvants.

Keywords: Cholecalciferol, phylloquinone, biofilm, pyocyanin, protease, antibiotic interaction

Introduction

Antimicrobial resistance has become a major public health concern worldwide. It has been estimated that antimicrobial resistance is responsible for approximately 700,000 deaths worldwide every year, and this number may increase markedly by 2050 if effective control strategies are not implemented. [1]

Among antimicrobial-resistant pathogens, *Pseudomonas aeruginosa* is considered one of the most clinically significant opportunistic bacteria. It is an aerobic, Gram-negative, nonfermenting bacterium, a member of the ESKAPE pathogens, which are well known for their ability to cause healthcare-associated infections and resist multiple classes of antimicrobial agents. [2] Drug-resistant *P. aeruginosa* is associated with high global mortality burden, and the World Health Organization has included carbapenem-resistant *P. aeruginosa* in the bacterial priority pathogens list because of its clinical importance and limited therapeutic options. [3]

Biofilm formation is one of the most important virulence mechanisms; bacterial cells adhere to biotic and abiotic surfaces and protect themselves from antibiotics and host immune responses by embedding within an extracellular matrix; therefore, infections resulting from this way are more persistent, recurrent, and difficult to eradicate.[4] In addition, extracellular proteases contribute to tissue damage, degradation of host proteins, interference with immune mediators, inactivation of antibodies, facilitating bacterial invasion and persistence. [5]

Pyocyanin is a pigment and virulence factor that contributes to oxidative stress, immune cell dysfunction, Inflammation, and host cell injury. Its production is closely regulated by quorum-sensing systems, which also control biofilm formation and protease production.

With the increasing rate of antibiotic resistance, anti-virulence strategies have emerged as supportive alternatives to conventional antimicrobial therapy. By targeting virulence factors rather than bacterial growth, these approaches reduce the severity of infection. [6] Among the compounds that have recently attracted interest, vitamins D3 and K1 may have a supportive role in reducing some virulence-associated features *of P. aeruginosa*, inhibiting biofilm formation in Gram-negative bacteria. [7] Vitamin D3 is also known for its immunomodulatory role, enhancing macrophage bactericidal activity against *P. aeruginosa* [8] and Vitamin K1 interferes with quorum sensing and reduces virulence factors such as biofilm formation, pyocyanin, and protease production. [9]

However, limited information is available regarding the individual and combined effects of vitamins D3 and K1 on the virulence-associated characteristics of clinical P. aeruginosa isolates recovered from burn infection.

Aim: This study aimed to evaluate the effect of cholecalciferol (vitamin D3), phylloquinone (vitamin K1), individually and in combination on biofilm formation, pyocyanin production, protease production, antibiotic activity and phagocytic activity.

Methodology

Isolation of *Pseudomonas aeruginosa* Isolates

120 swab samples were collected from burn infections in patients at the Al-Mosul Center for Burns and Plastic Surgery from October to March 2026. All samples were inoculated onto selective and differential culture media, including blood agar, MacConkey agar, and cetrimide agar, and the inoculated plates were incubated aerobically at 37 °C for 24 h. Morphological features of *P. aeruginosa* were observed, and pale colonies were transferred to cetrimide agar, which is commonly used as a selective medium for the isolation of *P. aeruginosa*

Definitive identification of *Pseudomonas aeruginosa*

Purified isolates were examined microscopically after Gram staining with a Gram stain kit (Atom Scientific, UK) and were observed to be rod-shaped Gram-negative bacteria. Standard biochemical tests, including the oxidase test, were performed to confirm the preliminary identification. For definitive species-level identification, the isolate was analyzed using the VITEK 2 system (bioMérieux, France) according to the manufacturer's instructions, using the identification card designed for Gram-negative organisms. The recovered isolates were confirmed as *Pseudomonas aeruginosa* based on the identification profiles generated.

Vitamin Preparations Used in the Study

The vitamins tested were obtained as commercial injectable pharmaceutical preparations. Vitamin D3 was supplied as cholecalciferol DIBASE 300,000 IU/mL (7.5 mg/ml) solution for injection, manufactured by Abiogen Pharma S.p.A., Pisa, Italy, and Vitamin K1 was supplied as Phytomenadione K-VIT for adults (10 mg/mL) injectable preparation, manufactured by Ibn Hayyan Pharmaceuticals, Homs, Syria. Levofloxacin was obtained as a commercial intravenous infusion preparation (LEVOX NS, 0.5% levofloxacin in 0.9% sodium chloride, 100 mL), manufactured by Al-Mustaqbal Pharmaceutical Industries, Iraq. The tested treatments included vitamin D3, vitamin K1, the combination of vitamin D3 and vitamin K1, and levofloxacin. These treatments were applied throughout the subsequent experimental assays at their selected sub-MIC concentrations, unless otherwise stated.

Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered statistically significant at $P < 0.05$. Different letters indicate significant differences among treatments, whereas shared letters indicate no significant difference.

Determination of sub Minimum Inhibitory Concentratin

The minimum inhibitory concentrations (MICs) of Vitamin D3 and Vitamin K1 against the tested bacterial isolates were determined using the broth macro-dilution method with two-fold serial dilutions in sterile test tubes, with slight modifications according to CLSI guidelines. Mueller-Hinton broth (MHA; HiMedia, India) was used as the test medium, and the final volume in each tube was adjusted to 2 mL .The initial concentrations tested were prepared from the commercial vitamin preparations described above and the levofloxacin stock solution. The initial concentrations were 5 mg/mL for Vitamin D3, 5 mg/mL for Vitamin K1. The tubes were inoculated with bacterial suspension to obtain a final concentration of approximately 1 × 10^6 CFU/mL, then incubated aerobically at 37°C for 24 h. The MICs were calculated as the lowest concentration of vitamin D3and K1 that inhibited bacterial growth, and sub-MICs were calculated for further experiments. [10]

Biofilm Formation

Initial formation of biofilm was determined using a flat-bottom 96-well polystyrene microtiter plate assay with crystal violet staining. [11] Previously selected strong biofilm-producing clinical isolates were cultured in Tryptone Soy Broth (TSB; Neogen Culture Media, UK) overnight at 37 °C. The next day, the bacterial culture was adjusted to a turbidity equivalent to the 0.5 McFarland standard and then exposed to each vitamin at a sub-MIC concentration. The wells of the microtiter plate were inoculated with 200 µl of treated or untreated bacterial suspension; wells containing TSB only, without bacterial inoculation, were included as negative controls. The plate was then incubated at 37 °C for 24 h. After incubation, non-adherent planktonic cells were removed by aspiration, and the wells were washed twice with distilled water. Then, the attached biofilm was fixed with 250 µl of methanol for 15 minutes, removed, and the plate was left to air-dry. It was then stained with 1% (w/v) crystal violet for 20 minutes, and excess stain was removed using D.W. The bound crystal violet was dissolved in 250 µl of glacial acetic acid 33% (v\v). The optical density was measured at 570 nm; the ELISA plate was read using a CHROMATE microplate reader (Awareness Technology Inc., USA). The percentage of biofilm formation in treated cultures was calculated relative to untreated control cultures.

Protease Activity

Protease activity in *P. aeruginosa* was evaluated using skim milk [12] with slight modifications. Protease activity was assessed using skimmed milk as a protein substrate. Briefly, *Pseudomonas aeruginosa* isolates were first refreshed on nutrient agar and incubated at 37°C for 24 h. A bacterial suspension was then prepared in sterile normal saline and adjusted to 0.5 McFarland standard, approximately equivalent to 1.5×10^8 CFU/ml. The adjusted suspension was inoculated into nutrient broth containing the compounds under test, while the untreated bacterial culture served as a positive control. The cultures were incubated at 37°C for 24 h. After incubation, the cultures were centrifuged to obtain cell-free supernatants. For the assay, 200 µl of each cell-free supernatant was mixed with 1 ml of 1.25% skimmed milk solution and incubated at 37°C for 2 h. After incubation, proteolytic degradation of skimmed milk proteins was determined by measuring the change in turbidity at 500 nm using a spectrophotometer. The assay was carried out in triplicate for each treatment. Lower turbidity reflected greater protease activity, whereas higher turbidity indicated reduced proteolytic activity. Higher turbidity indicated lower proteolytic degradation of milk proteins, and lower turbidity indicated greater protease activity.

Pyocyanin Production

Pyocyanin production by *Pseudomonas aeruginosa* was evaluated by measuring the optical density of culture

supernatants, as described by Juhas *et al*. (2004), with slight modifications. The bacterial suspension was adjusted to a turbidity equivalent to that of the 0.5 McFarland standard. The adjusted bacterial cultures were then inoculated into BHI broth (HiMedia, India) containing sub-MIC concentrations of vitamin D3, vitamin K1, their combination, and Levofloxacin. Untreated bacterial cultures were used as positive controls. All cultures were incubated aerobically at 37°C for 24 h. After incubation, the bacterial cultures were centrifuged to obtain culture supernatants, which were then filtered through 0.22 µm membrane filters to obtain cell-free supernatants.

The concentration of pyocyanin in the supernatant of each culture was quantitatively determined by measuring the optical density at 695 nm. The instrument was blanked against sterile BHI broth. In addition, BHI broth containing each tested treatment, without bacterial inoculation, was used as a treatment-specific blank to minimize potential background interference from the color or turbidity of the tested vitamins. [13]

In vitro Phagocytic Activity

The phagocytic activity assay *in vitro* was performed according to van Furth *et al* (1986) in the Handbook of Experimental Immunology, with some modification. Fresh heparinized blood was used within 1-2 hours of collection to preserve the viability of phagocytic cells. The blood samples were collected from apparently healthy donors into sterile siliconized tubes containing heparin as an anticoagulant, and 1 mL of whole blood was mixed with 1 mL of bacterial suspension at a previously prepared concentration (approximately 10^6 CFU/mL) in sterile siliconized tubes. sub-MIC concentration of vitamin D3 was added directly to the tubes to evaluate its effect on phagocytic activity. same procedure was followed for vitamin K1-treated tubes and for tubes containing the combination of vitamin D3 and K1. Blood and bacterial suspension without vitamin treatment were used as control tubes. [14] All tubes were incubated in a water bath at 37 °C for 30 min with gentle shaking. After that, a drop of each mixture was placed on a clean glass slide to prepare a thin blood smear and was allowed to dry at room temperature, stained with Leishman stain for 10 min, gently washed with distilled water, and left to dry, examined under a light microscope using a 40× objective lens.

One hundred phagocytic cells were counted per slide. Phagocytic activity was expressed as the percentage of phagocytic cells according to the following formula

Phagocitic activity (%) =

$$\frac{Number\ of\ phagocytic\ cells\ showing\ bacterial\ phagocytosis}{Total\ number\ of\ counted\ cells} * 100$$

Evaluation of Vitamin-Antibiotic Interaction

The interaction between vitamins and antibiotics against *Pseudomonas aeruginosa* isolates was evaluated

using a modified disk diffusion assay based on the Kirby-Bauer method, as described by Shahzad *et al*. (2018), with reference to CLSI recommendations for antimicrobial susceptibility testing. [15]

Initially, the MIC values of vitamins were determined; fresh bacterial suspensions were adjusted to the 0.5 McFarland standard. The suspension was then uniformly inoculated onto Mueller-Hinton agar plates using sterile cotton swabs. After the agar surface had dried, antibiotic disks were placed on the inoculated agar. A volume of 10 µL of each vitamin solution at MIC concentration was aseptically applied directly onto each antibiotic disk using a sterile micropipette, and the combination of vitamins was applied onto the antibiotic disk while maintaining the same applied volume. The plate was incubated at 37 °C for 24 h to allow the vitamins to be absorbed. Following incubation, inhibition zone diameters around the disks were measured in millimeters and compared with antibiotic disks alone as a control.

Results

Isolation rate of *Pseudomonas aeruginosa*

A total of 120 swab samples were collected from patients with burn infections and examined for the presence of *Pseudomonas aeruginosa* (table 1). These findings indicate that *P. aeruginosa* represented a considerable proportion of the bacterial isolates recovered from wound and burn infections.

Table 1: Distribution of *Pseudomonas aeruginosa* isolates among clinical samples

Sample category	Number	Percentage (%)
Total examined samples	120	100
Positive for *P. aeruginosa*	64	53.33
Negative for *P. aeruginosa*	56	46.67

Definitive Identification of Pseudomonas aeruginosa Isolates:

The identification results confirmed that the examined isolates belonged to *Pseudomonas aeruginosa*. The isolates showed the characteristic features of this species, including Gram-negative bacillary morphology, non-lactose fermentation on MacConkey agar, green pigmentation on cetrimide agar, oxidase and catalase positivity, and motility. No hemolytic activity was detected on blood agar. Final confirmation by the VITEK system verified the isolates as *Pseudomonas aeruginosa*.

Sub-Minimum Inhibitory Concentration of the tested vitamins

The minimum inhibitory concentrations (MICs) of vitamin D3 and vitamin K1 against *Pseudomonas aeruginosa* were determined using the two-fold serial dilution method. The MIC value of vitamin D3 was 1.25 mg/ml, whereas that of vitamin K1 was 2.5 mg/ml. Based on these findings, sub-inhibitory concentrations (sub-MICs) corresponding to 0.625 mg/ml for vitamin D3 and 1.25 mg/ml for vitamin K1 were selected for all subsequent experiments. The MIC of levofloxacin was determined using the VITIK 2 automated system and the MIC value was 1 µg/ml. A sub-MIC was 0.5 µg/ml. These concentrations were used to investigate the effects of compounds on bacterial virulence factors without directly inhibiting bacterial growth.

Effect of Treatments at Sub-Mic on Biofilm Formation

Biofilm inhibition was calculated relative to the untreated control, which recorded the highest biofilm value, with a mean absorbance of 1.722, and was classified within statistical group A, which means strong biofilm formation under untreated conditions

Vitamin D3 showed a stronger reduction in biofilm formation. Vitamin K1 reduced the biofilm formation.

Vitamin K1 clearly reduced biofilm formation, its inhibitory effect was lower than that observed with vitamin D3, the combined treatment, and levofloxacin. The combination treatment of vitamin D3 with vitamin K1 produced a marked reduction in biofilm formation, suggesting that the combined treatment strongly suppressed biofilm biomass. Levofloxacin showed the lowest biofilm value among the tested treatments, the inhibition percentage reached 98.78%, the highest reduction rate in biofilm formation.
Overall, all treatments reduced biofilm formation compared with the untreated control (Table 2).

Table 2: Effect of Treatments at Sub-MIC on Biofilm Formation

Treatment	Biofilm value	Statistical group	Biofilm inhibition (%)
Un-treated	1.722	a	0.00
Vitamin D3	0.137	c	92.04
Vitamin K1	0.549	b	68.12
Vitamin D3+K1	0.029	d	98.32
Levofloxacin	0.021	d	98.78

Effect of Treatments at Sub-Mic on Pyocyanin Production

Pyocyanin inhibition was calculated relative to the positive control. Vitamin D3 treatment at sub-MIC showed the highest mean pyocyanin production. This indicates that Vitamin D3 did not inhibit pyocyanin production; rather, it increased pigment production by 57.81% compared with the positive control. In contrast, Vitamin K1 at sub-MIC showed the lowest level of pyocyanin production. This treatment recorded the highest inhibition percentage, indicating that Vitamin K1 was the most effective treatment in reducing pyocyanin production. The combined treatment of vitamin D3 with K1 showed an intermediate effect. Its shared statistical grouping with the positive control indicates that this reduction was not statistically distinct.
Levofloxacin also caused a marked reduction in pyocyanin production, Although the mean value under levofloxacin treatment was higher than that observed with Vitamin K1, both treatments were in the same statistical group, indicating that their effects were statistically comparable (Table 3).

Table 3: Effect of Treatments at sub-MIC on Pyocyanin Production

Treatment	Mean ± SD	Statistical group	Pyocyanin inhibition (%)
Un-treated	0.1107 ± 0.04562	b	0.00
Vitamin D3	0.1747 ± 0.00551	a	57.81%increase
Vitamin K1	0.0060 ± 0.00000	c	94.58
Vitamin D3+ K1	0.0837 ± 0.00231	b	24.39
Levofloxacin	0.0343 ± 0.00651	c	69.02

Effect of Treatments at Sub-mic on Protease Activity

The results of the protease activity assay showed clear differences among the tested treatments. In this assay, turbidity was used as an indirect indicator of protease activity. Protease inhibition was calculated in comparison with the positive control and the milk-only control.
The milk-only control recorded the highest turbidity reading. This treatment represented the milk-containing medium without bacterial inoculation and was therefore considered a reference for the absence of proteolytic degradation. The low turbidity observed in this group indicates high protease activity, as untreated *Pseudomonas aeruginosa* hydrolyzes milk proteins, thereby markedly reducing turbidity. Among the treated groups, Vitamin D3 showed the highest numerical inhibitory effect on protease activity. This finding indicates that Vitamin D3 markedly reduced proteolytic activity, as the medium remained more turbid than that of the untreated positive control. Vitamin K1 reduced protease activity compared with the positive control, it showed the lowest numerical inhibitory effect among the tested treatments. The combined treatment reduced protease activity compared with the positive control. Levofloxacin also caused a clear reduction in protease activity, indicating substantial inhibitory effect. All treatments reduced protease activity to compared with the positive control (table4).

Table 4: Effect of Treatments at sub-MIC on Protease Activity

Treatment	Mean ± SD	Statistical group	Protease inhibition (%)
Skimed Milk only	*1.8270 ± 0.00000	a	Reference no proteolysis
Untreated	**0.4537 ± 0.01872	d	0.00
Vitamin D3	1.3867 ± 0.13467	b	67.94
Vitamin K1	1.2323 ± 0.01629	c	56.70
VitaminD3+K1	1.2963 ± 0.03958	bc	61.36
Levofloxacin	1.3287 ± 0.07298	bc	63.72

*Higher turbidity indicates lower protease activity**Lower turbidity indicates higher proteolytic activity

Effect of Treatments at Sub-Mic on Phagocytic Activity

The phagocytic activity assay showed variable responses among tested treatments. Phagocytic activity was expressed as a percentage, and the relative change was calculated relative to the control group. The control group recorded a mean phagocytic activity of 44.33±5.03% and was classified within statistical group AB. This value was used as the reference for describing the relative changes induced by the different treatments.
Treatment with vitamin D3 produced the highest phagocytic activity and significantly increased it compared with the group control. In contrast, vitamin K1 showed the lowest phagocytic activity. Similarity, the combined treatment of vitamin D3 with vitamin K1 caused reduction. Both vitamin K1 and the combined treatment were classified within the same statistical group (Table 5).

Table 5: Effect of treatments at sub-MIC on phagocytic activity

Treatment	Phagocytic activity Mean ± SD (%)	Statistical group	Relative change compared with control (%)
Untreated	44.33 ± 5.03	ab	0.00
Vitamin D3	51.67 ± 3.51	a	+16.56 increase
Vitamin K1	36.33 ± 6.66	b	−18.05 reduction
Vitamin D3+K1	37.67 ± 6.03	b	−15.03 reduction

Effect of Treatments on Antibiotic Activity

The modified disk diffusion assay was used as a preliminary phenotypic screening method to evaluate possible interactions between the tested vitamins and antibiotics. Most antibiotics showed no meaningful enhancement. The only notable effect was observed with tigecycline and vitamin K1, where the mean inhibition zone diameter increased from 9mm in the positive control to 16mm, indicating enhanced antibacterial activity.

In contrast, vitamin D3 slightly reduced the inhibition zone to 8 mm, while the combined treatment produced a value of 9 mm, similar to the positive control. For the remaining antibiotics, ciprofloxacin and imipenem maintained stable activity, levofloxacin and ceftazidime showed only minor variations, and ampicillin remained inactive. Therefore, the observed enhancement was limited to the interaction between vitamin K1 and tigecycline and should be interpreted cautiously as a preliminary antibiotic-dependent phenotypic effect (Table 6).

Table 6: Effect of Treatments on Antibiotic Activity

Antibiotic	Un-treated	VitaminD3	Vitamin K1	Vitamin D3 + K1	Interpretation
Tigecycline (TG)	9 f	8 f	16 d	9 f	Vitamin K1 enhanced
Ciprofloxacin (CIP)	34 a	34 a	34 a	34 a	No change
Levofloxacin (LEVO)	15 de	15 de	15 de	14 e	No improvement
Imipenem (IPM)	20 c	20 c	20 c	20 c	No change
Ampicillin (AMP)	0 g	0 g	0 g	0 g	No antibacterial activity
Ceftazidime (CAZ)	23 b	21 c	21 c	23 b	No improvement

Discussion

Biofilm formation: The antibiofilm effect of D3 is consistent with Lutfi *et al*. (2024), who reported that vitamin D3 reduced biofilm formation in Gram-negative bacteria, including *P. aeruginosa*. This agreement may be due to the ability of vitamin D3 to interfere with early adhesion, extracellular polymeric substance production, intra or intercellular communication, or other environmental conditions. [16, 17] Biswas *et al*. (2025) support this interpretation, as vitamin D3 has been shown to influence virulence-associated factors, particularly biofilm formation and secretion of virulence factors. [18] The effect of vitamin K1 agrees with Jia *et al*. (2022), who demonstrated that phylloquinone can inhibit the pqs quorum-sensing system in *Pseudomonas aeruginosa*. This explains why vitamin K1 may reduce biofilm formation without acting as a bactericidal agent. [19] Similarly, Soltani *et al* (2021) support the anti-quorum-sensing role of vitamin K1 against biofilm formation and also pyocyanin and protease as virulence factors. [9] The combination of vitamin D3 with vitamin K1 treatment may reflect an enhanced anti-biofilm trend; each vitamin may target a different aspect of virulence regulation. Levofloxacin is consistent with She *et al*. (2019), who demonstrated significant dose-dependent inhibition of biofilm formation during the early stages of biofilm development. [20]

Pyocyanin production: The response to vitamin D3 does not fully align with its expected anti-virulence role, as it stimulates pyocyanin production under non-lethal conditions. This discrepancy may be explained as a stress-related bacterial response. Amyl *et al*. (2021) reported that subinhibitory concentrations of some bioactive compounds increase pyocyanin production in *Pseudomonas aeruginosa*. [21] Since pyocyanin is regulated by RhI and PQS quorum-sensing systems, this response may represent adaptive virulence activation rather than direct inhibition. [22, 23]

In contrast, vitamin K1 strongly agrees with Soltani *et al*. (2021), who reported that vitamin K1 reduces quorum-sensing-regulated virulence factors, including pyocyanin. [9] Because the inhibition of the pqs system can directly affect pyocyanin regulation [19], vitamin K1 appears more consistent as a quorum-sensing inhibitor than vitamin D3 in this specific trait.

The combined treatment dose not clearly agree with an expected synergistic inhibition of pyocyanin. This may be because the stimulatory effect of Vitamin D3 could partially mask the inhibitory effect of Vitamin K1. Therefore, the combination should be interpreted as treatment-dependent rather than universally synergistic.

Levofloxacin agrees with Zeki and Atiyea (2022), who reported that levofloxacin can reduce pyocyanin production in clinical isolates of *P. aeruginosa*. This may result from its effect on physiology and virulence expression in addition to its antibacterial activity. [24]

Protease activity: Vitamin D3 reduced protease activity; this finding may be interpreted in light of previous reports showing that vitamin D3-containing combinations attenuated QS-associated phenotypes of *P. aeruginosa*, including extracellular protease secretion. [18] Since extracellular proteases in *P. aeruginosa*, particularly LasA, LasB, and protease IV, are strongly regulated by quorum-sensing systems, the observed reduction may reflect suppression of QS-regulated protease production rather than direct inhibition of the enzymes themselves [24, 26]

In contrast, vitaminK1 shows strong agreement with Soltani *et al*. (2021), who reported that vitamin K1 acts as an anti-quorum-sensing agent against *P. aeruginosa* and can reduce the regulation of quorum-sensing virulence factors, including protease activity. [9] Extracellular proteases in *P. aeruginosa*, including elastase and alkaline proteases, are regulated by quorum-sensing systems. Therefore, inhibition of quorum sensing by vitamin K1 reduces protease secretion and weakens the bacterium's tissue-damaging potential. [23, 25]

The combined treatment may reflect overlapping effects on virulence regulation, especially because Vitamin D3 may contribute to broader anti-virulence modulation, whereas Vitamin K1 may interfere more directly with quorum-sensing-regulated protease production

The reduced protease activity observed after levofloxacin treatment may indicate that this antibiotic can modulate protease-related virulence in *Pseudomonas aeruginosa*. Yang *et al*. (2024) showed that levofloxacin-mediated selective pressure was linked with the development of extracellular protease-deficient strains, suggesting that levofloxacin may affect production of extracellular proteases. Based on this, the present finding may indicate a reduction in protease production. [26]

Phagocytic activity: Vitamin D3 agrees with Chandra *et al*. (2004), who report that this vitamin enhanced macrophage phagocytosis. [27] It also agrees with Nouari *et al*. (2016), who showed that the active form of vitamin D3 improved macrophage bactericidal activity against *P. aeruginosa*. [8] Small *et al*.

(2021) further support this mechanism by showing that vitamin D3 upregulates CRIg receptors on macrophages, thereby enhancing microbial recognition and uptake. [28]

Vitamin K1 does not fully agree with the immune-enhancing pattern of vitamin D3. This difference may be due to the anti-inflammatory and anti-virulence effects of vitamin K1. Jia *et al*. (2022) and Soltani *et al*. (2021) showed that vitamin K1 reduces quorum-sensing-regulated virulence factors, which may decrease bacterial stimulation of phagocytic cells [9, 19] In addition, Kieronska-Rudek *et al*. (2021) and Ohsaki *et al*. (2010) reported that Vitamin K can suppress macrophage inflammatory responses in macrophage-like cells, reducing mediators such as NOS-2/NO, COX-2, IL-6, and TNF-α, mainly through inhibition of NF-κB. Therefore, the lower phagocytic activity observed with K1 reflects lower inflammatory stimulation rather than harmful suppression of phagocyte function. [29, 30]

The combination treatment appears closer to vitamin K1 than vitamin D3. This may indicate that the anti-inflammatory or virulence-attenuating effect of K1 predominated over the stimulatory immune effect of D3.

Levofloxacin agrees with Smith *et al*. (2000), who reported that levofloxacin penetrates human monocytes and enhances intracellular killing of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This suggests that levofloxacin may support bacterial clearance both extracellularly and intracellularly. [31]

Interaction with antibiotics: The interaction results showed that the interaction between the tested vitamins and antibiotics was antibiotic-dependent. The clearest enhancement is observed with vitamin K1 in combination with tigecycline; the inhibition zone is larger than with tigecycline alone. This finding suggests that vitamin K1 improves the antibacterial activity of tigecycline against *P. aeruginosa*. This is supported by Celebi *et al*. (2024), who reported that certain vitamins can enhance antibiotic activity against multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. [32] The improvement observed with tigecycline is particularly important because *P. aeruginosa* is known to show reduced susceptibility to tigecycline, partly due to intrinsic resistance mechanisms such as efflux pump activity. The efflux pump contributed to tigecycline resistance in *P. aeruginosa*, as reported by Dean *et al*. (2003). [33] Therefore, the increase in inhibition zone in the vitamin K1-tigecycline combination may indicate a possible adjuvant effect.

Conclusion

At sub-MIC concentration, Vitamin D3 and K1 affect *P. aeruginosa* in different but complementary ways.

Vitamin D3 showed the greatest effect on protease activity and phagocytic activity. In contrast, vitamin K1 markedly reduced pyocyanin production and increased the *in vitro* activity of tigecycline under the tested conditions. The combined treatment may provide supportive anti-virulence effects against biofilm formation, but it does not show a uniform synergistic pattern across all virulence factors. Therefore, these vitamins may be considered promising supportive anti-virulence or antibiotic-adjuvant candidates, while further molecular studies, gene-expression analysis and *in vivo* validation are required to confirm their therapeutic relevance**.**

Ethical approval

The study protocol was approved by the Scientific Research and Ethics Committee of the Ninevah Health Directorate, Ministry of Health, Iraq, under approval letter No. 2781 dated 1 October 2025 and committee resolution No. 272. Clinical isolates were collected and processed in accordance with the approved research protocol.

Author Contributions

Haneen Haitham Al-Saraj: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft.

Rasmia Omar Al-Jobory: Supervision, Validation, Writing – review & editing.

Both authors read and approved the final version of the manuscript.

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