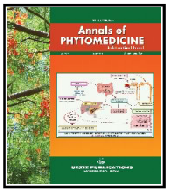
Annals of Phytomedicine 13(1): 764-769, 2024

DOI: http://dx.doi.org/10.54085/ap.2024.13.1.80

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

Repurposing aceclofenac: Antibacterial and antibiofilm activities of the NSAIDs against healthcare-associated infections

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Article Info

Article history
Received 13 February 2024
Revised 1 April 2024
Accepted 2 April 2024
Published Online 30 June 2024

Keywords
Aceclofenac
Antibacterial
Antibiofilm
Drug repurposing
Healthcare-associated infections
Multidrug resistance
Synergism

Abstract

The multidrug resistance development in many of the bacterial species is directly related to their ability to form three-dimensional structured biofilms and it brings about serious threat to public health in various aspects. Therefore, there is a need for alternative antimicrobial agents to fight against such bacteria. As it is a unique process of developing antibacterial agents, the process of drug repurposing has attracted the wide attention of scientists across the globe, and in the present study, we tried to repurpose an NSAIDs (non-steroidal anti-inflammatory drug) aceclofenac for its antimicrobial potentials against *Enterococcus faecalis*, *Staphylococcus aureus*, and *Escherichia coli* which contribute to hospital-associated infection. It was found that aceclofenac showed significant antibacterial activities at a concentration of 200 µg/ml against all the tested microbes and also, and it has also effectively inhibited biofilm formation. The antibiofilm activities of aceclofenac were evaluated and it effectively eradicated the mature biofilm at 89%, 86%, and 76% against *E. faecalis*, *S. aureus*, and *E. coli*, respectively. Aceclofenac also showed a synergistic effect with the antibiotic rifampicin. Overall, aceclofenac showed antibacterial and antibiofilm activities and it could represent a better alternative as an antimicrobial drug against the mentioned bacteria.

1. Introduction

Many of the microorganisms that cause life-threatening infections gain multidrug resistance leading to serious menace to public health with complications that are extremely challenging for treatment with existing antibiotics, which in turn result in severe socio-economic burden with higher morbidity and mortality rates (Santajit and Indrawattana, 2016; Leão *et al*., 2020). Drug resistance is accountable for more than a million death cases globally due to their drastic increase which indicates that if there are no novel antimicrobials on the Table by 2050, there will be numerous deaths encountered per year with life-threatening bacterial infections which may be the underlying cause of mortality (Farha *et al*., 2019; Yssel *et al*., 2017; Magill *et al*., 2014). The multidrug resistance increase is strictly limiting and delaying the options for treatment and management of the disease due to its biofilm-forming ability creating treatment challenges that lead to resistance development to existing antibiotics and also escape from host defence mechanisms (Muhammad *et al*., 2022; Li *et al*., 2019; Aslam *et al*., 2018; Nathan *et al*., 2014; Borges *et al*., 2013). Apart from that, the formation of an extracellular polymeric matrix substance can decrease the penetration and diffusion of antibacterial agents and also protect the microbes from external environmental stresses which, in turn, promotes drug resistance (Meireles *et al*., 2016). Moreover, biofilms are not easily treated by antimicrobials due to the presence of EPX matrix which prevents the biofilms from proper oxygen and nutrient absorption, making the biofilms unapproachable for antimicrobial treatment. The antimicrobial resistance can also be directed by the resistance gene expression that makes enzymes neutralize or degrade the antibiotics, and overexpression of efflux pumps which eliminate the antigen elements from the intracellular, space resulting in the initiation of cell apoptosis (Singh *et al*., 2017; Abreu *et al*., 2014). Several biofilm-forming, life-threatening microbes especially *E. faecalis, S. aureus,* and *E. coli* generally cause healthcare-associated infections which significantly contribute to higher morbidity and death rates (Caldara *et al*., 2021; Cassini *et al.,* 2019; Gajdács, 2019). To overcome drug resistance, there is an urgent requirement to develop novel antibacterial compounds that can eliminate biofilms. However, the development of antimicrobial agents is an enormously time-consuming and expensive process, and as a consequence of this, the idea of old drugs for their new use against microorganisms including viruses is gaining popularity among researchers because their efficacy and safety have been approved and even promoted (Poyil *et al*., 2023; Poyil and Bari, 2023; Kaustubh and Nupur, 2022; Kunz Coyne *et al*., 2022; Paes Leme *et al*., 2021). Nowadays, many studies have supported the antimicrobial potentials of non-steroidal anti-inflammatory drugs against many medically significant gram-negative and gram-positive organisms (Kaul *et al*., 2019; Chan *et al*., 2017; Zimmermann and Curtis, 2017). So, in this investigation, aceclofenac a non-steroidal anti-inflammatory drug that has been widely studied (Shanthi *et al.,* 2023) and in use for many decades, was examined for its antibiofilm

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and antibacterial potentials against three major healthcare-associated infection-causing bacterial pathogens, *viz*., *E. faecalis, S. aureus,* and *E. coli.*

2. Materials and Methods

2.1 Chemicals, reagents, bacterial cultures

Bacteriological media like heart infusion agar (BHA), Mueller-Hinton agar (MHA), *etc.,* and antibiotics like rifampicin, ampicillin, *etc*., were purchased from the Hi-Media company, and the bacterial cultures used (*E. coli* (ATCC 25922, *E. faecalis* (ATCC 29212) and *S. aureus* (ATCC 25923) from ATCC. For all the experiments, the overnight cultures were adjusted to 1X 10^6 CFU/ml and used for the study. Aceclofenac (1 mg/ml), ampicillin (5 µg) and rifampicin (5 µg) were used for all the experiment.

2.2 Determination of the antimicrobial potentials of the drug aceclofenac

To understand the antimicrobial potential of aceclofenac against *E. faecalis, S. aureus,* and *E. coli,* the well-diffusion method (Meiyazhagan *et al*., 2015) was used. Overnight cultural specimens of the selected bacterial pathogens were inoculated onto the MHA plate and BHI agar plates and the wells were prepared to receive the various concentrations of aceclofenac. They were subjected to 24 h incubation. The diameter of growth inhibition zones around wells indicates the antimicrobial activities of aceclofenac against *E. faecalis, S. aureus,* and *E. coli.* The positive controls used were ampicillin and rifampicin.

2.3 Determination of MIC (minimal inhibitory concentration) of the drug aceclofenac

To investigate the aceclofenac MIC against *E. faecalis, E. coli,* and *S. aureus* the method of micro-dilution (Meiyazhagan *et al*., 2016) was used. In short, MHB and BHI broth were added to 96 well plates, and 200 µg/ml of aceclofenac was subjected to serial dilution to make it to 1.55 µg/ml. Later, overnight bacterial cultures were inoculated to the respective broth and incubated, and using a spectrophotometer, at 600 nm, the plate was read.

2.4 Effect of the drug aceclofenac on biofilm formation

The efficiency of the drug aceclofenac on biofilm formation by *E. faecalis, S. aureus,* and *E. coli* was studied by the crystal violet staining method (Meiyazhagan *et al*., 2015). In brief, MHB and BHI broth were added to a polystyrene plate and 200 µg/ml of aceclofenac was diluted serially to 1.55 µg/ml. Then, the test organisms were inoculated to respective broths, followed by an incubation for 5 days. Later, the biofilms were washed, fixed by methanol, and then subjected to crystal violet staining for 45 min. Then, the biofilms were destained with an ethanol acetone mixer. Then, the attained purple color compound was spectrophotometrically analyzed at 570 nm. The untreated wells were the negative controls.

2.5 Effect of the drug aceclofenac on matured biofilms

To study the aceclofenac effect on mature biofilms of *E. faecalis, E. coli,* and *S. aureus,* the biofilm-formation assay was performed by crystal violet (Gowri *et al*., 2020). In brief, cultures of test microbes were added and allowed for 96 h to form biofilms. Then, the mature biofilms were washed and treated with three various concentrations (1X MIC, 2X MIC, and 3X MIC) of aceclofenac for 24 h. After that, to detach the non-adherent cells, the biofilms were washed and then subjected to methanol-fixation. Then, the cells were subjected to crystal violet staining followed by destaining with the complex of ethyl alcohol and acetone. The attained purple-coloured final compound was measured spectrophotometrically at 570 nm. The negative controls were untreated wells.

2.6 Synergistic activities of the drug and selected antibiotics

To examine the synergistic effect of aceclofenac with commonly available antibiotics like ampicillin and rifampicin against *S. aureus, E. coli,* and *E. faecalis,* the checkerboard was used as standardized by Meiyazhagan *et al*. (2016). For the assay, the combinations of aceclofenac and antibiotics were added at one concentration above its MIC, MIC, and three concentrations below its MIC, followed by the addition of cultures of *E. faecalis, S. aureus*, and *E. coli* and incubated. The OD was spectrophotometrically analyzed at a wavelength of 600 nm. The synergistic effect was estimated based on the fractional inhibitory concentration index (FICI).

3. Results

3.1 Antibacterial activity of the drug aceclofenac

The antibacterial potentials of the drug aceclofenac were determined against *S. aureus, E. faecalis,* and *E. coli,* and the findings are shown in Figure 1. The zones of growth inhibition were found around the well dictating the aceclofenac antibacterial activity against tested microbes, and also, the zone size was increased when increasing the aceclofenac concentrations.

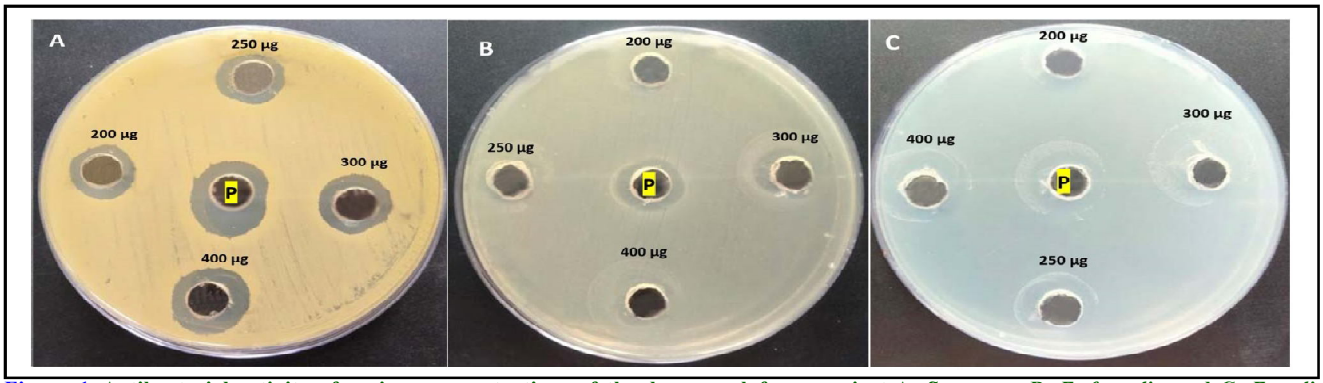


Figure 1: Antibacterial activity of various concentrations of the drug aceclofenac against A. *S. aureus,* B. *E. faecalis,* and C. *E. coli.* "P" denotes the positive control taken.

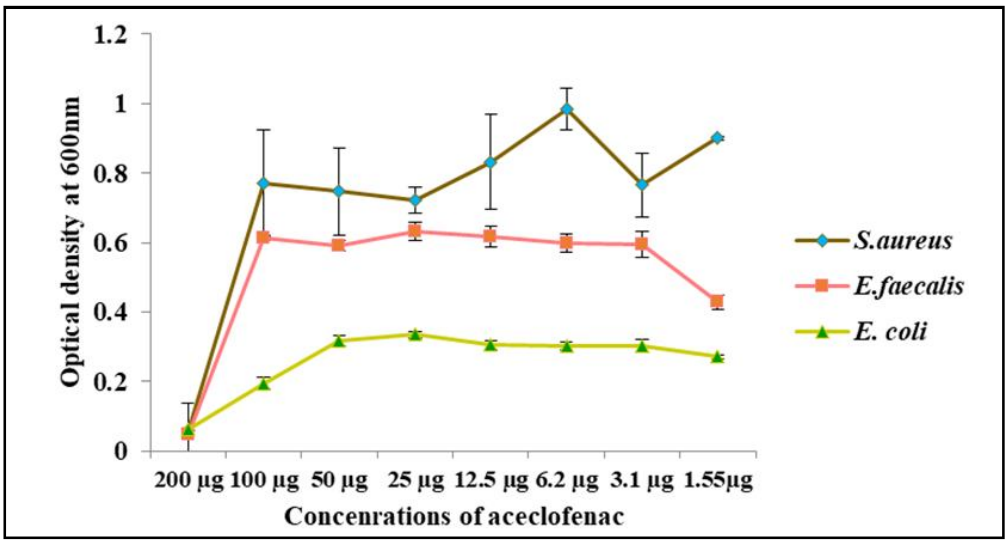


Figure 2: Aceclofenac MIC determination against *S. aureus, E. faecalis,* and *E. coli.*

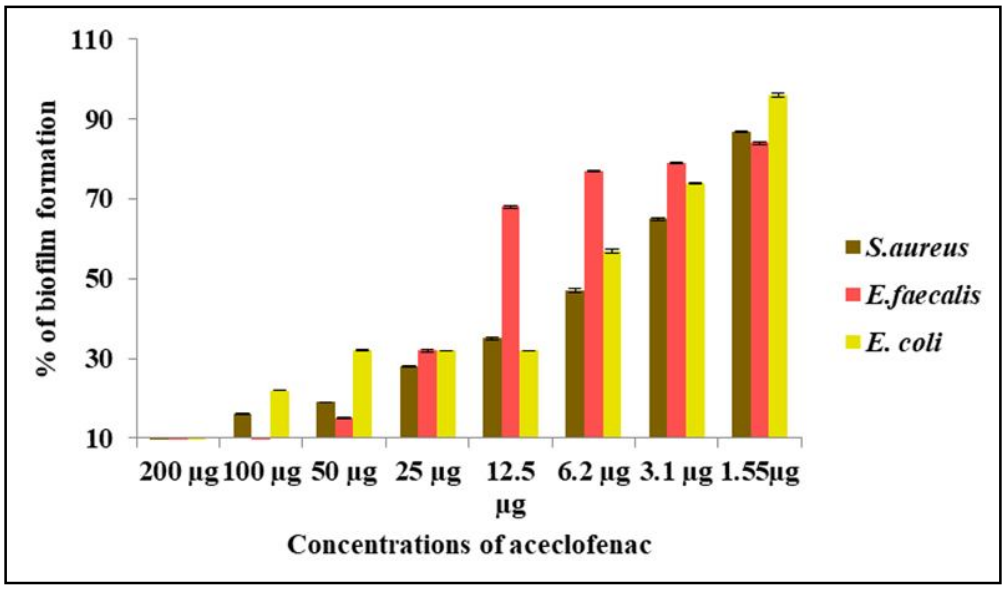


Figure 3: Aceclofenac effect on *S. aureus, E. faecalis,* and *E. coli* biofilm formation.

3.2 Determination of the MIC (minimal inhibitory concentration) of aceclofenac

Against the selected bacteria, *viz*., *S. aureus, E. faecalis,* and *E. coli*, the MICs of aceclofenac were found out by the micro-dilution protocol, and the calculated MICs are interpreted in Figure 2. As shown in the graph, 200 µg/ml of aceclofenac was required to stop *S. aureus, E. faecalis,* and *E. coli* growth.

3.3 Effect of aceclofenac on biofilm formation

The potentials of aceclofenac on *E. faecalis, S. aureus,* and *E. coli* biofilm formation were evaluated using the crystal violet technique, and the attained result is shown in Figure 3. The drug aceclofenac inhibited the biofilm formations of *E. faecalis, S. aureus,* and *E. coli* at their MIC level. A gradual increase in biofilm formation was observed below its MIC against all the tested microbes representing aceclofenac capability to prevent the biofilm formation.

3.4 Aceclofenac effect on biofilm eradication

The antibiofilm activities of aceclofenac were investigated against *E. faecalis, S. aureus,* and *E. coli* on polystyrene surfaces by crystal violet method, and the attained percentage of biofilm reductions after treatment with 1X MIC (200 µg /ml), 2X MIC (400 µg/ml) and 3X MIC (600 µg/ml) aceclofenac is presented in Figure 4. All the tested concentrations of aceclofenac effectively eradicated the biofilms of tested microbes**.** The aceclofenac effectively eradicated 81%, 85%, and 86% of *S. aureus* biofilm after aceclofenac treatment. Sameway, aceclofenac eradicated 83%, 87%, and 89% of *E. faecalis* biofilm, and also, 68%, 70%, and 76% of *E. coli* biofilm was eradicated after aceclofenac treatment which represents the aceclofenac antibiofilm activity.

3.5 Synergistic activities of aceclofenac and antibiotics

The synergistic activities of aceclofenac were studied with ampicillin and rifampicin using a checkerboard assay and the results are shown

in Figure 5. When, the *S. aureus* and *E. faecalis* were treated with aceclofenac in combination with ampicillin, there was no difference in their MIC level indicating no synergism. Whereas, *E. coli* was treated with aceclofenac in combination with rifampicin, the reduction in their original MIC level was observed indicating synergism. FICI calculated for aceclofenac and rifampicin proved the synergistic effect which represents aceclofenac can be used in combination to treat infections.

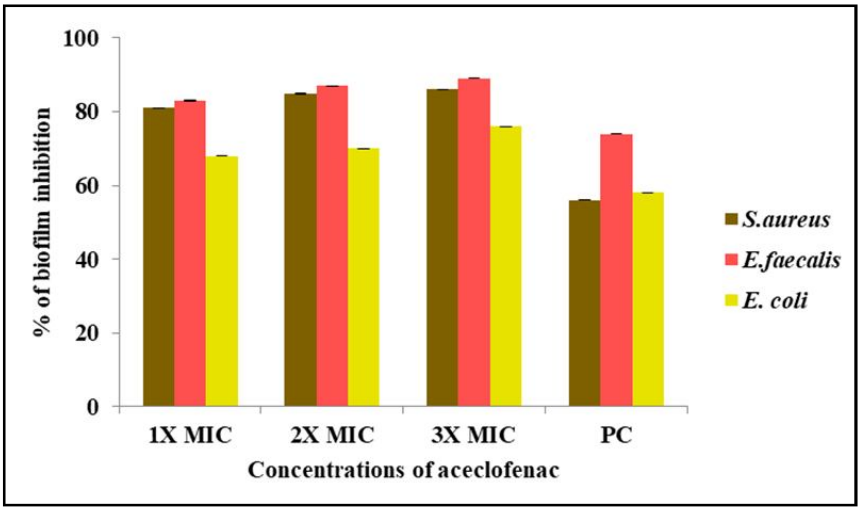
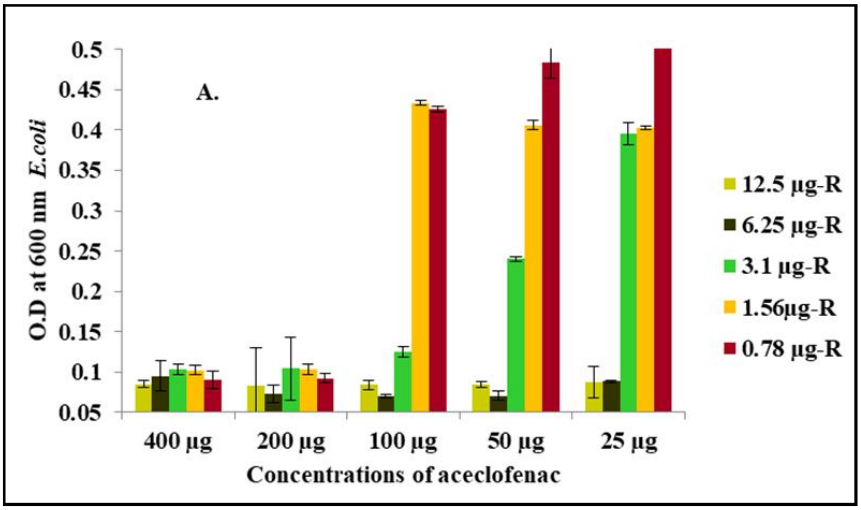
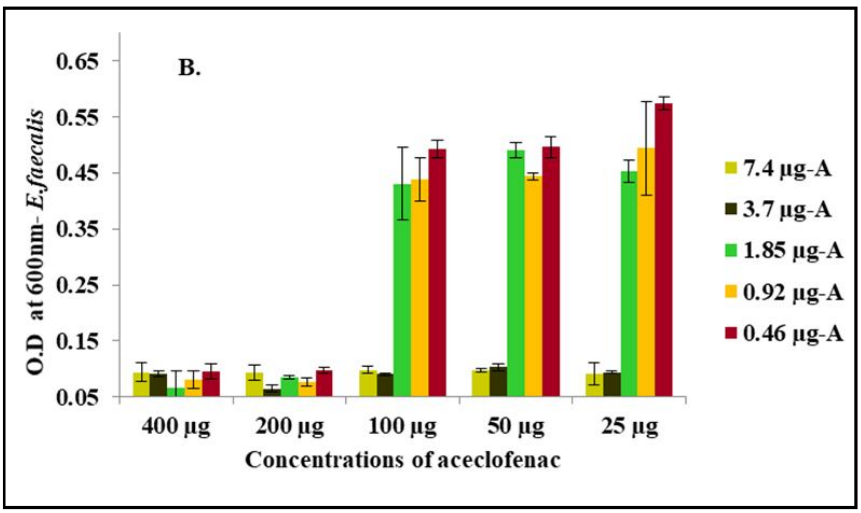


Figure 4: Antibiofilm activity of aceclofenac *S. aureus*, *E. faecalis* and *E. coli*.





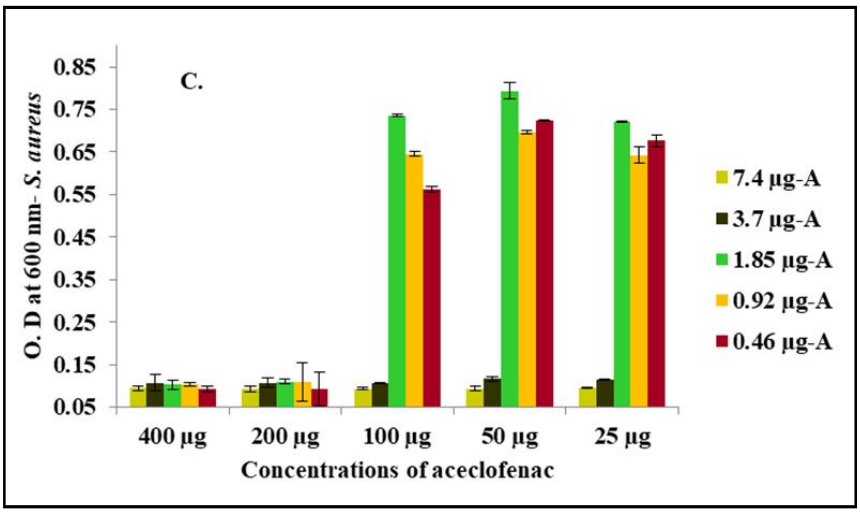


Figure 5: Synergistic activities of aceclofenac with ampicillin and rifampicin.

4. Discussion

The rise of multidrug resistant microorganisms to different classes of antibiotics creates a severe threat to human health resulting in higher morbidity and death rates. Mostly, the development of multidrug resistance is primarily related to biofilm formation which makes treatment challenging. Therefore, in this investigation, the antimicrobial and antibiofilm activity of a nonsteroidal anti-inflammatory drug, aceclofenac was investigated against *E. faecalis, S. aureus,* and *E. coli* which have involvement in healthcare facilities. The study showed the antimicrobial and antibiofilm potentials of aceclofenac against *E. faecalis, S. aureus,* and *E. coli* with minimum inhibitory concentration and also reduced the biofilms. The study was correlated with a recent report wherein the antimicrobial potentials of aceclofenac were investigated against *S. mutant, E. faecalis, S. aureus,* and *C. albicans* involved in root canal infection and showed better antimicrobial activity at 20 µg/ml (Ashwin and Sandeep, 2022). Similarly, diclofenac and ibuprofen were analyzed for their antimicrobial potential against *E. faecalis* and showed the least inhibitory concentration at 50 µg/ml (Salem-Milani *et al*., 2013). In support of this, many scientific investigations have revealed the antimicrobial activities of repurposed drugs against various microbes. Recently, hexestrol, selamectin, paroxetine, amodiaquine, and amlodipine were evaluated for antibacterial and antibiofilm effects against methicillin-resistant *S. aureus,* and they displayed excellent antimicrobial activities. Moreover, it has effectively reduced the biofilm and showed synergism with antibiotics (Liu *et al*., 2023; Folliero *et al*., 2023; Cabral *et al*., 2023; Barbosa *et al*., 2023). Based on the earlier reports, our findings showed the antimicrobial and antibiofilm potentials suggesting that aceclofenac could be a promising antimicrobial agent against *E. faecalis, S. aureus,* and *E. coli.*

5. Conclusion

The antimicrobial and antibiofilm activity of a repurposing drug, aceclofenac was analyzed against *E. faecalis, S. aureus,* and *E. coli* and showed antibacterial activity with the least inhibitory concentration. The aceclofenac effectively prevented biofilm formation and also could eradicate the matured biofilms by tested organisms. The drug had a synergistic effect with rifampicin suggesting that the aceclofenac can be used in combination to treat bacterial infection associated with health care settings.

Acknowledgements

The authors are grateful to the Deanship of Scientific Research, Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia for its support and encouragement in conducting the research and publishing this report.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **Tahane Bashir M. Ahmed, Areeg S. Aziz and Nagat Siednamohammeddeen (2024). Repurposing aceclofenac: Antibacterial and antibiofilm activities of the NSAIDs against healthcare-associated infections. Ann. Phytomed., 13(1):764-769. http://dx.doi.org/10.54085/ap.2024.13.1.80.**