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Article

Anti-infective Efficacy of Duloxetine against Catheter-Associated Urinary Tract Infections Caused by Gram-Positive Bacteria

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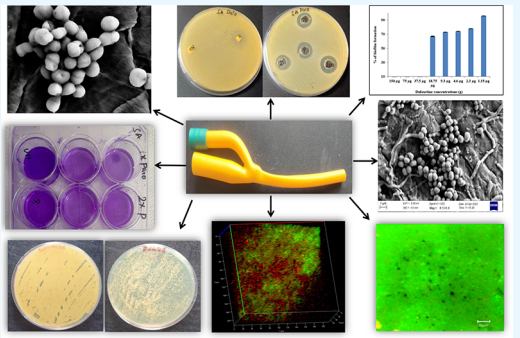
ABSTRACT: Catheter-associated urinary tract infections (CAUTIs) frequently occur following the insertion of catheters in hospitalized patients, often leading to severe clinical complications. These complications are exacerbated by biofilm-forming organisms such as *Staphylococcus aureus*, contributing to the emergence of multidrug-resistant (MDR) strains, which complicates treatment strategies. This study aims to investigate the antibacterial, antibiofilm, and antiadhesive properties of duloxetine against *S. aureus* in the context of CAUTI. Our findings demonstrate that duloxetine exhibits significant antibacterial activity, as evidenced by the agar diffusion method. A minimal inhibitory concentration (MIC) of 37.5 μg/mL was established using the microdilution method. Notably, duloxetine displayed inhibitory effects against biofilm formation on polystyrene surfaces up to its MIC level, as demonstrated by the crystal violet method. Intriguingly, the study also revealed that duloxetine could prevent biofilm formation at lower concentrations and reduce mature biofilms, as confirmed by scanning electron microscopy (SEM) and quantitative biofilm assays. Furthermore, duloxetine-coated silicone catheter tubes exhibited antibacterial properties against *S. aureus* in a bladder model, visualized by confocal laser scanning microscopy (CLSM) and corroborated through FDA and PI staining, highlighting noticeable morphological changes in *S. aureus* post-treatment. In conclusion, this study presents duloxetine as a promising alternative agent with antibacterial and antiadhesive properties against *S. aureus* in the prevention and management of CAUTI, warranting further exploration in the clinical setting.



Antibacterial, anti-biofilm, and anti-adhesive properties of duloxetine against *S. aureus* in the context of CAUTI

1. INTRODUCTION

The recent technology used in modern medicine has improved the knowledge of physicians to overcome problems related to untreated diseases. In the current scenario, hospital-acquired or nosocomial infections, particularly associated with medical devices, are gaining much attention due to their social burden. However, several medical devices have been used for healthcare improvement, and expected or unexpected complications have occurred from such life-saving devices. Particularly, indwelling catheters play a dynamic role in the medical era by supporting hospitalized patients to overcome illnesses arising due to various reasons.[1−3] Medical devices present in the human body provide the appropriate microenvironment for suitable microbial colonization resulting in the emergence of hospital-acquired infections.[4] Indwelling catheters are mainly used for urine drainage in hospitalized patients; in certain circumstances, the occurrence of infections through catheter usage plays a serious role in catheter-associated urinary tract infections (CAUTIs), which affect more than a million people globally.[5,6] In accordance, CAUTIs rank as the third most significant contributor to nosocomial infections, accounting for a substantial 40% of total healthcare-related infections. This alarming prevalence is associated with elevated rates of morbidity and mortality.[7,8] Long-term catheter usage provides the opportunity for uropathogen colonization in the urinary tract region, leading to various difficulties such as bacteriuria, sepsis, extended stay in the hospital, high treatment cost, chances for antibiotic resistance, and, in some cases, high mortality rates, which cause a severe economic burden.[9−11] Most importantly, CAUTI favors polymicrobial structures, including Gram-positive as well as Gram-negative etiological agents. Of these, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus mirabilis* stand out as the predominant organisms responsible for microbial colonization, facilitating the formation of biofilms on catheter surfaces. This highlights the critical nature of CAUTI treatment, given the emergence of antibiotic-resistant strains.[12,13]

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Indeed, CAUTIs are primarily linked to the formation of biofilms on catheter surfaces, which significantly diminish the efficacy of antibiotics by various mechanisms such as efflux pumps, which expel the antimicrobial agents, or through the transfer of antibiotic-resistant genes within bacterial and extracellular polymeric substances, thus making CAUTI treatment ineffective, and thereby lead to the development of antibiotic-resistant organisms.[14,15] Therefore, there is a very urgent need for the development of antimicrobial agents that possess antibiofilm as well as antiadhesive properties to prevent further infection. Our research group reported the risk factors of nosocomial infections and *in vitro* studies of dispiro-3-phenylpyrrolothiazoles for a wide range of uropathogenic pathogens.[16] In recent years, strategies have been reported in the literature to combat polymicrobial biofilms of uropathogens with biopolymer, organic, and metal complex-coated catheters.[17−20] Frieboes and his research group discussed the infusion of antibiotics and antimicrobial compounds with modifications of the catheter to control the colonization of pathogens in the catheter and bladder. Interestingly, their report concludes that coating the catheter with nonpathogenic bacteria results in the effective control of colonization by uropathogenic organisms.[21]

Nonetheless, the development of novel antimicrobial agents is a time-consuming and expensive process, and they have to clear many preclinical studies before reaching public use. Amidst diverse strategies for drug development, repurposing old drugs for novel applications has garnered significant attention. This approach has gained prominence due to its successful passage through various clinical trials, ensuring safety, establishing pharmacological profiles, and assessing the impact on humans. This, in turn, has reduced the time, cost, and risks associated with new antibiotic discoveries.[22] Considering these compelling factors, this study explores the antibacterial, antibiofilm, and antiadhesive properties of a repurposed drug, duloxetine (originally an antidepressant), against the highly prevalent bacterium *S. aureus* implicated in CAUTIs.

2. MATERIALS AND METHODS

2.1. Chemicals and Media. All chemicals used in this study were purchased from Sigma-Aldrich, and the medium was procured from HiMedia Pvt Ltd., Mumbai, India. The strain used in the study was procured from the American Type Culture Collection. Throughout the study, ampicillin was used as a positive control, and an overnight culture adjusted to 0.5 McFarland was used throughout the study. All experiments were performed in triplicates.

2.2. Antibacterial Activity of Duloxetine. The antibacterial efficacy of duloxetine against *S. aureus* was assessed by the agar diffusion method following established protocols.[23] In brief, an overnight culture of the *S. aureus* strain was swabbed onto sterile Mueller−Hinton agar (MHA) plates, creating wells. Different concentrations (125.00, 150.00, and 200.00 μg/well) of duloxetine were loaded into each well and incubated. Subsequently, the antibacterial activity was evaluated by calculating the inhibition zone surrounding the well, with ampicillin serving as a positive control.

2.3. Minimum Inhibitory Concentration (MIC) Determination. The MIC of duloxetine was determined against *S. aureus* by the microdilution method using a 96-well plate, as mentioned earlier.[23] In brief, each well was loaded with sterile Muller−Hinton broth (MHB), and 2-fold serial dilutions of duloxetine were made from 150 μg/mL, which was serially diluted up to 1.15 μg/mL. Later, the instant *S. aureus* strain was added into each well and incubated. The turbidity of the plate was visually examined, and the optical density (OD) was read at 600 nm with a Thermo Evolution 600 UV−vis spectrophotometer (Thermo Fisher Scientific).

2.4. Time−Kill Assay. The time−kill kinetics of duloxetine against *S. aureus* was determined by a well-known method described in the literature.[24] In brief, an overnight culture of *S. aureus* (1×10^6 CFU) was added into the BHI broth, duloxetine was added at its MIC concentrations, and the mixture was incubated at various time intervals such as 0, 1, 2, 4, 6, and 12 h. After incubation, a 10-fold serial dilution was performed for each time point, and spread plating was carried out for treated as well as untreated samples. After incubation, the inhibitory effect of duloxetine against *S. aureus* was calculated by counting the CFUs.

2.5. Effect of Duloxetine on *S. aureus* Biofilm Formation. To study the effect of duloxetine on *S. aureus* colony formation, the microdilution method was adopted as described earlier.[24] In brief, duloxetine was serially diluted from 150 to 1.15 μg/mL using BHI broth in 96-well plates, and the overnight *S. aureus* culture was added and incubated for 96 h. Later, the impact of duloxetine on *S. aureus* colony formation was assessed using the crystal violet method. This involved washing each well with phosphate-buffered saline (PBS) to remove nonadherent cells. The remaining adherent cells were fixed with methanol and stained with 0.1% crystal violet. After removing the excess stain and allowing the wells to air-dry, the plate was read at 570 nm following the addition of an ethanol/acetone mixture. These experiments were conducted in triplicates for consistency.

2.6. Qualitative and Quantitative Biofilm Assay. A qualitative biofilm assay was conducted to investigate the impact of duloxetine on *S. aureus* biofilm formation, following the method previously described by Gowri et al.[25] In this assay, an overnight culture of *S. aureus* was spread on Whatman No. 1 filter paper strips and incubated for 96 h. Subsequently, the filter paper strips were subjected to treatment with a concentration of 37.5 μg/mL duloxetine for a duration of 1 h, followed by thorough washing with PBS to eliminate nonadherent bacterial cells. These strips were then fixed by using glutaraldehyde and dehydrated through an ethanol gradient. Both treated and untreated filter paper strips were coated with gold, and their images were captured using SEM (Supra 55, Carl Zeiss). As a control, untreated filter paper strips were employed. To quantify the extent of biofilm inhibition following duloxetine treatment, the crystal violet method was utilized, in accordance with the procedure previously outlined.[25] In brief, *S. aureus* biofilm formation was achieved by culturing *S. aureus* in 96-well plates for 96 h. After this incubation period, the biofilm was subjected to treatment with duloxetine at concentrations of 37.5 and 75.00 μg/mL for 24 h, followed by the removal of nonadherent cells. The adherent cells were subsequently fixed using methanol, stained with a crystal violet solution, and allowed to air-dry. The plate was then read at 570 nm after the addition of an ethanol/acetone mixture. These experiments were conducted in triplicate for reliability.

2.7. *In Vitro* Qualitative Bladder Model. To assess the antibacterial efficacy of the duloxetine-coated silicone catheter against *S. aureus*, an *in vitro* bladder model was employed, following the methodology described in a previous study.[26]

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Concisely, small pieces of the silicone catheter tube were immersed in a duloxetine solution (37.5 mg/mL) for 1 h. The air-dried, drug-coated pieces were then placed onto sterile Mueller−Hinton agar (MHA) plates that had been swabbed with an overnight *S. aureus* culture, and the plates were subsequently incubated. The presence of an inhibition zone around the well indicated the antibacterial activity of the drug-coated catheter tube.

2.8. Quantification of the Bacterial Load in the Bladder Model. The quantification of the bacterial load in a coated catheter tube was achieved when small pieces of catheter tubes were immersed in BHI broth cultured with *S. aureus* for 120 h. Then, the catheter tubes with the formed biofilm were immersed in the duloxetine solution (37.5 mg/mL) for 1 h, and the viable bacterial count was obtained for the catheter tubes with and without drug coating, by diluting 10-fold using 100 μL from the drug-coated and uncoated suspensions and using the spread plate technique on BHI plates, followed by incubation. Later, the viable count was determined by counting the CFUs,[27] and the untreated tube served as the control.

2.9. Confocal Laser Scanning Microscopy (CLSM) of the Bladder Model. To visualize the biofilm formation on a silicone catheter, the silicone catheter tube was cut into small square pieces, then immersed in a BHI broth containing an overnight *S. aureus* culture, and incubated for 96 h to allow biofilm formation. Subsequently, the small catheter tube was washed with PBS and subjected to a 1 h treatment with duloxetine. Following this treatment, the catheter tube was stained with FDA and PI for 10 min and allowed to air-dry. The stained catheter piece was subjected to CLSM (Zeiss LSM 900, U.K.), and the untreated tube served as the control.[27]

2.10. Morphological Changes Observed by Scanning Electron Microscopy (SEM). To examine the morphological changes of *S. aureus* after treatment with duloxetine, scanning electron microscopy was performed.[25] In brief, an overnight *S. aureus* culture was loaded on Whatman No. 1 filter paper strips, and biofilm formation was allowed for 96 h. Then, the paper strip was washed and treated with duloxetine for 1 h, washed with PBS, fixed with glutaraldehyde, and dehydrated with ethanol gradient. Gold coating was performed for the treated and untreated paper strips, and the images were captured using a scanning electron microscope (Zeiss SUPRA 55-VP FEGSEM, U.K.).

3. RESULTS

3.1. Antibacterial Activity of Duloxetine. The antibacterial activity of duloxetine against *S. aureus* was assessed, and the zone of inhibition around the well reflected the antibacterial effectiveness across different concentrations of duloxetine (125, 150, and 200 μg), as displayed in Figure 1. As observed in Figure 1, the antibacterial activity was evident even at the lowest concentration of 125 μg/well of duloxetine against *S. aureus*. Furthermore, the activity increased as the concentration of duloxetine was increased against *S. aureus*.

3.2. Determination of MIC. The MIC of duloxetine was determined against *S. aureus* using the microdilution method, and the results are illustrated in Figure 2. As displayed in Figure 2, the calculated MIC of duloxetine against *S. aureus* was 37.5 μg/mL.

3.3. Time−Kill Assay. The killing effect of duloxetine was investigated against *S. aureus* using the time−kill assay, and the growth inhibitory effect was calculated based on the colony-forming units. As observed in Figure 3, *S. aureus* initially receiving duloxetine treatment showed no viable cells after 1 h, whereas the cells that received no treatment produced viable cells. The growth inhibitory effect of duloxetine proved the interaction with *S. aureus*.

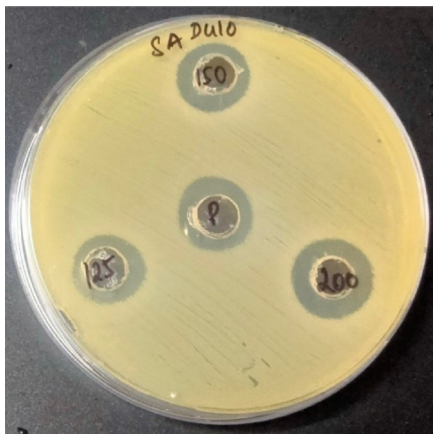

Figure 1. Antibacterial activity of duloxetine against *S. aureus*.

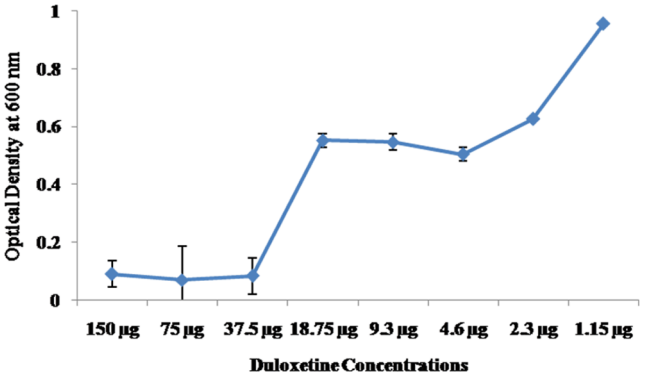

Figure 2. Determination of the MIC of duloxetine against *S. aureus*.

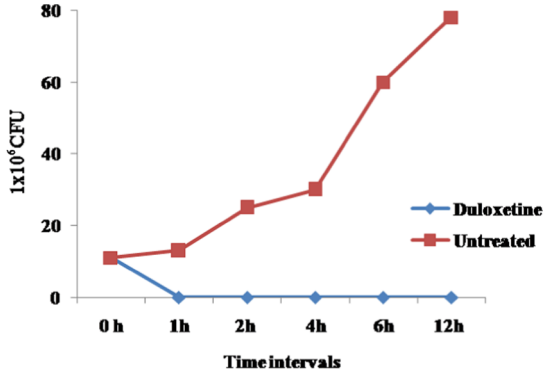

Figure 3. Time−kill assay of duloxetine against *S. aureus*.

3.4. Impact of Duloxetine on Biofilm Formation. The impact of duloxetine on *S. aureus* biofilm formation was assessed using the crystal violet method. Figure 4 displays the calculated percentage of biofilm formation following treatment with various concentrations of duloxetine ranging from 150 to 1.15 μg/mL. As depicted in Figure 4, biofilm formation was not observed up to the MIC level of duloxetine (37.5 μg/mL) in the polystyrene plate, whereas a gradual increase in the percentage of biofilm formation was observed above the

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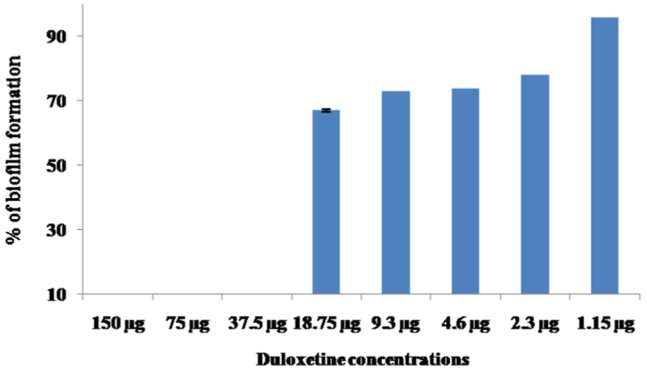

Figure 4. Effect of duloxetine on *S. aureus* biofilm formation.

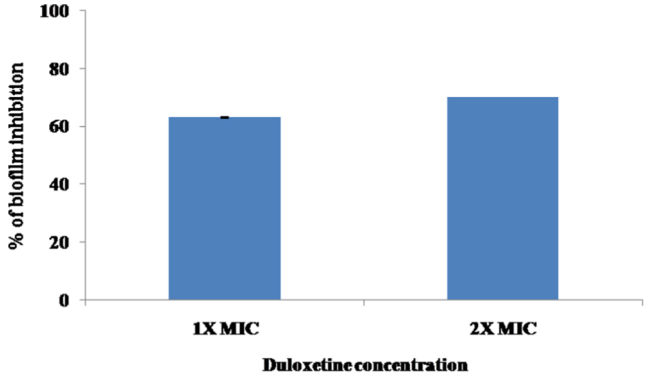

Figure 6. Effect of duloxetine on a mature *S. aureus* biofilm.

duloxetine MIC level, indicating that duloxetine present in the well at low concentrations can reduce the biofilm-forming potency of *S. aureus*.

3.5. Impact of Duloxetine on Mature Biofilms. A qualitative analysis of the inhibitory effect of duloxetine on the *S. aureus* biofilm was conducted using cellulose matrices. The visual evidence of biofilm inhibition following treatment with duloxetine is illustrated in Figure 5, wherein the SEM image shows a negligible number of adherent cells on their surface after treatment with 37.5 μg/mL duloxetine against *S. aureus*; thus, the biofilm was inhibited, whereas untreated matrices showed a greater number of adherent cells on their surfaces, indicating no biofilm inhibition. Further, the biofilm inhibition was calculated quantitatively by the crystal violet method, and the biofilm inhibition (%) after treatment with duloxetine (1 and 2× MIC) is displayed in Figure 6, wherein 37.5 μg/mL duloxetine was able to reduce 63% of the biofilm after treatment, but an increase of up to 70% biofilm inhibition was observed when treated with a 2× MIC level of duloxetine, whereas untreated cells showed no biofilm inhibition.

3.6. *In Vitro* Bladder Model. The antibacterial efficiency of the duloxetine-coated silicone catheter tube against *S. aureus* was examined under suitable conditions, and the inhibition zones are illustrated in Figure 7. In the figure, the presence of an inhibition zone around the drug-coated catheter tube clearly signifies the antibacterial activity of duloxetine against *S. aureus*, in contrast to the uncoated catheter tube, which exhibited no such inhibition zone. Furthermore, the quantification of the bacterial load from the silicone catheter tube after drug coating was performed. The growth inhibitory effect of duloxetine in the bladder model was calculated based on the colony-forming units (CFUs). Notably, Figure 8 reveals the minimal number of viable colonies in the drug-coated catheter tube, while viable colonies were observed in the

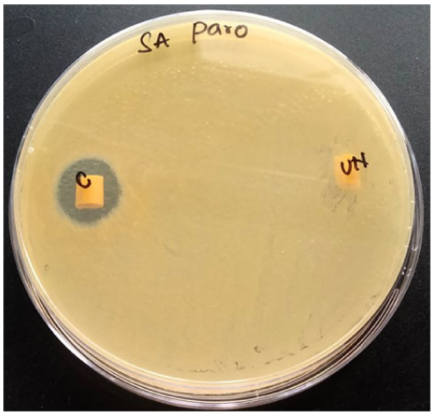

Figure 7. Antibacterial activity of the drug-coated silicone catheter tube against *S. aureus*.

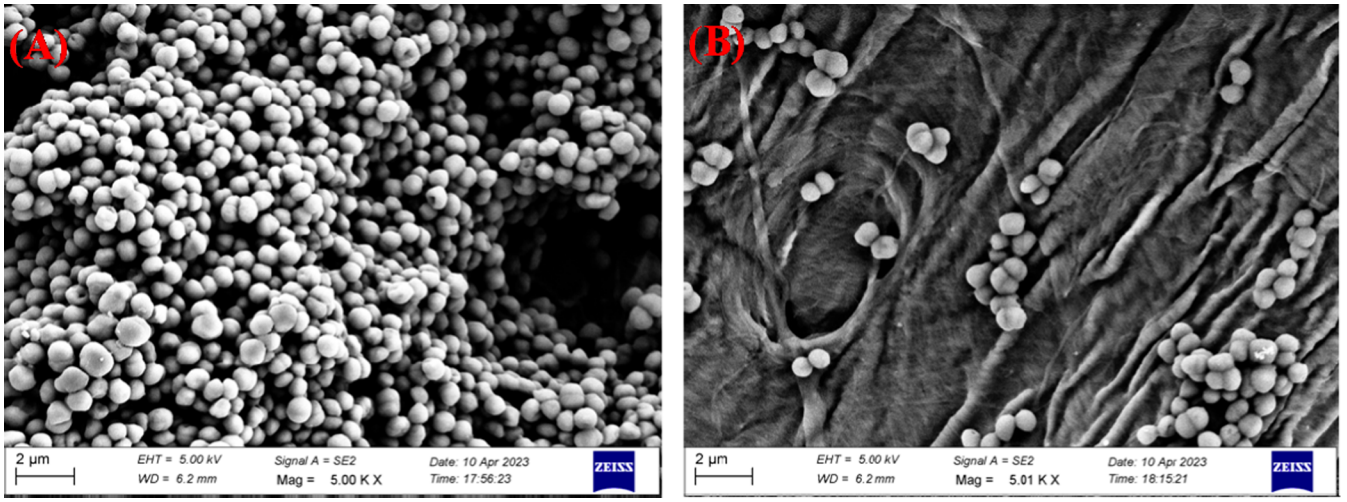

Figure 5. Biofilm inhibition was assessed by SEM. (A) More adherent *S. aureus* cells on cellulose matrices. (B) Adherent cells were reduced after treatment with duloxetine.

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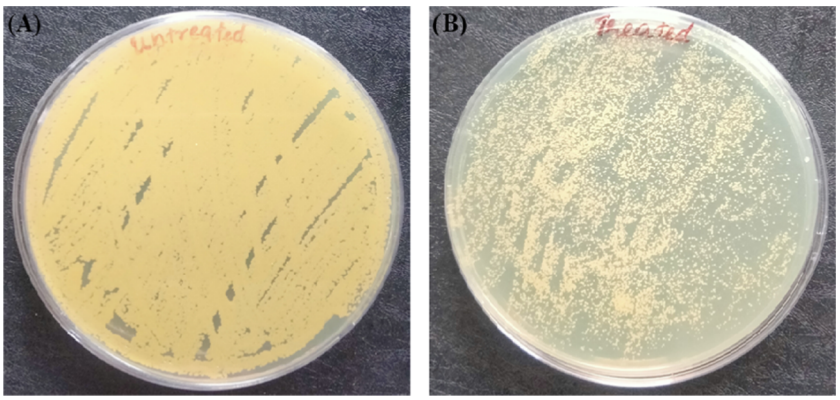

Figure 8. Quantification of bacterial load from a catheter tube treated with duloxetine.

uncoated catheter tube, emphasizing the potent antibacterial impact of duloxetine.

3.7. CLSM of the Bladder Model. The growth inhibitory effect of duloxetine against *S. aureus* in the bladder model was visualized using CLSM after 1 h treatment, and the calculated live/dead percentage is presented in Figure 9A−E. As depicted in the figure, the silicone catheter tube, which had been contaminated with *S. aureus* for a period of 21 days, was observed after staining with fluorescein diacetate (FDA), which emits green fluorescence in live cells, and propidium iodide (PI), which emits red fluorescence upon binding to DNA. Here, the green fluorescence indicates live cells in the silicone catheter tube, and also, Figure 9B indicates the three-dimensional structure of the *S. aureus* biofilm thickness (90 μM) on a silicone catheter tube. However, Figure 9C shows red fluorescence, indicating the treatment of the contaminated silicone catheter with duloxetine, which disrupts the *S. aureus* cell membrane and binds to the DNA, thereby enhancing the fluorescence observed. Figure 9D shows that the three-dimensional structure of the silicone catheter tube reduced the biofilm thickness after treatment with duloxetine (60 μM). Here, duloxetine can disrupt the cell membrane when present at its MIC. The combination of FDA and PI offers visual evidence of live and dead cells on the silicone catheter tube. By utilizing the FDA and PI combination, we calculated the percentage of live and dead cells based on the disruption of the cell membrane within the entire cell population (Figure 9E). The result revealed that the drug-coated tube showed 76% dead cells and 24% live cells, indicating the potency of duloxetine against *S. aureus* in the bladder model.

3.8. Morphological Changes Observed by SEM. The morphological changes of *S. aureus* after treatment with duloxetine were examined by SEM and are presented in Figure 10, wherein the cell surface of *S. aureus* analyzed after treatment with duloxetine showed cell shrinkage, indicating internal component leakage when compared with the smooth surface of untreated *S. aureus*.

4. DISCUSSION

One of the most common and significant hospital-associated infections is CAUTI, caused by indwelling catheters, which results in severe clinical complications and leads to high morbidity and mortality rates due to the presence of polymicrobial structures that make the treatment ineffective and also due to the prevalence of antibiotic-resistant strains, resulting in severe socioeconomic burden.[28,29] Taking all of these factors into account, our study delves into the investigation of duloxetine, a repurposed drug, for its antibacterial, antibiofilm, and antiadhesive properties against *S. aureus*, a prominent contributor to CAUTIs. Duloxetine exhibited promising antibacterial activity against *S. aureus*. Additionally, a previous study documented the antibacterial efficacy of fluoxetine and its impact on the growth of *S. aureus*, *P. aeruginosa*, and *E. coli*. Furthermore, it was noted that the antibacterial activity of fluoxetine was enhanced when used in combination with commercially available antibiotics.[30] Similarly, the phosphate prodrug, an anticancer drug, was repurposed for its antibacterial properties against *S. aureus* and *E. faecalis*; the potent antibacterial activities of ebselen, amlodipine, sertraline, and azelastine against *S. aureus* were was also proven.[31,32] Likewise, a number of studies by various groups have reported antibacterial activities of various repurposed drugs, such as quercetin, curcumin, duloxetine, ibuprofen, amodiaquine, mitomycin-C, ellargic acid, and diiodohydroxyquinoline, against *S. aureus*, *E. faecalis*, *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, and *Clostridium difficile*.[33,34] These studies have promoted the importance of drug repurposing for various new applications.

In addition to assessing its antibacterial properties, our study investigated the impact of duloxetine on various stages of biofilm formation by *S. aureus*. This investigation encompassed qualitative and quantitative analyses of biofilm formation on cellulose matrices and polystyrene surfaces using *in vitro* models. Catheter insertion allowed the entry of uropathogens for microbial colonization, resulting in urinary tract infection and microbial attachment on the surface of the catheter, in turn providing an opportunity to form complex structures that can shelter the bacteria from various external sources such as antibiotic treatment, thus leading to the emergence of antibiotic-resistant strains, which made CAUTI management crucial.[35,36] Therefore, our study mainly concentrated on every stage of biofilm formation, from attachment to the formation of matured biofilms, to prevent biofilm formation on the catheter surface.[37] Initially, duloxetine was studied for its effect on *S. aureus* biofilm formation and inhibition using cellulose matrices and polystyrene surfaces. The results exhibited the effectiveness of duloxetine in inhibiting *S. aureus* biofilm formation, as corroborated by SEM imaging and the crystal violet method. Similarly, the antibacterial and antibiofilm activities of three repurposed drugs, such as etoposide-A, sertraline, and penfluridol, were investigated against *S. aureus* biofilm formation on cellulose matrices and hydroxyapatite pellets. The results revealed that the tested repurposed drugs demonstrated the ability to both reduce and inhibit biofilm

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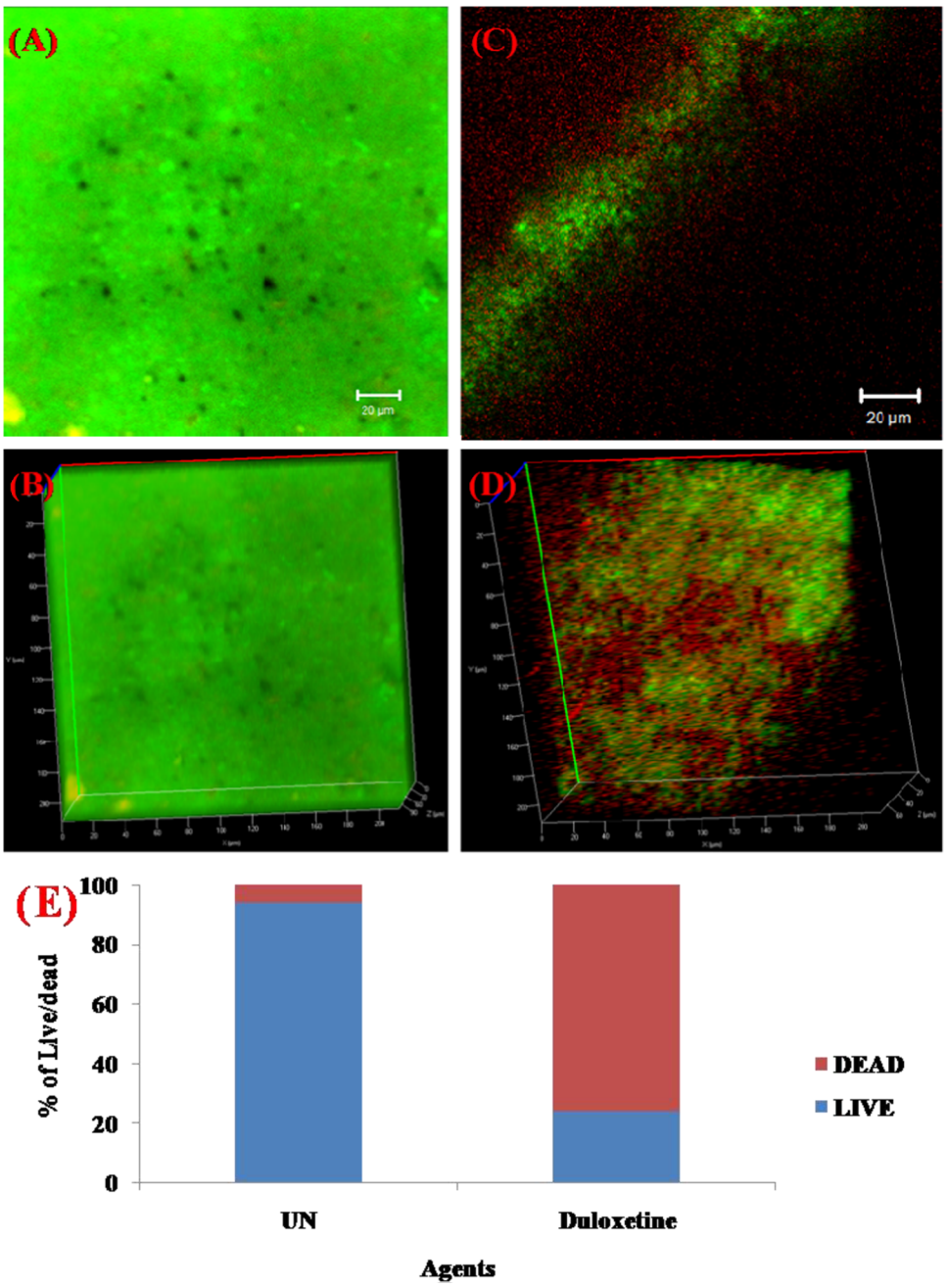

Figure 9. CLSM analysis of the catheter tube: (A) *S. aureus* biofilm on the uncoated catheter tube. Here, green fluorescence indicates live cells. (B) Three-dimensional structure of the *S. aureus* biofilm on a catheter tube. (C) Biofilm reduction after treatment with duloxetine was observed from 100 to 60 μM. Here, red fluorescence indicates dead cells. (D) Three-dimensional structure of a silicone catheter tube upon reduced biofilm thickness after treatment with duloxetine (60 μM). (E) Graph indicating the percentage of live/dead cells.

formation on matrices and hydroxyapatite after treatment with duloxetine.[38] Various studies reported the antibiofilm potency of repurposed drugs against *S. aureus* biofilm formation.

Later, duloxetine was investigated for its antiadhesive properties against *S. aureus* using an *in vitro* bladder model, which was used to study the prevention of biofilm formation on the catheter surface. The catheter surface was coated with an antibacterial agent for some time to delay or prevent bacterial attachment on the surface, and this method also prevented the attachment of uropathogens. Therefore, this study aimed to mitigate biofilm formation on catheter surfaces by applying a duloxetine coating. The antiadhesive quality was validated through the inhibition zone observed around the drug-coated silicone catheter tube. Additionally, we assessed catheter tubes with ZnO and Ag NPs against *S. aureus*, *E faecalis*, and *E. coli* over a span of 7 days and found sustained and remarkable antibacterial activity throughout this duration.[39] Also, CLSM was employed to visually corroborate the impact of duloxetine on biofilm formation on catheter surfaces. This analysis, after staining with FDA and PI, provided compelling evidence of the excellent activity of duloxetine, offering insights into the live and dead microbial presence on the catheter surface. Similarly, a silver poly-(tetrafluoroethylene) nanocomposite-coated catheter showed enhanced antibacterial and antiadhesive properties against two CAUTI strains, *S. aureus* and *E. coli*; when compared to the

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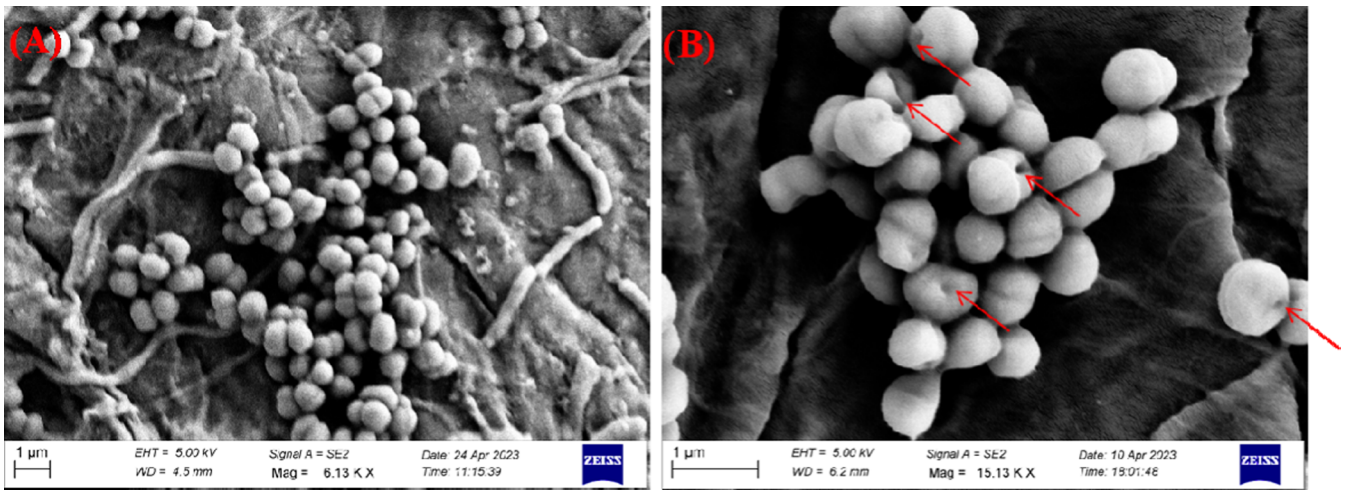

Figure 10. Morphological changes observed by SEM: (A) SEM image of *S. aureus* with a smooth surface indicating no cell damage. (B) Morphological changes such as cell shrinkage observed in *S. aureus* after treatment with duloxetine.

uncoated catheter, the silver polytetrafluoroethylene nanocomposite-coated catheter showed a reduction of bacterial adhesion up to 55.2 and 60.3% for the two strains, respectively.[40] Overall, this study confirmed the bioactivity of the repurposed drug for new applications.

5. CONCLUSIONS

The antibacterial and antibiofilm activities of duloxetine, a repurposed drug, were investigated against *S. aureus*, a predominant microorganism involved in CAUTIs. The study efficaciously determined the antibacterial activity and the minimum inhibitory concentration of duloxetine. Remarkably, duloxetine demonstrated the ability to inhibit biofilm formation and reduce mature biofilms on both cellulose and polystyrene surfaces. This antibiofilm activity was confirmed through SEM imaging and crystal violet methods. In addition, the antiadhesive properties of a duloxetine-coated catheter tube were assessed against *S. aureus*. Furthermore, the antibiofilm activity of duloxetine was substantiated through CLSM. Altogether, duloxetine presents a viable alternative as both an antibacterial and an antibiofilm agent against *S. aureus* implicated in CAUTIs.

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Author Contributions

G.P.: conceptualization, investigation, methodology, formal analysis, data curation, and writing—original draft. P. Karuppiah: conceptualization, investigation, methodology, formal analysis, data curation, and writing—original draft. P. Karthikeyan: investigation, methodology, visualization, data curation, formal analysis, writing—original draft, and writing—review and editing. S.P.: conceptualization, investigation, methodology, formal analysis, data curation, writing—original draft, and review of the final draft.

Notes

The authors declare no competing financial interest.

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