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ZnO-Rutin nanoparticles: a potent formulation to inhibit *Staphylococcus aureus* biofilm

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Abstract

Staphylococcus aureus is a major human pathogen. This opportunistic pathogen is responsible for a variety of infections in humans. Biofilm formation is a key factor in infection development and drug resistance. Biofilm acts as a barrier and reduces bacterial exposure to antibacterial drugs. Targeting bacterial biofilm is a promising strategy to overcome drug resistance. In this work, the antibiofilm potential of ZnO-rutin nanoparticles was characterized. Growth inhibitory potential was studied using the broth microdilution method. Biofilm inhibitory effects of ZnO and ZnO-rutin nanoparticles were investigated by crystal staining and electron microscopy imaging was used to visualize the treated and untreated biofilms. The minimum inhibitory concentration (MIC) of the ZnO and ZnO-rutin nanoparticles for *S. aureus* strains was 0.5-1.0 mg/mL. Treatment of *S. aureus* with ZnO and ZnO-rutin nanoparticles inhibited biofilm formation by 68.2 and 81.2%, respectively. In addition, exposure of *S. aureus* with ZnO-rutin nanoparticles considerably disrupted biofilm architecture, inhibited cell adhesion to the surface, and caused morphological alteration of biofilm architecture. Additionally, damage to cell surface was evident in the treated cells. This study shows that ZnO-rutin can be a promising candidate for combating biofilm-associated infection and drug resistance caused by *S. aureus*.

Key words: Biofilm, Drug resistance, Rutin, ZnO

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Introduction

As one of the most prevalent causes of human infection, *Staphylococcus aureus* remains a major health threat. These non-motile, Gram-negative cocci are associated with many human infections, particularly in patients with underlying diseases and immunocompromised patients. Additionally, *S. aureus* is one of the most abundant causes of nosocomial infections. The infections caused by this pathogen vary from topical infections, such as pimples, to more extensive infections such as urinary tract infections, and even life-threatening ones such as endocarditis and septicemia (Cheung et al., 2023).

The emergence of drug-resistant strains of *S. aureus* has resulted in complications of treatment approaches, leading to therapeutic failure in many cases. The increasing trend in the prevalence of drug-resistant strains, such as MRSA (methicillin-resistant *S. aureus*) and VRSA (vancomycin-resistant *S. aureus*) is a major threat to human health (Liu et al., 2021). Therefore, finding novel antibiotics to combat *S. aureus* infection has been the goal of many research studies.

Several factors contibute to drug resistance in *S. aureus*, such as the activity of efflux pumps, drug inactivation by β-lactamases, and alterations in the target sites of the antibiotics. Biofilm formation is also considered a major cause of drug resistance in many pathogenic bacteria, including *S. aureus* (Parastan et al., 2020; Mlynarczyk-Bonikowska et al., 2022). Biofilm is a dense population of bacteria attached to surfaces and embedded in self-produced extracellular polymeric substances (EPS). In contrast to planktonic growth, in biofilm growth, the bacteria are considerably more resistant to antimicrobials, including antibiotics. In biofilm growth, the biofilm matrix, which is composed of extracellular polymeric carbohydrates and DNA, as a physical barrier reduces bacterial exposure to the drugs (Senobar Tahaei et al., 2021). In addition, many antibiotics are less efficient for treating the bacteria embedded in a biofilm, as they target metabolically active bacteria, while in a biofilm growth, the metabolic activity of the cells is considerably reduced (Parastan et al., 2020; Senobar Tahaei et al., 2021). As a result, targeting bacterial biofilm can be a potent auxiliary treatment to eradicate bacterial infections.

Nanotechnology offers efficient and potent drug-delivery systems to be used in clinical applications. A large number of nanoparticles with antibacterial properties have been introduced (Rizzello et al., 2013). In addition, nanoparticles can be used to deliver bioactive substances to infection sites by improving their stability and bioavailability. ZnO nanoparticles have good biocompatibility and have shown promising antibacterial properties. In our previous study, we found that ZnO nanoparticles coated with rutin have considerable antibacterial properties against *S. aureus* (Alidoust et al., 2024). However, their antibiofilm feature has not been investigated. Due to the antibacterial properties of ZnO and rutin, which is a plant flavonoid, their combination may offer efficient antibiofilm potential against bacterial pathogens. In this regard, in this work, the antibiofilm potential of ZnO-rutin nanoparticles was investigated.

Material and Methods

Materials and bacterial strains

Synthesis and characterization of ZnO-rutin nanoparticles were performed in our previous study (Alidoust et al. 2024). A clinical *S. aureus* strain, isolated from a clinical specimen, and *S. aureus* ATCC 25923 were included in this study.

Minimum inhibitory concentration (MIC)

To determine the MIC of ZnO-rutin nanoparticles, a fresh culture of each strain (1.5×106 CFU/mL) was prepared, and treated with a gradient concentration of the ZnO and ZnO-rutin nanoparticles in a 96-well palte. The bacteria without exposure to nanoparticles were regarded as a control group. The plate was incubated at 37 ˚C for 18 h, after which bacterial growth was evaluated. The minimum concentration of the nanoparticles that can abolish bacterial growth was regarded as MIC (Zahmatkesh et al., 2023).

Biofilm inhibition assay

A crystal violet staining assay was used to investigate the effect of ZnO and ZnO-rutin nanoparticles on biofilm formation by *S. aureus*,. Bacterial cultures with a density of 1.5×106 CFU/mL

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were prepared in a 96-well plate and treated with ZnO and ZnO-rutin nanoparticles at a ½ MIC concentration. The plate was incubated at 37˚C for 72 hours. The untreated wells were regarded as a control group. After incubation, the medium was removed, and the wells were washed with distilled water and stained with 0.1% crystal violet solution for 15 min. Next, the excess dye was removed and the biofilm-attached stain was solubilized with 30% acetic acid. Finally, the OD550 was measured and the biofilm formation level in the treatment groups was calculated (Das and Dash, 2014).

Scanning electron microscoy

To visualize bacterial biofilm by a scanning electron microscope (SEM), glass coverslips were placed in the wells of a 24-well plate, and fresh bacterial cultures (1.5×106 CFU/mL) were added. Next, an equal volume of a well-disperced suspension of ZnO-rutin nanoparticles was added to the wells to adjust the nanoparticle concentration to ½ MIC. The plate was incubated for 48 h at 37°C, and next, the content of the wells was removed. The coverslips were gently rinsed with PBS to remove unattached cells and bacterial biofilm was fixed using 2.5% glutaraldehyde for 12 h at 4°C. After washing the cells, dehydration was done using serial concentrations of ethanol (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%). Finally, the coverslip was dried at room temperature, and bacterial biofilm was visualized using the SEM MIRA2 TESCAN microscope (Czech Republic). The untreated biofilm was regarded as a control group (Khan et al., 2021).

Results

Growth inhibition

Evaluating the inhibitory effect of ZnO and ZnO-rutin nanoparticles on *S. aureus* strains showed that ZnO nanoparticles inhibited bacterial growth at a concnetration of 0.5 mg/mL for *S. aureus* ATCC25923 and 1.0 mg/mL for clinical *S. aureus* strain. The MIC of ZnO-Rutin for both *S. aureus* strains was 1 mg/mL (Figure 1).

Biofilm inhibition

The biofilm levels produced by *S. aureus* treated with ZnO and ZnO-rutin nanoparticles

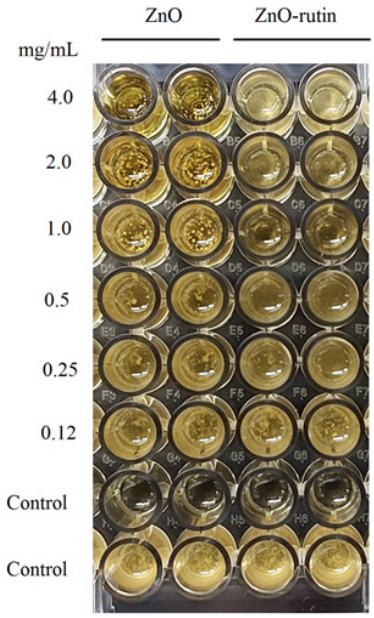

Figure 1. Growth inhibitory effects of different concentrations of ZnO and ZnO-rutin nanoparticles on S. aureus

were investigated by crystal violet staining assay. According to the results, ZnO nanoparticles efficiently inhibited biofilm formation of the clinical and standard *S. aureus* strains to 31.1 and 34.2%, respectively. ZnO-rutin nanoparticles showed more potent inhibitory effects on biofilm formation of *S. aureus* strains. Treatment of the clinical and standard *S. aureus* with ZnO-rutin nanoparticles reduced biofilm levels to 22.9 and 14.6% respectively, compared to the untreated bacteria. The results are displayed in Figure 2.

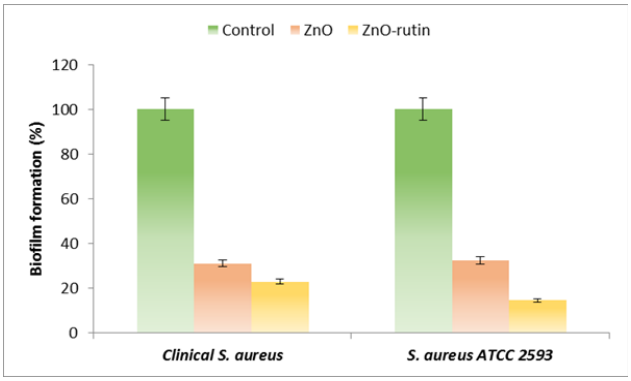

Figure 2. Inhibitory effects of ZnO and ZnO-rutin nanoparticles on biofilm formation by *S. aureus* strains

Electron microscop imaging of bacterial biofilm

The inhibitory effects of ZnO-rutin nanoparticles on biofilm formation by *S. aureus* were visualized by SEM imaging. According to the

results, treatment of *S. aureus* with ZnO-rutin nanoparticles considerably disrupted biofilm architecture, inhibited extensive cell adhesion to the surface, and caused morphological alterations of biofilm. Additionally, damage to the cell surface was evident in the treated cells. In contrast, in the control group, a dense biofilm with highly aggregated architecture with a compact layer of adhered cells was observed. In addition, the generation of extracellular matrix, which is a typical characteristic of bacterial biofilm, was evident in the control group. Figure 3 displays the biofilm architecture in the control and treated groups.

Discussion

Biofilm formation by *S. aureus* strain is a major factor in the infection initiation and development of drug resistance. Targeting biofilm tive oxygen species (ROS). ROS are considered unstable molecules that can interact with various macromolecules such as lipid membranes and proteins, causing damage to cell components (Konkuri et al., 2024). Additionally, through the overproduction of ROS, ZnO nanoparticles can interfere with bacterial adhesion with surfaces and disrupt the biofilm matrix, which results in inhibition of biofilm development and maturation (Alves et al., 2017). As observed in this work, treatment with ZnO-rutin nanoparticles significantly inhibited biofilm formation which was evident by SEM imaging.

In addition to ZnO, rutin can exert inhibitory effects on the *S. aureus* growth and biofilm formation. The antibacterial properties of rutin may contribute to damage to the bacterial membranes and biofilm matrix components. In addition, treatment of bacteria with rutin causes alterations in bacterial metabolic functions and gene expression (Arima et al., 2002; Negahdari et al., 2021; Alidoust et al., 2024). In agreement with our study, it has been reported that rutin can enhance the antibacterial effects of antibiotics on pathogenic bacteria (Alvarez et al., 2006; Amin et al., 2015). Therefore, a synergic antibacterial activity between ZnO nanoparticles and rutin can efficiently inhibit biofilm formation and cause cell death.

Conclusion

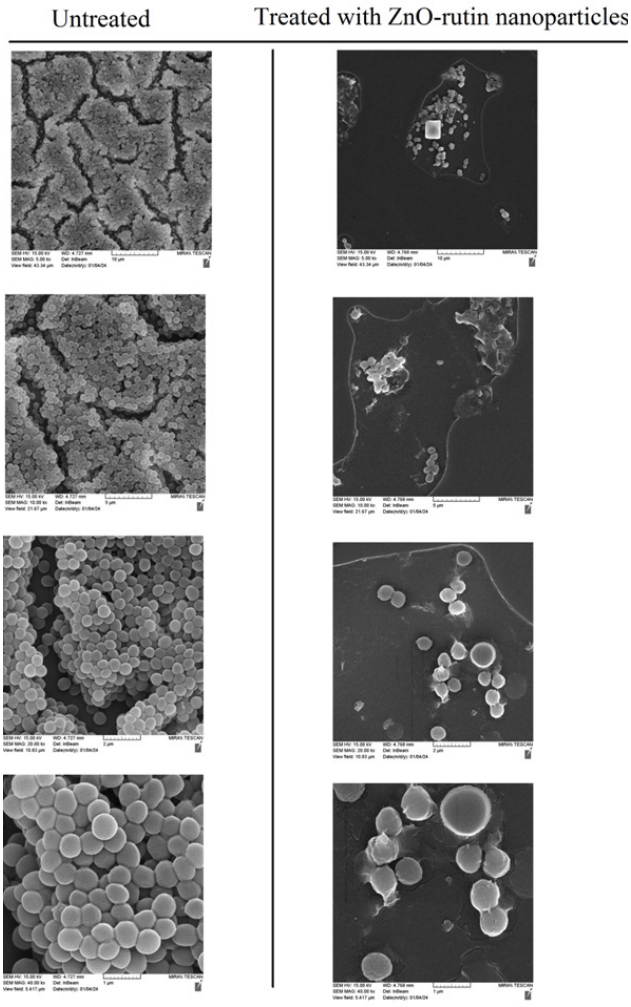

Figure 3. Effects of ZnO-rutin nanoparticles on the biofilm architecture and cell structure of S. aureus strain. Left: untreated, Right: treated with nanoparticles.

This study was done to characterize the antibiofilm potential of ZnO-rutin nanoparticles against *S. aureus* strains. Our results showed that ZnO-rutin nanoparticles inhibit bacterial growth, minimize biofilm formation, and disrupt biofilm architecture. By targeting biofilm formation, ZnO-rutin can be considered a potent antibacterial for the auxiliary treatment of *S. aureus* with antibiotics.

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Competing interests

The authors have no conflict of interest to declare.

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