Research Article

Assessment Of The Effect Of 3-Caffeoyl Quinic Acid On Biofilm Formation By Kocuria Kristinae Clinical Isolates From Ear Infections

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Abstract:

Background: Kocuria kristinae (earlier known as Rothia kristinae) is a relatively new opportunistic pathogen responsible for ear infections. The ability of this organism to form biofilms increases its resistance to therapy and promotes the development of antimicrobial resistance, necessitating new treatment methods. **Objective:** To assess the effect of 3-caffeoylquinic acid on K. kristinae isolated from patients with ear infections. **Methods:** Isolation of five clinical strains was done by conventional microbiological procedures. The ability of K. kristinae to form biofilms was determined by the crystal violet method (absorbance at OD_{570}) before and after 3-caffeoylquinic acid exposure; statistical data analysis was done using a paired t-test ($p < 0.05$). **Results:** All strains showed the presence of strong biofilm production ability before treatment (OD 0.54–0.80). After treatment, it was significantly reduced (0.642 ± 0.108 vs 0.124 ± 0.025), by 80.7%. All the isolates changed from being strong biofilm formers to weak ones ($p < 0.05$). **Conclusion:** 3-caffeoylquinic acid exhibited strong antibiofilm activity against K. kristinae isolates, suggesting it could be used as an alternative treatment for ear infections. In vivo studies need to be further carried out.

Keywords: Kocuria kristinae; ear infections; 3-caffeoylquinic acid; antibiofilm.

Introduction:

Infections in the ear (otitis media and externa) have emerged as an important health problem worldwide and are a source of morbidity to people of all ages. While common pathogens such as Staphylococcus aureus, Streptococcus pneumoniae, and Haemophilus influenzae are well-documented etiological agents, emerging opportunistic pathogens are increasingly recognised in clinical settings, particularly among immunocompromised patients and those with indwelling medical devices (Saleh et al., 2020; Ibraheem et al., 2023; Usta et al., 2025).

Kocuria kristinae (recently reclassified as Rothia kristinae) is a Gram-positive, catalase-positive, coagulase-negative coccus belonging to the family Micrococcaceae (Usta et al., 2025). First described by Kloos and colleagues in 1974 as Micrococcus kristinae, the organism was later reclassified into the genus Kocuria in 1995 and subsequently into Rothia in 2018 based on chemotaxonomic and phylogenetic studies ,This bacterium is a commensal of human skin, oropharynx, and upper respiratory tract, and is generally considered a low-virulence organism (Golla et al., 2022).

However, accumulating evidence indicates that K. kristinae can act as an opportunistic pathogen, particularly in immunocompromised hosts. Documented infections include bacteremia, infective endocarditis, peritonitis, and catheter-related bloodstream infections (Golla et al., 2022; Poyser et al., 2024; Usta et al., 2025). A systematic review of Rothia spp. infections identified R. kristinae as the most commonly isolated species, with bacteremia (36.3%), infective endocarditis (13.7%), and skin and soft tissue infections (18.6%) being the most frequent clinical presentations (Usta et al., 2025). Risk factors include haematological malignancies, solid organ malignancies, dialysis-dependent end-stage renal disease, and the presence of indwelling long-term devices (Golla et al., 2022; Poyser et al., 2024).

The ability of K. kristinae to form biofilms represents a critical virulence factor that complicates clinical management and contributes to persistent infections (Ananieva etal.,2018; Saleh et al., 2020). Biofilm formation facilitates adherence to host tissues and medical devices, protects the bacteria from antimicrobial agents, and enables evasion of host immune responses(Ananieva etal.,2018). Studies have demonstrated a direct correlation between the adhesion index of K. kristinae clinical strains and their biofilm-forming capacity, highlighting the importance of these properties in pathogenesis (Ananieva etal.,2018).

In the context of ear infections, Kocuria spp. have been isolated from patients with otitis media and peri-implant mucositis (Ananieva etal.,2018; Saleh et al., 2020). A study investigating bacterial isolates from otitis media infections identified Kocuria kristinae among the pathogenic species recovered from clinical specimens (Saleh et al., 2020). Furthermore, many studies have documented Kocuria kristinae isolated from different clinical isolates, with antibiotic resistance and biofilm-forming (Ziogou et al., 2023).

Natural products have become promising due to their alternative or complementary therapeutic potential. Among such compounds, caffeoylquinic acids, which are polyphenolic compounds found in many plants, including coffee and medicinal plants, possess antibacterial and antibiofilm activity (Igarashi et al., 2021; Zhang et al., 2024). Caffeoylquinic acids were proved to interfere with biofilm-forming processes of bacteria by affecting such important mechanisms as c-di-GMP and YcgR protein interaction (Zhang et al., 2024). Hence, the derivatives of caffeoylquinic acid may be considered a promising option for treating biofilm-related infections, such as Kocuria spp. (Igarashi et al., 2021; Zhang et al., 2024).

The current research aims to study the antibacterial and antibiofilm activity of 3-caffeoylquinic acid against Kocuria kristinae clinical strains isolated from ear infections.

Materials and Methods :

3.1 Bacterial Isolates

Five clinical isolates of Kocuria kristinae were collected from patients with otitis media. Identification of isolates was done through Gram staining, biochemical tests, and the Vitek system. The bacteria were cultured in nutrient agar at 37oC for 24-48 hours.

3.2 Test Compound

3-Caffeoylquinic acid (chlorogenic acid) was obtained from Sigma-Aldrich and dissolved in sterile distilled water to prepare stock solutions.

3.3 Antibiofilm Activity Assay

Bacterial biofilm was examined using the crystal violet microtiter plate method (Christensen et al., 1985):

1. Bacterial cultures (200 µL, 0.5 McFarland) were inoculated into 96-well plates and incubated at 37oC for 24 hours.
2. The wells were washed with PBS, fixed with methanol, and stained with 0.1% crystal violet for 15 minutes.
3. Excess dye was washed off, and plates were air-dried. Crystal violet was then dissolved in 95% ethanol.
4. The optical density was read at 570 nm with an ELISA reader. For treatment, biofilms were exposed to 3-caffeoylquinic acid at MIC concentration for 24 hours, and biofilm mass was reassessed.

3.4 Biofilm Classification: Isolates were classified based on OD_{570} values:

Category	OD_{570}
Strong	> 0.480
Moderate	0.240 - 0.480
Weak	0.120 - 0.240
Non-producer	≤ 0.120

3.5 Statistical Analysis: Data were expressed as mean ± standard deviation (S.D.). Comparison before and after treatment was performed using paired t-test. A p-value < 0.05 was considered significant. All experiments were performed in triplicate.

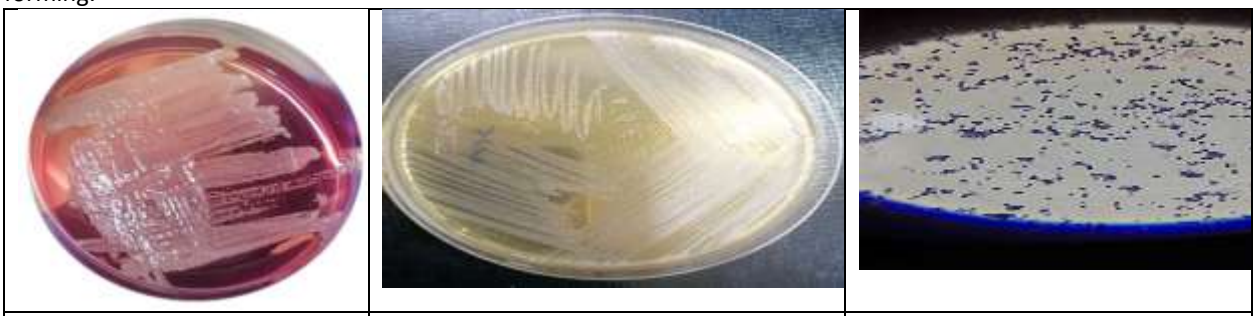
3.6 Quality Control

- Positive control: S. aureus ATCC 25923
- Negative control: Sterile culture medium

Results:

Results in Figure shows Kocuria kristinae clinical isolates after 24–48 hours at 37°C.

A. On Blood Agar: Small, circular, convex, smooth colonies with creamy-white to pale yellow pigmentation, showing γ-hemolysis(Non-Hemolytic).
B. On Nutrient Agar: Similar colony morphology with creamy-white to pale yellow pigmentation, characteristic of Kocuria species.
C. Gram-positive Kocuria kristinae under Microscope: Gram-positive cocci arranged predominantly in tetrads and irregular clusters, occasionally in pairs or short chains. Cells are 0.8–1.2 µm in diameter, non-motile, and non-spore-forming.



A- On Blood on agar	**B-** On Nutrient agar	**C-** Gram positive kocuria kristinae under microscope

Figure 1. Kocuria kristinae isolates on agar

Antibiofilm Activity of 3-Caffeoylquinic Acid Against Kocuria kristinae Isolates
The antibiofilm activity of 3-caffeoylquinic acid against Kocuria kristinae clinical isolates obtained from ear infections was evaluated. Biofilm formation was measured spectrophotometrically before and after treatment, and the results are presented below.

Table 1. Quantitative assessment of biofilm formation by Kocuria kristinae isolates before and after treatment with 3-caffeoylquinic acid.

Isolate No.	Before Treatment	After Treatment	Reduction (%)
	(Mean ± S.D.)		
1	0.80 ± 0.03	0.12 ± 0.01	85.0%
2	0.69 ± 0.02	0.09 ± 0.01	86.9%
3	0.62 ± 0.01	0.11 ± 0.01	82.3%
4	0.56 ± 0.02	0.15 ± 0.01	73.2%
5	0.54 ± 0.02	0.15 ± 0.01	72.2%

Data are presented as mean ± standard deviation (S.D.) at optical density (OD) 570 nm.

Table 2. Classification of biofilm-forming ability of Kocuria kristinae isolates before and after treatment.

Isolate No.	Biofilm Category	
	Before Treatment	After Treatment
1	S	W
2	S	W
3	S	W
4	S	W

5	S	W

Note: S: Strong, W: Weak.
Biofilm-forming ability was classified as strong or weak based on optical density cutoff values.

Table 3. Statistical comparison of biofilm formation before and after treatment.

Parameter	Before Treatment	After Treatment	p-value
	Mean ± S.D.		
Biofilm formation	0.642 ± 0.108	0.124 ± 0.025	< 0.05*

Data are expressed as mean ± standard deviation (S.D.). Statistical analysis was performed using paired t-test. Asterisk () denotes statistically significant difference ($p < 0.05$).*

- All five clinical isolates of Kocuria kristinae demonstrated strong biofilm-forming ability prior to treatment with 3-caffeoylquinic acid.
- A decrease in biofilm formation was seen for all isolates after the administration of 3-caffeoylquinic acid, changing them from strong to weak biofilm formers.
- The average level of biofilm formation reduced from 0.642 ± 0.108 before administration to 0.124 ± 0.025 after administration, which amounts to an 80.7% decrease.
- The decrease in biofilm formation is statistically significant ($p < 0.05$).

Discussion

In the current study, the antibiofilm properties of 3-caffeoylquinic acid were assessed for Kocuria kristinae clinical strains isolated from ear infections.

Biofilm Formation in Kocuria kristinae Clinical Isolates

Before being subjected to any treatment, all five strains exhibited high biofilm-forming ability, with optical density measurements ranging from 0.54 to 0.80. Such findings have been supported by other studies which show the biofilm-forming ability of Kocuria spp. clinical isolates (Saleh et al., 2020). Biofilm formation is an important virulence factor that helps bacteria to attach to host cells and medical devices, creating resistance to antibiotics and host immunity (Ibraheem et al., 2023; Donlan & Costerton, 2002).

In the process of biofilm formation in Kocuria species, there is an excretion of extracellular polymeric substance (EPS) that includes polysaccharides, proteins, and extracellular DNA that act as the matrix of the biofilm (Flemming & Wingender, 2010). Adherence index of the K. kristinae isolates from clinical samples has been found to be directly proportional to the ability of the bacteria to form biofilm (Ananieva et al., 2018).

Activity of 3-Caffeoylquinic Acid on Antibiofilm

Exposure to 3-caffeoylquinic acid led to significant inhibition in the development of biofilms by all five isolates. The average optical density was reduced from 0.642 ± 0.108 before exposure to 0.124 ± 0.025 after exposure, which indicated that there is an overall inhibition of about 80.7%. The five isolates were classified as weak biofilm producers after exposure, and the reduction was highly significant ($p<0.05$).

This experiment supports the mounting scientific literature that suggests that caffeoylquinic acid derivatives have antibiofilm activity. Caffeoylquinic acids are phenolic compounds in coffee, artichoke, and medicinal herbs (Igarashi et al., 2021**;** Zhang et al., 2024). Studies have demonstrated that these compounds exert antibacterial and antibiofilm effects through multiple mechanisms.

One of the primary mechanisms of action involves the disruption of bacterial biofilm formation pathways. Zhang et al., (2024) reported that caffeoylquinic acid compounds target the interaction between c-di-GMP and the YcgR protein, which are critical regulators of biofilm formation. By interfering with this signaling pathway, caffeoylquinic acid derivatives inhibit the production of EPS and promote the dispersion of pre-formed biofilms (Zhang et al., 2024). Additionally, caffeoylquinic acids have been shown to disrupt bacterial cell membranes, leading to increased permeability and leakage of intracellular contents, ultimately resulting in bacterial cell death (Igarashi et al., 2021).

The ability of 3-caffeoylquinic acid to significantly reduce biofilm formation by K. kristinae isolates is of clinical importance. Biofilm-associated infections are notoriously difficult to treat due to the intrinsic resistance of biofilm-embedded bacteria to antimicrobial agents (Costerton et al., 1999and Hussain etal.,2026). The shift from a good to a poor biofilm-producing strain in this experiment indicates the possibility that 3-caffeoylquinic acid increases the susceptibility of K. kristinae to regular antibiotics.

Clinical Implications and Therapeutic Potential

There have been a number of experiments conducted on the therapeutic value of plant compounds in treating biofilm infections.Usta et al., (2025) highlighted the emerging threat of Rothia kristinae infections and the need for alternative therapeutic approaches. Similarly, Poyser et al., (2024) reported a case of Kocuria kristinae-induced infective endocarditis, underscoring the clinical significance of this organism in invasive infections. The demonstration of antibiofilm activity by 3-caffeoylquinic acid in the present study adds to the growing body of evidence supporting the use of natural products in the management of emerging opportunistic pathogens.

Furthermore, the synergistic effects of caffeoylquinic acid compounds with conventional antimicrobial agents have been documented (Zhang et al., 2024). Combination therapy involving 3-caffeoylquinic acid and standard antibiotics may not only enhance antibacterial efficacy but also reduce the selection pressure for antimicrobial resistance (Zhang et al., 2024).

Limitations of the Study

The outcomes from the study seem encouraging; however, there are certain limitations to consider. To begin with, the antibiofilm efficacy of 3-caffeoylquinic acid does not necessarily extend to in vivo settings. It is necessary to conduct further research using animal models and clinical trials to assess the compound's efficacy and safety in vivo. Secondly, this research does not consider the mechanism of action of 3-caffeoylquinic acid on K. kristinae. Even though the effectiveness of caffeoylquinic acids in targeting pathways responsible for biofilm formation in other bacteria is established, it would be beneficial to confirm that it applies in this case as well. Lastly, the sample size of K. kristinae clinical isolates involved in the study (n=5) is rather small, especially considering the variation in the antibiofilm effect.

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