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Quorum-Sensing inhibition by furanone compounds and therapeutic effects on Pseudomonas aeruginosa keratitis rabbit model

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Abstract

Background To investigate Quorum-Sensing inhibition by furanone compounds in Pseudomonas aeruginosa keratitis rabbit model.

Methods Thirty adult New Zealand White rabbits were used. Anesthetized rabbits were intrastromally injected with Pseudomonas aeruginosa (P. aeruginosa). The rabbits were divided into six groups: the control group (infected only with P. aeruginosa), group A (50 mg/mL ceftazidime), group B (0.1 mg/mL furanone), group C (0.2 mg/mL furanone), group D (0.3 mg/mL furanone), and group E (20% dimethyl sulfoxide). One drop of the treatment was applied every hour for 3 days, starting 1-h post-inoculation. Rabbits were then sacrificed, and corneas were analysed clinically, microbiologically, histologically, and biochemically. One-way analysis of variance was used for the mean comparison of independent groups. The Least Significant Difference method was used as a post-hoc test for pairwise comparisons.

Results In all evaluations, the antibiotic group (group A) showed the best therapeutic response. The slit-lamp examination score of group C was significantly lowered than those compared of to the control ($p=0.009$) and E groups ($p=0.014$). Histological evaluation showed that inflammation is decreased in groups B, C, and D. Levels of cyclooxygenase-2 (COX-2), superoxide dismutase-1, and reactive oxygen species (ROS) were lowest in the antibiotic-treated group, whereas the highest levels were detected in the control group. Notably, the COX-2 levels in group B and ROS levels in groups B and C were significantly lower than in control group. ($p=0.045$, $p=0.039$ and $p=0.045$, respectively).

Conclusion Furanone compounds may have minimal therapeutic effects against Pseudomonas keratitis. Its therapeutic effect has not been observed to be sufficient compared with that of antibiotics. Further studies are needed to investigate their protective effects and mechanisms.

Keywords Furanone, Keratitis, Pseudomonas aeruginosa, Quorum-Sensing

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Background

Keratitis is an inflammation of the cornea and is associated with both infectious and non-infectious diseases. Infectious keratitis is a major cause of visual impairment and blindness globally [1]. Among all types of infectious keratitis, bacterial keratitis is the most common type and it consists for approximately 65–90% of all microbial keratitis [2]. Pseudomonas aeruginosa (P. aeruginosa) is a versatile Gram-negative pathogen that causes a wide range of infections and is the most frequent agent among bacterial keratitis [3]. Nowadays, the occurrence of multidrug resistant (MDR) microbial pathogens threatens the foundation upon which standard antibacterial treatment is based. Therefore, alternative treatment methods to limit bacterial infections have been investigated. There is a new strategy to control the infections that aims to affect and stop the adaptability of microbes to the host environment and prevent their communication with each other rather than affecting their growth [4]. A mechanism called Quorum-Sensing (QS) is communication between bacterial cells that relies on the cell density and the concentration of specific signalling molecules [5]. Many Gram-negative bacterial species, such as P. aeruginosa, utilize acyl homoserine lactone (AHL) molecules as signals to coordinate their population behaviour during invasion and colonization. After host colonization, P. aeruginosa causes tissue damage by various virulence factors and QS is responsible for the regulation of these factors, such as the ability of bacteria to form biofilms [6, 7]. Some quorum sensing inhibitors attenuate bacterial pathogenicity by creating an antagonistic effect on these signalling molecules. Inhibitors of QS are an advanced strategy to decrease P. aeruginosa pathogenesis and virulence. Two decades ago, furanones were the first class of chemical compounds identified as Pseudomonas aeruginosa QS inhibitors (QSIs) [8]. Based on the previous studies and the proven effects of furanones as a quorum sensing inhibitors on Pseudomonas infection both in vivo and in vitro [9], this study aimed to investigate the effects of Quorum-Sensing inhibition by furanone compounds on bacterial restriction, without directly killing bacteria or inhibiting their growth, in a *Pseudomonas aeruginosa* keratitis rabbit model.

Materials and methods

Bacterial strain

The bacterial strain used in this study was P. aeruginosa (ATCC 27853). A single colony of P. aeruginosa was grown in tryptic soy broth (TSB) overnight at 37 C. Then, to determine the amount to be used in inoculation, the sample was diluted (1:1000) in 2 mL saline over 0.5 McFarland (10^8) to form 100,000 colony-forming units (CFU) per ml. For the amount to be inoculated into corneas, saline was prepared with 1000 CFU of bacteria in 0.01 mL.

Animals

Specific pathogen-free, 30 adult New Zealand White rabbits were used ($n = 5$ corneas per group). Their weights ranged from to 4–5 kg. The animals used in this study were obtained from the Bezmialem Vakıf University Experimental Application and Research Center (Istanbul, Turkey). All procedures involving animals were conducted in accordance with institutional guidelines and approved by the relevant ethics committee. The animals were housed in accordance with the institutional guidelines and tenets of the Association of Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research. The animals were free of food and water. Animals were allocated to experimental groups using simple random allocation based on the order in which they were housed and handled. There were no systematic differences between groups at baseline. All clinical, histological, and biochemical assessments were performed by observers who were blinded to the group assignments.

Experimental keratitis

All procedures were designed in accordance with the generally accepted ethical standards for animal experimentation, the guidelines established by the National Institutes of Health for the care and use of laboratory animals. The study was approved by Laboratory Animals Local Ethics Committee at the Bezmialem Vakıf University. Only the left eye of the rabbits was included in the study. Animals were anesthetized with intramuscular injections of ketamine hydrochloride (10 mg/kg) and xylazine hydrochloride (5 mg/kg). Prior to infection, proparacaine hydrochloride (0.5%; Alcaine®; Alcon, Puurs, Belgium) was topically applied to the left eye. The central corneas of anesthetized rabbits were intrastromally injected with 10^3 bacteria in 0.01 mL of saline (using a 26-gauge needle; Berika, Konya, Turkey). At the time of sacrifice, all animals were anesthetized and administered a lethal overdose of pentobarbitol (Sigma- Aldrich, St. Louis, MO).

Furanone

Commercially purchased synthetic (Z-)− 4-Bromo-5-(bromomethylene)− 2(5H)-furanone (Furanone C-30) (Sigma-Aldrich®) was used in this study and stored at − 20 C as a long-term stock. It was dissolved in 20% dimethyl sulfoxide (DMSO) to be dripped onto the rabbit's eyes and placed in sterile droppers at predetermined doses following all decontamination procedures. It was stored at +4 C degrees to be use in the experiment.

Table 1 Scoring system for slit lamp examinations

Anatomical Site	*Parameter Graded*	*Range of Grades per Parameter*	*Grade for a Normal Eye*	*Grade for a Maximally Inflamed Eye*
Conjunctiva	Injection	0–4	0	4
	Chemosis	0–4	0	4
Cornea	Infiltrate	0–4	0	4
	Swelling	0–4	0	4
Anterior Chamber	Hypopyon	0–4	0	4
Total			0	20

Furanone concentrations (0.1, 0.2, and 0.3 mg/mL) were calculated based on the ratio of the furanone dose reported by Wu et al. [9] in the treatment of lung infection to the ceftazidime dose used in human *Pseudomonas* pneumonia, as well as considering the anatomical difference in eye volume between humans and New Zealand rabbits [10].

Treatment regimen

Rabbits were randomly divided into six groups, with five rabbits per group after inoculation. Group N was determined as the control group and was infected with P. aeruginosa only. In group A ($n=5$), 50 mg/mL ceftazidime (ZIDIM®, Tüm Ekip Ilaç A.Ş) treatment will be given topically per hour to investigate the effectiveness of furanone treatment compared to antibiotic treatment. Starting one hour after inoculation, 0.1 mg/mL dose furanone in group B ($n=5$), 0.2 mg/mL dose furanone in group C ($n=5$), 0.3 mg/mL dose furanone in group D ($n=5$) were topically dripped on the left eyes with one drop per hour. Group E ($n=5$) was administered 20% DMSO alone as a solvent for furanone. Furanone solutions and ceftazidime were dripped 12 times a day every hour for 3 days. After 3 days, the rabbits were sacrificed, and their corneas were collected. The collected corneas were divided into three equal parts, with the centre of the infected area positioned in the central region. The pieces were randomly separated and stored for histopathological, biochemical, and microbiological examination.

Slit lamp examination (SLE)

Three days post-inoculation (PI), slit-lamp examination (SLE) of pathological changes in rabbit eyes was performed by two blinded observers using a Leica M620 F18 biomicroscope. Each of the five parameters was graded on a scale ranging from 0 (none) to a maximum of 4 (severe) as follows: injection, chemosis, hypopyon, corneal infiltrate, and corneal edema. The sum of these grades for an eye after averaging determines the SLE score. The SLE score ranges from 0 (normal eye) to a theoretical maximum of 20 [11]. A summary of the scoring system is presented in Table 1.

Quantitative colony count

At 3 days PI, rabbits were anesthetized and sacrificed, and corneas were collected to quantify the colony count per cornea. Corneas were placed in sterile saline solution (NaCl 0.45%- pH:4.5–7.0) and homogenized. After the samples were homogenized by vortex for 10 min, the corneal homogenates were plated in triplicate on blood agar for quantitative colony counting. Plates were incubated at 37 C° with CO_2 overnight and the colony count per cornea was determined.

Biochemical evaluations

Corneas were immediately placed in sterile centrifuge tubes. The corneal tissues were weighed and homogenized in phosphate-buffered saline (PBS, 10% w/v) at 30 frequencies for 3 min using a ball mill homogenizer. Homogenates were centrifuged at 16,000 ×g for 5 min at 4 °C. Protein quantification in each supernatant was performed using a Commassie Plus-The Better Bradford Assay reagent (Thermo, USA). The supernatants were transferred to centrifuge tubes and stored at − 80 °C until the experiments. Enzyme-Linked ImmunoSorbent Assay (ELISA) kits were used to detect superoxide dismutase- 1 (SOD- 1) and cyclooxygenase- 2 (COX- 2) levels. Experiments were performed according to the manufacturer's instructions, subsequently the absorbance was read at 450 nm wavelength in a microplate reader (Varioskan Flash, Multiplate Reader, Thermo, USA). Reactive oxygen species (ROS) levels in the tissues were analysed using a fluorescent probe, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich). Cornea tissue homogenates and 10 µM DCFH-DA were incubated for 30 min at 37 °C, afterwards washed twice with PBS. After the conversion of DCFH to the fluorescent product DCF, ROS levels were detected using a Varioskan Flash Multiplate Reader (Thermo, USA) with excitation at 485 nm and emission at 530 nm.

Histopathological evaluation

Corneal samples were fixed in 10% neutral buffered formalin for one day. Fixed tissue samples were dehydrated by passing through graded alcohol according to the routine light microscope tissue processing method. After clearing in xylol, the sections were embedded in paraffin. Sections of 3–4 μm thickness were taken from the paraffin blocks with a rotary microtome on positively charged slides. The slides were deparaffinized at 70 °C, they were then rehydrated by decreasing alcohol series. After rehydration, slides were stained with hematoxylin–eosin (HE) and examined using a Nikon Eclipse i5 (Tokyo, Japan) with a Nikon DS-Fi1c camera attachment (Tokyo, Japan) and analysed using the NIS Elements version 4.0 image analysis system (Nikon Instruments Inc., Tokyo, Japan). The histopathological changes were evaluated.

Statistical analysis

In this study, descriptive statistics of quantitative variables were presented as mean and standard deviation (SD). Assumptions with normal distribution were evaluated by Shapiro–Wilk test. One-way analysis of variance (ANOVA) was used for the mean comparison of independent groups. The Least Significant Difference (LSD) method was used as a post-hoc test for pairwise comparisons. For non-normally distributed variables, the Kruskal–Wallis test was applied. When a significant difference was found, pairwise group comparisons were conducted using the Mann–Whitney U test. All tests were two-sided, with 95% confidence level; $p < 0.05$ was considered significant. All statistical analyses were performed using the SPSS statistical analysis software (IBM SPSS Statistics for Windows, Version 26.0). The power analysis for this study used a sample size calculation with 80% statistical power and 95% confidence level.

Results

Effects of furanone on clinical examination

Clinical observations were evaluated by SLE (Table 1). At 3 days PI, while the untreated eye group had an average SLE score of 19.2 ± 1.30, the eyes treated with Ceftazidime had an average SLE score of 2.4 ± 1.87, a difference which was statistically significant across all groups ($p \leq 0.001$). The eyes treated with 0.2 mg/mL Furanone had an average SLE score of 2.8 lower than the untreated control group ($p = 0.009$) and 2.6 lower than the DMSO group ($p = 0.014$), both of which were statistically significant differences. No statistically significant differences were found in the other comparisons between the groups in terms of SLE scores. (Table 2).

Effects of furanone on bacterial density

Untreated eyes had approximately 186.4 ± 120.22 CFU per cornea (Table 2). Eyes treated with Ceftazidime had significantly fewer colony counts per cornea than the other groups ($p < 0.05$). The average colony count in the DMSO group was not significantly different from that of the untreated group. ($p > 0.05$). Also, there was no statistically remarkable difference between the furanone groups. ($p > 0.05$). (Table 3).

The histology of centrally sliced 1/3 of corneas after 3 days of PI is shown in Fig. 1. Especially in the control and DMSO groups, vast infiltration of P. aeruginosa was seen covering the epithelium and entire stroma and infiltrating the Descemet's membrane. While the entire epithelium was intact in the ceftazidime group, no diffuse cell infiltration was observed in the furanone groups. Although there were areas of inflammatory cell infiltration in particular sections. When the corneal stroma was evaluated as a whole, it was observed that the damage in the furanone groups was less than in the control and DMSO groups. Therefore, histological evaluation showed that inflammation is decreased in groups B, C, and D.

Effects of furanone on SOD1-COX2-ROS levels

Statistically significant differences were observed between groups in the mean levels of COX- 2 and ROS ($p = 0.029$ and $p = 0.044$, respectively), whereas no statistically significant difference was found in SOD- 1 levels ($p = 0.846$). In Tables 2 and 3, the differences in COX- 2, SOD1, and ROS levels between the groups were analysed, and the corresponding descriptive statistics and p-values were provided. The lowest mean COX- 2 level was measured in the antibiotic-treated group (71.66 ± 12.91), whereas the highest mean level was observed in Group N (control group) (99.39 ± 6.54).

COX- 2 levels in the corneas of the antibiotic-treated group were significantly lower than those in the 0.1 mg/mL furanone ($p = 0.012$), 0.3 mg/mL furanone ($p = 0.01$), DMSO ($p = 0.024$) and control ($p = 0.007$) groups. However, when compared to the control group and other groups, COX- 2 levels were significantly higher in the control group than in the 0.2 mg/mL furanone group ($p = 0.045$). According to the statistical analysis, ROS levels in the antibiotic-treated group, 0.1 mg/mL furanone and 0.2 mg/mL furanone group were significantly lower than those in the control group. ($p = 0.028$, $p = 0.039$ and $p = 0.045$, respectively) (Table 3).

Discussion

In this study, we investigated the efficacy of three different doses of furanone (0.1 mg/mL, 0.2 mg/mL, and 0.3 mg/mL) on P. aeruginosa keratitis in a rabbit model.

Table 2 Clinical and laboratory outcomes in antibiotic, furanone, DMSO, and control groups for pseudomonas keratitis

	Group	*Mean ± S.D*	*Range*	*P-value*
Slit-Lamp Examination (SLE) Score	*Control (N)*	19.20 ± 1.30	17–20	< 0.001*
	Antibiotic-treated (A)	2.00 ± 1.87	0–4	
	0.1 mg/mL Furanone (B)	17.80 ± 1.78	16–20	
	0.2 mg/mL Furanone (C)	16.40 ± 1.81	14–18	
	0.3 mg/mL Furanone (D)	17.60 ± 1.51	16–20	
	% 20 DMSO (E)	19.00 ± 0.70	18–20	
Colony Forming Unit (CFU) Count	*Control (N)*	186.4 ± 120.22	112–400	0.014**
	Antibiotic-treated (A)	0.4 ± 0.89	0–2	
	0.1 mg/mL Furanone (B)	155 ± 83.64	92–300	
	0.2 mg/mL Furanone (C)	161.4 ± 82.59	91–300	
	0.3 mg/mL Furanone (D)	149.6 ± 141.19	65–400	
	% 20 DMSO (E)	141.4 ± 42.36	100–200	
SOD- 1	*Control (N)*	32.67 ± 8.46	22.32–40.27	0.846*
	Antibiotic-treated (A)	28.50 ± 6.33	20.31–35.36	
	0.1 mg/mL Furanone (B)	33.44 ± 6.25	22.72–38.91	
	0.2 mg/mL Furanone (C)	32.83 ± 7.46	24.72–40.73	
	0.3 mg/mL Furanone (D)	31.73 ± 6.65	22.98–37.86	
	% 20 DMSO (E)	30.12 ± 4.30	25.64–36.98	
COX- 2	*Control (N)*	99.39 ± 6.54	90.27–106.06	0.029*
	Antibiotic-treated (A)	71.65 ± 12.91	62.21–95.64	
	0.1 mg/mL Furanone (B)	97.41 ± 12.48	86–111.05	
	0.2 mg/mL Furanone (C)	82.46 ± 19.60	66.57–116.50	
	0.3 mg/mL Furanone (D)	97.98 ± 8.88	89.78–111.37	
	% 20 DMSO (E)	94.97 ± 11.38	83.78–111.55	
ROS	*Control (N)*	0.44 ± 0.20	0.24–0.70	0.044*
	Antibiotic-treated (A)	0.26 ± 0.11	0.16–0.41	
	0.1 mg/mL Furanone (B)	0.27 ± 0.12	0.17–0.38	
	0.2 mg/mL Furanone (C)	0.28 ± 0.05	0.22–0.36	
	0.3 mg/mL Furanone (D)	0.31 ± 0.08	0.20–0.48	
	% 20 DMSO (E)	0.39 ± 0.07	0.34–0.52	

* One way ANOVA Test

** Kruskal–Wallis Test, DMSO: dimethyl sulfoxide

The outcomes were compared with those of a group treated with fortified ceftazidime and an untreated control group. While virtually no clinical or histological abnormalities were observed in the antibiotic-treated group and bacterial growth was minimal, none of the furanone doses significantly reduced bacterial colonies. However, the furanone-treated groups showed less ophthalmic pathology, both clinically and histologically compared to the control group.

The MDR pathogens are a significant threat to antibiotic therapy. Consequently, alternative treatment strategies have been explored [2]. Many pathogens utilize intercellular communication mechanisms to convey their overall bacterial population density, one of which is Quorum-Sensing. When high concentrations are detected, pathogens can modify their transcriptional patterns by adopting invasive phenotypes. Experimental models of systemic infections have identified a wide range of organic and synthetic chemical compounds that are capable of inhibiting quorum sensing and improving therapeutic outcomes [12, 13]. Due to the limited effects of natural furanone compounds on the Quorum-Sensing systems of P. aeruginosa, we selected the synthetic Furanone C- 30 compound for this study, as it has been reported to exhibit greater efficacy in both in vivo and in vitro studies [9, 12, 14]. However, QSIs have demonstrated varying levels of success in animal models, with both successful and unsuccessful applications documented. [15–22] Therefore, the

Table 3 Statistical Analysis of Treatment Effects: Post Hoc Comparisons Among Study Groups

P-values						
Group		***SLE Score****	***CFU Count*****	***COX- 2****	***SOD- 1****	***ROS****
Antibiotic-treated (A)	*0.1 mg/mL Furanone (B)*	< 0.001	0.008	0.012	0.255	0.881
	0.2 mg/mL Furanone (C)	< 0.001	0.008	0.403	0.318	0.749
	0.3 mg/mL Furanone (D)	< 0.001	0.008	0.010	0.453	0.470
	% 20 DMSO (E)	< 0.001	0.007	0.024	0.706	0.092
	Control (N)	< 0.001	0.006	0.007	0.335	0.028
0.1 mg/mL Furanone (B)	*0.2 mg/mL Furanone (C)*	0.167	0.841	0.074	0.886	0.865
	0.3 mg/mL Furanone (D)	0.841	0.310	0.944	0.690	0.566
	% 20 DMSO (E)	0.234	0.845	0.763	0.441	0.122
	Control (N)	0.167	0.548	0.807	0.857	0.039
0.2 mg/mL Furanone (C)	*0.3 mg/mL Furanone (D)*	0.234	0.314	0.064	0.798	0.686
	% 20 DMSO (E)	0.014	0.931	0.131	0.529	0.166
	Control (N)	0.009	0.690	0.045	0.970	0.045
0.3 mg/mL Furanone (D)	*% 20 DMSO (E)*	0.167	0.310	0.710	0.707	0.318
	Control (N)	0.117	0.151	0.862	0.827	0.121
% 20 DMSO (E)	*Control (N)*	0.841	0.811	0.586	0.553	0.563

DMSO dimethyl sulfoxide

* Post hoc test (LSD)

** Mann–Whitney U test

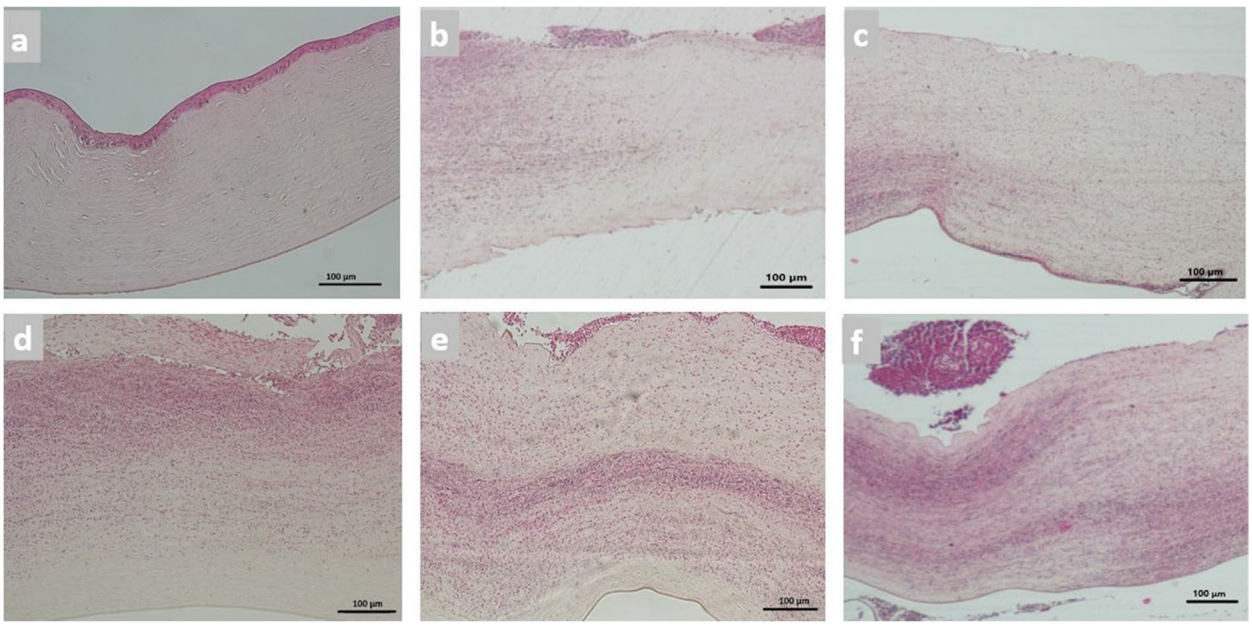

Fig. 1 Cross-section of the corneas of each group (histological evaluation). **a** Group A (antibiotic-treated), **b** Group B (0.1 mg/mL Furanone), **c** Group C (0.2 mg/mL Furanone), **d** Group D (0.3 mg/mL Furanone), **e** Group E (% 20 DMSO), **f** Group N (Control) H&E stain; X100

clinical utility of QSIs in the treatment of MDR pathogens remains a subject of debate [12, 13].

In studies investigating the therapeutic effects of furanones, several parameters have been examined, including the inhibition of QS mechanisms to suppress bacterial virulence factors [6, 9, 17, 23], prevention or disruption of biofilm formation [9, 15, 17, 21], efficacy of combination treatments with antibiotics [15–17], reduction of bacterial load and effects on clinical and histological improvement of infections [9] and support of immune

system functions [16, 17, 23]. In our study, we evaluated the therapeutic potential of furanone compounds in a rabbit keratitis model by analysing their effects on clinical outcomes, histological inflammation, bacterial load reduction, and biochemical parameters, such as ROS levels and SOD- 1 activity, in comparison to standard antibiotic therapy. We considered the clinical outcomes to be the most significant among these factors.

P. aeruginosa induces cellular death and tissue destruction in the cornea through ExoU and ExoS toxins while exacerbating infection severity via excessive immune inflammation and biofilm formation, resulting in clinical parameters such as corneal opacity, corneal epithelial defect, chemosis, and conjunctival hyperemia, as observed in many studies [24]. McCormick et al. evaluated the clinical status of microbial keratitis cases using a scoring system. In our study, we assessed the clinical condition of subjects using SLE based on a total score obtained by grading each parameter—conjunctival injection and chemosis, corneal infiltrate and swelling, and anterior chamber hypopyon—on a scale of 0 to 4. At three days PI, a significant reduction in the SLE score was observed in eyes treated with furanone (Group C) compared to the control and DMSO groups. However, this reduction was substantially less pronounced than in the ceftazidime group. Similar findings were noted in the histological corneal sections. Inflammation and cellular infiltration levels in the furanone groups were slightly lower compared to the control and DMSO groups. Specifically, epithelial damage, inflammation, and cellular infiltration were minimal in the ceftazidime group but severe in the control and DMSO groups. In furanone groups, inflammation appeared to be somewhat lower than in the control and DMSO groups. These findings align, to some extent, with the histological results of an in vivo study by Wu et al. [9], which investigated the effects of furanone on Pseudomonas lung infections in mice.

In the study by Wu et al. [23], severe tissue damage characterized by lung abscesses was typically found in the placebo and low dose furanone treatment groups. In contrast, the medium- or high-dose furanone treatment groups demonstrated lower inflammatory cell density, with polymorphonuclear leukocyte (PMN) infiltration largely confined to the bronchi. In the placebo and low-dose groups, lung abscesses, marked inflammation, and severe tissue damage with haemorrhage were observed in both the bronchi and lung parenchyma. In our study, although minor differences were observed between the furanone groups and other groups in clinical and histological evaluations, no superiority of furanone over the other groups was detected in terms of bacterial density in corneal sections. The inability to achieve a significant reduction in bacterial density may be attributed to the lack of direct bactericidal effects of the furanones [16]. This is consistent with numerous studies reporting that QS inhibitors do not affect bacterial growth or viability, but instead specifically target QS-controlled gene expression [23], suppress the production of virulence factors in P. aeruginosa [6, 9, 23], prevention of biofilm formation [9, 21], enhance the sensitivity of biofilms to antibiotics [15, 17], and support the host immune response [16, 17, 23]. Quorum sensing inhibitors have been shown to support the host immune system through multiple mechanisms, including suppression of NF-κB-mediated pro-inflammatory cytokine production, prevention of immune cell apoptosis via neutralization of AHL signals , reduction of COX- 2 and ROS levels, and modulation of Th1/Th2 immune balance [20, 25]. This suggests that quorum sensing inhibition might be more appropriate as an adjuvant therapy in combination with antibiotic treatment [15, 17]. On the other hand, some studies have found that certain QSIs, even when used alone without antibiotics, can significantly reduce CFU counts compared to the placebo group [9, 23].

To justify the dosing strategy applied in this research, both previously reported systemic dosing and species-specific ocular anatomy were taken into account. The final concentration was calculated based on the ratio of the furanone dose reported by Wu et al. [9] in the treatment of lung infection to the ceftazidime dose used in human *Pseudomonas* pneumonia, while also considering the difference in eye volume between humans and New Zealand rabbits [10]. A dose-escalation study could provide more insight into the optimal concentration of furanone for ocular use.

The most notable findings from the biochemical tests in our study were as follows: COX- 2, SOD- 1, and ROS levels were lowest in the antibiotic-treated group and highest in the control group. COX- 2 levels were significantly lower in the 0.2 mg/mL furanone group compared to the control group, and ROS levels were significantly lower in the antibiotic-treated, 0.1 mg/mL furanone, and 0.2 mg/mL furanone groups than in the control group. However, the SOD- 1 levels did not differ significantly between the groups.

COX- 2 is an enzyme that promotes the production of pro-inflammatory prostaglandins, and is significantly upregulated during infection and inflammation. SOD-1 acts as an antioxidant, converting superoxide radicals into less reactive species such as hydrogen peroxide to manage oxidative stress. ROS, which are elevated in inflammatory and infectious states, contribute to tissue damage but also play a role in signalling pathways. COX-2 expression can be further amplified by ROS. SOD- 1 levels may also rise in response to increased ROS levels,

aiming to neutralize oxidative stress. However, in chronic inflammation or overwhelming oxidative stress, antioxidant defenses, such as SOD- 1 can become depleted, leading to sustained high ROS levels, persistent COX- 2 expression and perpetuating inflammation [26].

In our study, low levels of ROS, COX- 2, and SOD- 1 in the antibiotic-treated group were expected. However, despite the much more pronounced differences between the antibiotic-treated group and the other groups in clinical, microbiological, and histological assessments, the differences in biochemical tests were less significant. This discrepancy may be attributed to several factors. Firstly, these biochemical markers are influenced by a wide array of stimuli beyond infection and inflammation, including mechanical stress, tissue handling, hypoxia, and cellular redox status. For instance, COX- 2 expression can be triggered by cytokines, growth factors, and oxidative stress, while SOD- 1 levels are sensitive to fluctuations in intracellular oxidative load, metal ion availability, and mitochondrial activity [27, 28].

Secondly, these markers may not exhibit changes that are temporally aligned with histological or clinical outcomes. Unlike structural damage observed in histopathology, the levels of COX- 2, ROS, and SOD- 1 can vary rapidly and are subject to regulatory feedback mechanisms. SOD- 1, for example, may spike early in response to oxidative stress and return to baseline despite ongoing inflammation [29].

Thirdly, it is important to recognize that these markers, although associated with inflammation, are not absolute indicators of its presence or severity. ROS also function as signaling molecules under physiological conditions, and both COX- 2 and SOD- 1 participate in processes such as wound healing and cellular adaptation [30, 31].

This discrepancy may also be partially explained by differences in the anatomical scope and sampling methods used across the various assessments. SLE evaluations were based on findings from the entire cornea, conjunctiva, and anterior segment structures, providing a broad and clinically reliable overview. Similarly, histological sections were obtained from the central focus of infection, likely yielding robust results due to the high concentration of pathological changes in that area. In contrast, microbiological and biochemical analyses required the corneas to be processed differently, necessitating their division into multiple parts. Although the corneas were sectioned equally, with the central infected area placed in the middle segment, potential heterogeneity in the distribution of infection and inflammation across the tissue may have contributed to variability or relative weakening in these specific outcomes.

The finding that ROS levels in the 0.1 mg/mL furanone and 0.2 mg/mL furanone groups and COX- 2 levels in the 0.2 mg/mL furanone group were lower than those in the control group may indicate that, although furanone does not possess bactericidal effects, it exhibits a degree of anti-inflammatory activity reflected in biochemical parameters. Furthermore, the fact that the same response was not observed across all doses suggested a dose-dependent effect.

Despite studies reporting better outcomes for furanone efficacy in other organs or tissues, the limited success in our study may be related to inadequate corneal penetration or bioavailability of furanone. Future studies measuring corneal absorption rates or employing alternative formulations to enhance penetration could help clarify these issues. In addition, two other limitations should be acknowledged. First, the small sample size of each group limits the statistical power and generalizability of the findings, highlighting the need for larger cohorts in future studies to validate these preliminary results. Second, this study did not include the assessment of bacterial virulence factors (e.g., pyocyanin production, elastase activity) or specific inflammatory mediators and cellular responses that could confirm quorum-sensing inhibition. Future investigations incorporating the measurement of virulence determinants and proinflammatory cytokines such as TNF-α and IL- 6 may offer more comprehensive mechanistic insights into the immunomodulatory properties of furanone treatment. Fourth, quantitative histological analysis, such as inflammatory cell counts, was not performed, which may have limited the objectivity of tissue-level inflammatory assessment. Addressing these limitations in future studies will provide a more robust evaluation of the therapeutic effects of furanone.

Conclusion

According to the findings of our study, while ceftazidime demonstrated a strong effect in P. aeruginosa keratitis, no significant antimicrobial effect was observed with furanone at doses of 0.1 mg/mL, 0.2 mg/mL, and 0.3 mg/mL. However, the better clinical and histological outcomes compared with the control group highlight the need for further studies with a larger number of participants to determine the significance of these findings.

Abbreviations

P. aeruginosa	Pseudomonas aeruginosa
COX- 2	Cyclooxygenase-2
SOD- 1	Superoxide dismutase-1
ROS	Reactive oxygen species
MDR	Multi-drug resistant
QS	Quorum-Sensing
AHL	Acyl homoserine lactone
QSIs	Quorum-Sensing inhibitors
TSB	Tryptic soy broth
CFU	Colony-forming units
DMSO	Dimethyl sulfoxide
SLE	Slit-lamp examination
PI	Post-inoculation

PBS	Phosphate-buffered saline
ELISA	Enzyme-Linked ImmunoSorbent Assay
DCFH-DA	2,7-Dichlorodihydrofluorescein diacetate
HE	Hematoxylin–eosin
SD	Standard deviation
ANOVA	One-way analysis of variance
LSD	Least Significant Difference

Authors' contributions
A.D. conceived the work and she acquired and organized the data. A.E. designed the work that led to the submission and he played an important role in interpreting the results. A.D. and A.E. played a leading role in drafting the manuscript. R.H., B.S. and F.B.B. played an important role in interpreting the results and provided revisions to the manuscript. Z.Ö.G. and E.A. were responsible for acquiring the data and contributed to revising the manuscript. A.D., R.H., B.S., F.B.B., Z.Ö.G., E.A. and A.E. approved the final version of manuscript, and they agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Data availability
Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate
All procedures were designed in accordance with the generally accepted ethical standards for animal experimentation, the guidelines established by the National Institutes of Health for the care and use of laboratory animals. The study was approved by Laboratory Animals Local Ethics Committee at the Bezmialem Vakıf University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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