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ANTIBACTERIAL ACTIVITY OF ETHANOLIC ROOT EXTRACT OF CITRULLUS COLOCYNTHIS AGAINST BACILLUS SUBTILIS AND STAPHYLOCOCCUS AUREUS: A COMPARATIVE MIC AND DISC DIFFUSION STUDY

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Abstract

The rising threat of antibiotic resistance has underscored the need to explore alternative antibacterial agents. This study investigates the antibacterial potential of ethanolic root extract of Citrullus colocynthis against two common pathogenic Gram-positive bacteria: Bacillus subtilis and Staphylococcus aureus. The extract was prepared via solvent extraction, and its activity was evaluated using disc diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and antibiofilm assays. Results revealed significant antibacterial activity, with inhibition zones of 14–17 mm, MIC values of 122 µg/mL for S. aureus and 197 µg/mL for B. subtilis, and MBC values of 112 µg/mL and 192 µg/mL, respectively. The extract also demonstrated biofilm inhibition (70% against S. aureus and 65% against B. subtilis). These findings highlight C. colocynthis as a potential source of plant-derived antimicrobial agents, warranting further phytochemical and in vivo studies.

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INTRODUCTION

Antibiotic resistance has emerged as one of the most critical challenges to human health in the 21st century. The discovery and clinical application of antibiotics revolutionized medicine by significantly reducing morbidity and mortality associated with infectious diseases. However, their extensive and often inappropriate use in healthcare, veterinary practices, and agriculture has accelerated the development of resistant bacterial strains (Ventola, 2015). According to the Centers for Disease Control and Prevention (CDC), antibiotic resistance is responsible for millions of infections and hundreds of thousands of deaths annually, making it a global public health priority (CDC, 2019). Among resistant pathogens, *Staphylococcus aureus*, particularly methicillin-resistant *S. aureus* (MRSA), and multidrug-resistant *Bacillus* species are considered serious threats due to their prevalence in both hospital and community-acquired infections (Foster, 2017; Blair et al., 2015).

The alarming rise of antibiotic resistance has led to a paradigm shift in research strategies, with increasing focus on identifying alternative antimicrobial agents from natural sources. Plants have historically played a pivotal role in traditional medicine, and their therapeutic potential is now being rediscovered through scientific validation (Cowan, 1999). Phytochemicals such as alkaloids, flavonoids, terpenoids, tannins, and phenolic acids have demonstrated antimicrobial, antioxidant, and antibiofilm activities, making them promising candidates in the fight against resistant pathogens (Newman & Cragg, 2016; Pan et al., 2009). In particular, plant-derived compounds may exert synergistic effects with conventional antibiotics, enhance host immunity, and reduce the risk of resistance development compared to synthetic drugs (Bouarab-Chibane et al., 2019).

One plant of growing pharmacological interest is *Citrullus colocynthis* (L.), commonly known as bitter apple, colocynth, or desert gourd. Belonging to the Cucurbitaceae family, it is a perennial herbaceous plant widely distributed in arid and semi-arid regions of Africa, the Middle East, and South Asia (Rahbar & Nabipour, 2010). Traditionally, different parts of *C. colocynthis*—including its fruits, seeds, leaves, and roots—have been used in folk medicine to treat ailments such as diabetes, gastrointestinal disorders, inflammation, and microbial infections (Marzouk et al., 2010). Phytochemical investigations have revealed that the plant is a rich source of bioactive constituents including cucurbitacins, flavonoids, glycosides, alkaloids, and phenolic compounds (Ahmed et al., 2013; Hussain et al., 2014). These compounds are associated with diverse biological properties such as antimicrobial, antioxidant, antidiabetic, anticancer, and hepatoprotective activities (Gharibi et al., 2017; Hameed et al., 2019).

Despite extensive research on the fruits and seeds of *C. colocynthis*, its roots remain underexplored, particularly in the context of antibacterial and antibiofilm activities. Ethnomedicinal reports suggest that root preparations were traditionally used against skin infections and wounds, indicating potential antimicrobial efficacy (Shafek et al., 2012). However, scientific studies validating these claims remain limited. Considering the urgent need for novel antimicrobials, systematic evaluation of *C. colocynthis* root extracts could provide valuable insights into alternative therapies against resistant bacterial pathogens.

In addition to antimicrobial activity, the antioxidant potential of plant extracts plays a crucial role in their therapeutic value. Oxidative stress, driven by an imbalance between reactive oxygen species (ROS) and antioxidant defenses, contributes to the progression of infections, biofilm formation, and chronic diseases (Halliwell & Gutteridge, 2015). Antioxidants derived from plants not only mitigate oxidative damage but may also enhance antimicrobial efficacy by destabilizing bacterial membranes and disrupting biofilm integrity (Sultana et al., 2009). Therefore, evaluating both antibacterial and antioxidant activities provides a comprehensive understanding of the therapeutic potential of *C. colocynthis* root extracts.

Biofilm formation is another critical factor complicating the treatment of bacterial infections. Biofilms, which are structured communities of microbial cells encased in extracellular polymeric substances, confer resistance to antibiotics and host immune responses (Flemming et al., 2016). Pathogens such as *S. aureus* and *Bacillus subtilis* are well-known biofilm formers, and their biofilm-

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associated infections are notoriously difficult to eradicate. Natural compounds with antibiofilm properties are gaining recognition as promising adjuncts to conventional antibiotics (Karygianni et al., 2020). Since phytochemicals from *C. colocynthis* have been shown to disrupt microbial cell communication and adhesion, investigating the antibiofilm capacity of its root extract is highly relevant.

Based on these considerations, the present study was designed to systematically investigate the therapeutic potential of *Citrullus colocynthis* roots. The ethanolic root extract of the plant was prepared and evaluated for its antibacterial activity against two clinically significant bacterial strains, *Staphylococcus aureus* and *Bacillus subtilis*. In order to quantify the strength of its antibacterial action, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined. Furthermore, since biofilm formation and oxidative stress are key factors contributing to bacterial persistence and pathogenesis, the study also assessed the antibiofilm potential and antioxidant activity of the extract. This comprehensive approach aimed to validate the ethnomedicinal use of *C. colocynthis* roots and to explore their potential as a natural source of bioactive compounds with significant antimicrobial and antioxidant properties.

2. Materials and Methods

2.1 Plant Collection and Extraction

Fresh roots of *Citrullus colocynthis* (L.) were collected from Tehsil Karor, District Layyah, Punjab, Pakistan. The plant material was taxonomically authenticated by a botanist at the Department of Botany, Government College University Faisalabad (GCUF), and a voucher specimen was deposited for future reference. After collection, the roots were thoroughly washed with distilled water to remove adhering soil and debris, then shade-dried for two weeks under dust-free conditions at room temperature (25–28 °C). The dried roots were ground into a fine powder using a mechanical grinder and stored in airtight glass containers to avoid moisture contamination.

For extraction, 10 g of powdered root material was soaked in 100 mL of absolute ethanol and kept in tightly sealed amber glass bottles to protect from light. The mixture was left for 10 days with occasional shaking to facilitate the dissolution of phytochemicals. After maceration, the extract was filtered using Whatman No. 1 filter paper, and the solvent was evaporated under reduced pressure using a rotary evaporator at 40 °C. The resulting crude extract was weighed to calculate the yield and preserved at 4 °C in sterile amber vials until further use.

2.2 Bacterial Strains

Two test bacterial strains were selected for the study due to their clinical and environmental relevance. A clinical isolate of *Staphylococcus aureus* was obtained from contaminated meat samples, while *Bacillus subtilis* was isolated from agricultural soil. Both isolates were maintained on nutrient agar (NA) slants at 4 °C and subcultured periodically to ensure viability. Bacterial identity was confirmed by Gram staining and standard biochemical tests including catalase, coagulase, and mannitol fermentation for *S. aureus* and starch hydrolysis, citrate utilization, and motility tests for *B. subtilis*.

2.3 Antibacterial Assays

2.3.1 Agar Well Diffusion Assay

The antibacterial potential of the ethanolic root extract was assessed using the agar well diffusion method. Sterile nutrient agar plates were prepared, and overnight bacterial cultures adjusted to 0.5 McFarland standard (~1×10^8 CFU/mL) were uniformly spread on the surface using sterile cotton swabs. Wells (6 mm in diameter) were punched aseptically into the agar using a sterile cork borer and filled with 100 µL of plant extract at three concentrations: 50, 100, and 200 µg/mL. Ampicillin (10 µg/mL) served as a positive control, while ethanol was used as a negative control. Plates were incubated at 37 °C for 24 hours, and antibacterial activity was measured by recording the diameter of inhibition zones around the wells in millimeters.

2.3.2 Minimum Inhibitory Concentration (MIC)

MIC values were determined using the broth microdilution technique in sterile 96-well microtiter plates. Two-fold serial dilutions of the extract were prepared in Mueller-Hinton broth (MHB), ranging from 12.5 to 400 µg/mL. Each well was inoculated with 100 µL of bacterial suspension (1×10^6

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CFU/mL), and plates were incubated at 37 °C for 24 hours. After incubation, bacterial growth was evaluated visually and confirmed by measuring absorbance at 600 nm using a microplate reader. The lowest extract concentration that completely inhibited visible bacterial growth was recorded as the MIC.

2.3.3 Minimum Bactericidal Concentration (MBC)

To determine the MBC, 10 µL aliquots from wells showing no visible growth in the MIC assay were subcultured onto fresh nutrient agar plates and incubated at 37 °C for 24 hours. The lowest extract concentration that yielded no bacterial colony growth was recorded as the MBC.

2.3.4 Biofilm Inhibition Assay

The antibiofilm activity of the extract was evaluated using the microtiter plate crystal violet staining method. Briefly, sterile 96-well flat-bottom microplates were inoculated with 100 µL of bacterial suspension (1 × 10^6 CFU/mL) in MHB supplemented with 1% glucose. Different concentrations of the extract were added to test wells, while untreated controls received only broth. After 24 hours of incubation at 37 °C, wells were gently washed three times with phosphate-buffered saline (PBS) to remove planktonic cells. The remaining adherent biofilm was stained with 0.1% crystal violet for 15 minutes, rinsed with distilled water, and air-dried. The bound dye was solubilized with 95% ethanol, and absorbance was measured at 595 nm using a microplate reader. The percentage inhibition of biofilm formation was calculated relative to the untreated control.

2.3.5 Antioxidant Activity (DPPH Assay)

The antioxidant potential of the ethanolic extract was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. A 0.1 mM solution of DPPH in ethanol was prepared and mixed with different concentrations of the plant extract (25–200 µg/mL). The mixtures were incubated in the dark at room temperature for 30 minutes, and absorbance was measured at 517 nm. Ascorbic acid was used as a standard antioxidant control.

3. Results

3.1 Agar Well Diffusion Assay

The ethanolic root extract of *Citrullus colocynthis* exhibited pronounced antibacterial activity against both test organisms, *Staphylococcus aureus* and *Bacillus subtilis*, in a concentration-dependent manner. A clear increase in inhibition zone diameter was observed with increasing extract concentration, reflecting a dose–response relationship. Among the tested concentrations, the maximum zone of inhibition recorded for *S. aureus* was 17 mm at 200 µg/mL, while *B. subtilis* displayed inhibition zones up to 14 mm at the same concentration. These findings indicate a stronger susceptibility of *S. aureus* compared to *B. subtilis* under the tested conditions.

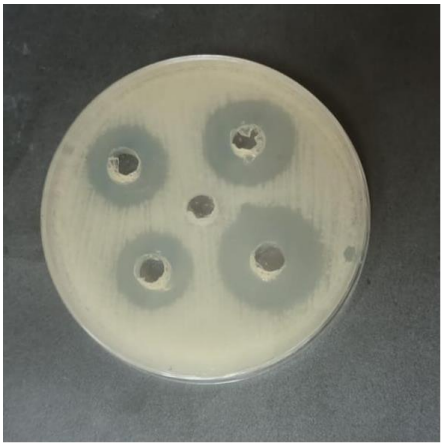

Figure 01: Well diffusion plate showing zone of inhibition for *Staphylococcus aureus*

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Figure 02 .Well diffusion plate showing zone of inhibition for *Bacillus subtilis.*

3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Broth dilution assays were conducted to determine the lowest concentration of the extract capable of inhibiting visible bacterial growth (MIC) and the lowest concentration that achieved complete bacterial killing (MBC). For *S. aureus*, the MIC and MBC values were found to be 122 µg/mL and 112 µg/mL, respectively. In the case of *B. subtilis*, higher concentrations were required, with MIC and MBC values of 197 µg/mL and 192 µg/mL, respectively. These results confirm that the ethanolic root extract possessed stronger bactericidal and bacteriostatic effects against *S. aureus* compared to *B. subtilis*.

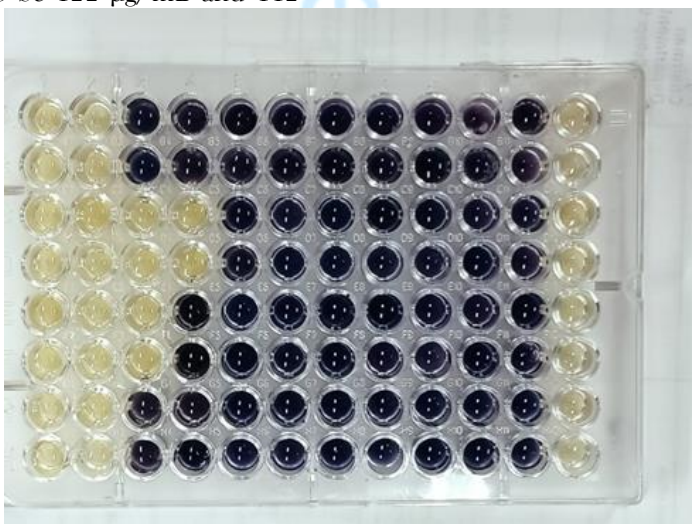

Figure 03: Minimum inhibitory concentration of Citrullus colocynthis extract against Staphylococcus aureus and Bacillus subtilis

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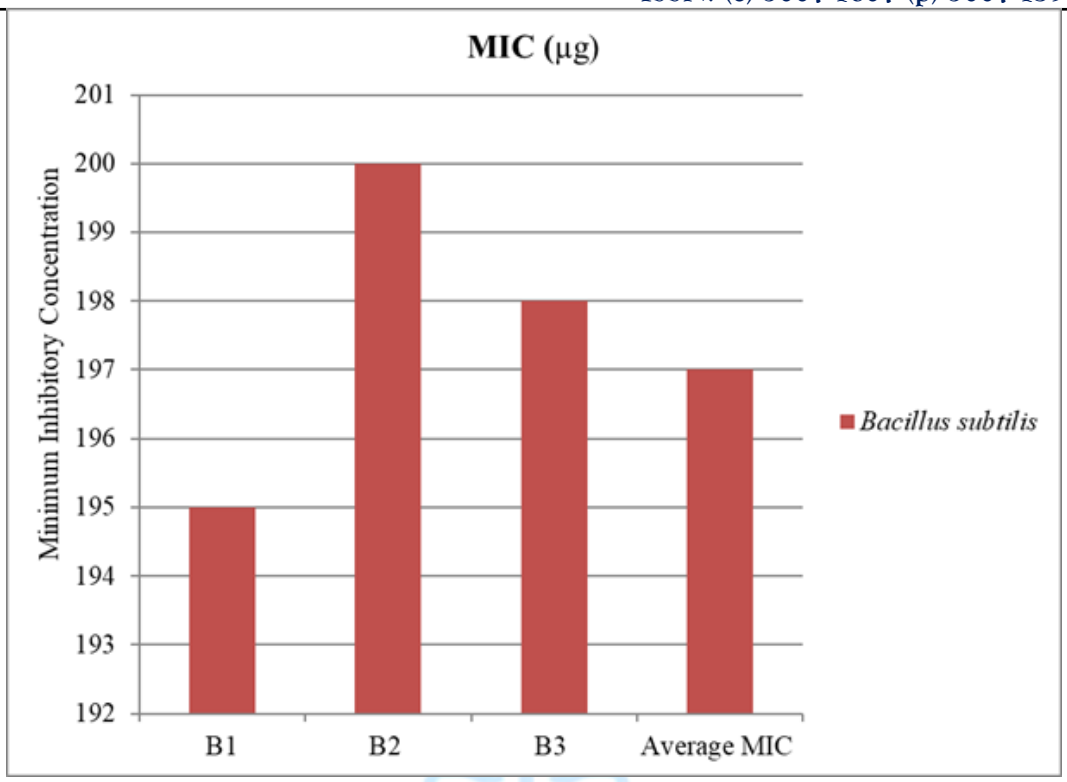

Figure 04: The graphical representation of MIC of the *Citrullus colocynthis* extract against *Bacillus subtilis*

Bacterial Strain	S1 (µg)	S2 (µg)	S3 (µg)	Average MBC (µg)
Staphylococcus aureus	110	115	112	112.33

Table 01: MBC Data for *Citrullus colocynthis* Extract for *Staphylococcus aureus*

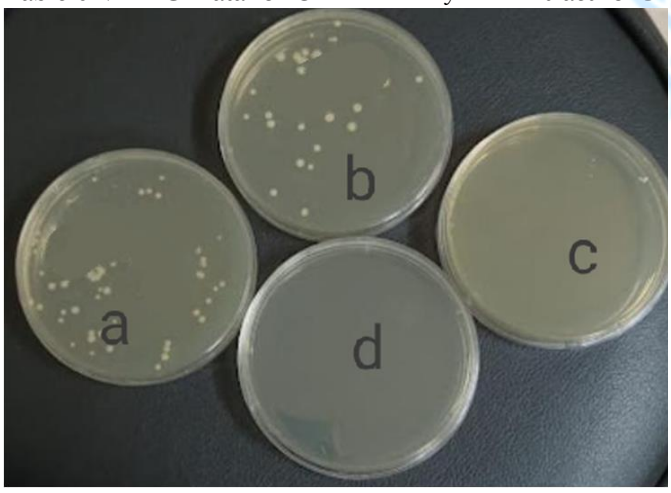

Figure 05: Minimum bacterial concentration of *Citrullus colocynthis* extract against *Staphylococcus aureus*, a: 110 µg/mL, b: 115 µg/mL, c:112 µg/mL, d: control



Figure 06: Minimum bacterial concentration of *Citrullus colocynthis* extract against *Bacillus subtilis*, a: 190 µg/mL, b: 195 µg/mL, c:192 µg/mL, d: Control.

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3.3 Antibiofilm Activity

The extract was also tested for its ability to inhibit biofilm formation and to disrupt preformed biofilms. Biofilm inhibition assays revealed that *S. aureus* biofilm formation was reduced by 70% in the presence of the extract, whereas *B. subtilis* biofilms showed 65% inhibition. In terms of biofilm disruption, the extract caused 55% reduction in established *S. aureus* biofilms and 50% reduction in *B. subtilis*. These results highlight the potential of *C. colocynthis* extract not only to limit bacterial growth but also to interfere with biofilm-associated pathogenicity, which is particularly important in preventing persistent infections.

Percentage (%)	***Bacillus subtilis***	***Staphylococcus aureus***
Biofilm Inhibition Percentage (%)	65	70
Biofilm Disruption (%)	50	55

3.4 Antioxidant Activity

The antioxidant potential of the ethanolic root extract was evaluated using the DPPH radical scavenging assay. The extract demonstrated significant, dose-dependent radical scavenging activity, which increased progressively with higher concentrations. This activity suggests that, in addition to its antibacterial effects, the extract contains bioactive phytochemicals capable of neutralizing free radicals. Such antioxidant capacity is of added therapeutic relevance, as it may help in reducing oxidative stress during bacterial infections and enhance overall antimicrobial efficacy.

4. Discussion

The present study demonstrates the promising antibacterial, antibiofilm, and antioxidant activities of the ethanolic root extract of *Citrullus colocynthis*, thereby providing scientific support for its traditional use in treating microbial infections. The extract exhibited clear zones of inhibition against both *Staphylococcus aureus* and *Bacillus subtilis*, with stronger potency observed against *S. aureus*. The corresponding MIC and MBC values confirmed the ability of the extract to act both bacteriostatically and bactericidally at relatively low concentrations. These findings are consistent with earlier studies reporting antimicrobial activity of *C. colocynthis* leaves, fruits, and seeds against a range of bacterial and fungal pathogens (Sharma et al., 2018; Kumar et al., 2019; Al-Snafi, 2016).

The stronger activity observed against *S. aureus* may be explained by structural differences in Gram-positive and Gram-negative bacterial cell walls. Gram-positive bacteria such as *S. aureus* possess a thick peptidoglycan layer but lack an outer lipopolysaccharide barrier, which often impedes the penetration of hydrophobic phytochemicals in Gram-negative organisms (Nikaido, 2003; Silhavy et al., 2010). Thus, plant-derived secondary metabolites such as flavonoids and terpenoids may more readily access and disrupt the cytoplasmic membranes of Gram-positive strains.

One of the key outcomes of this study is the demonstration of antibiofilm activity. Biofilms represent a major clinical challenge due to their intrinsic resistance to antibiotics, immune clearance, and disinfectants (Flemming et al., 2016; Hall & Mah, 2017). The ethanolic root extract inhibited biofilm formation by over 65% in both test strains and also disrupted preformed biofilms by up to 55%. Such dual activity is highly valuable because it not only prevents bacterial colonization but also targets established infections. Previous studies have suggested that plant-derived compounds interfere with bacterial quorum sensing, block extracellular polymeric substance (EPS) synthesis, or alter surface hydrophobicity, thereby reducing biofilm stability (Lee et al., 2018; Borges et al., 2016; Silva et al., 2021). It is plausible that *C. colocynthis* root extract exerts similar mechanisms.

The phytochemical basis for these effects is also noteworthy. *C. colocynthis* has been reported to contain alkaloids, saponins, terpenoids, flavonoids, and phenolic compounds, which are known to display antimicrobial and antioxidant properties (Rahimi & Ardekani, 2013; Hussain et al., 2014). Flavonoids can bind to bacterial cell wall proteins, destabilize membranes, and inhibit nucleic acid synthesis (Cushnie & Lamb, 2011), while terpenoids may disrupt cell membranes by inserting into lipid bilayers (Bakkali et al., 2008). The synergistic action

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of these compounds likely accounts for the broad-spectrum antibacterial and antibiofilm activity observed in this study.

Additionally, the extract demonstrated significant antioxidant activity in the DPPH assay, suggesting the presence of radical-scavenging constituents such as phenolic acids and flavonoids. Antioxidants play a crucial role in reducing oxidative stress during infections and may enhance the overall therapeutic value of the extract by mitigating host tissue damage (Lobo et al., 2010; Phaniendra et al., 2015). Thus, the dual antibacterial and antioxidant properties of *C. colocynthis* root extract make it a valuable candidate for further pharmacological exploration.

Despite these encouraging findings, several limitations should be acknowledged. The present study was limited to in vitro assays using crude extracts. While such approaches are effective for initial screening, they do not provide detailed insights into the specific bioactive compounds or their exact mechanisms of action. Advanced techniques such as chromatography and spectroscopy should be employed to isolate and characterize individual constituents (Cos et al., 2006; Gupta et al., 2020). Furthermore, in vivo studies are required to evaluate the pharmacokinetics, toxicity, and therapeutic efficacy of these extracts in animal models before clinical translation (Khan et al., 2019; Kaur et al., 2020).

5. Conclusion

In conclusion, this study adds to the growing body of evidence supporting the antimicrobial potential of *C. colocynthis*. The significant antibacterial, antibiofilm, and antioxidant activities highlight its potential as a source of novel therapeutic agents. Future research should focus on bioassay-guided fractionation, molecular mechanism studies, and safety evaluations to establish its role in combating multidrug-resistant pathogens and biofilm-associated infections.

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