




Repurposing of dibucaine and niflumic acid as antimicrobial agents in combination with antibiotics against *Staphylococcus aureus*

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Abstract

The versatile human commensal bacteria and pathogen *Staphylococcus aureus* cause several community and hospital-acquired illnesses associated with significant morbidity and death. Antibiotic therapy for *S. aureus* infections has grown increasingly difficult as the organism has developed a wide spectrum of antibiotic resistance mechanisms. This situation emphasizes the significance of developing and advocating new antimicrobials for preventative and therapeutic measures. Our study aimed to identify and evaluate new therapeutic options against *S. aureus*. We investigated the efficacy of two drugs, dibucaine, and niflumic acid, as potential adjuvant for anti-staphylococcal therapeutics. Dibucaine and niflumic acid found to have bactericidal activity against *S. aureus*. These drugs acted synergistically with antibiotics reducing the required dose of antibiotics up to 4 times. In combination with antibiotics, they were effectively and synergistically inhibited the formation of biofilms of *S. aureus*. The best synergistic partner of dibucaine was with kanamycin and tetracycline, whereas niflumic acid was with streptomycin and ampicillin. Both the drugs showed significant efflux inhibition in the bacteria. Moreover, the drugs are found to be safe at synergistic doses. Our findings suggest that dibucaine and niflumic acid could be potential adjuvant with antibiotics for the treatment of *S. aureus* infections. Their ability to significantly enhance the efficacy of antibiotics highlights their potential clinical significance as adjunct therapies.

Introduction

The rise of antibiotic resistance in pathogens like *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* has raised concerns about the effectiveness of broad-spectrum antimicrobial agents. Factors contributing to antimicrobial

resistance include misuse and overuse of antibiotics, their widespread use in animal feed, and increased global travel [1, 2]. *S. aureus* is particularly worrisome due to its virulence, ability to form biofilms, and capacity to adapt to environmental stress [3]. Despite advancements in antimicrobials, *S. aureus* infections still carry high fatality rates, especially in healthcare settings [4, 5]. Efforts to understand antimicrobial resistance have identified potential targets for new drugs, but progress in drug development remains limited. While candidates like Debio1452 show promise [6], the future of drugs such as platensimycin and FabF inhibitors is uncertain [7]. With few new antibiotics being discovered, there is a critical need for novel antimicrobials for both prevention and treatment.

The study aims to develop innovative therapies for treating *S. aureus* infections while minimizing antibiotic usage and resistance. It has been previously observed in several studies that various ion channel blockers and antagonists used in animal research have potential inhibitory activities against bacteria [8]. Previous research has shown that certain tertiary amine-type local anesthetics (procaine, lidocaine) [9, 10] and nonsteroidal anti-

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inflammatory drugs (NSAIDs) like aspirin, ibuprofen, and diclofenac, as well as fenamates like mefenamic acid possess antibacterial properties against *S. aureus* [11]. Additionally, from our preliminary in silico studies, we have identified two such drugs, dibucaine (FDA approved, local anesthetics from the tertiary amine type) and niflumic acid (NSAID from the fenamate group), that could have potential antibacterial activity against *S. aureus*. These drugs enhance the therapeutic effects of antibiotics by reducing their minimum inhibitory concentration (MIC) when used in combination. The combinations also effectively inhibit the formations of biofilms. Consequently, these drugs, in combination with antibiotics, offer promising alternative therapeutics for controlling *S. aureus* infections.

Materials and methods

Determination of minimum inhibitory concentration (MIC) of the drugs and antibiotics

MICs were calculated for the selected drugs (dibucaine and niflumic acid) and all the antibiotics (ampicillin, streptomycin, tetracycline, kanamycin, and chloramphenicol) following Clinical & Laboratory Standard Institute (CLSI) guidelines [12] using a broth-microdilution experiment in Mueller Hinton Broth (MHB) on *S. aureus* (MCC 2408). To prevent any inhibitory action of drugs and antibiotics due to the DMSO, a control with a concentration of DMSO comparable to the final dose per well was also tested. The bacterial inoculum was adjusted to $\sim 5 \times 10^5$ CFU ml⁻¹ and inoculated into 96 well microtiter plates containing a two-fold serial dilution of the antibiotics or drugs and incubated at 37 °C overnight. For quantification purposes, a 630 nm absorbance wavelength was used to measure the optical densities of the bacterial growth in the wells [13].

Time-kill kinetics

Time-kill investigations were used to evaluate the antibacterial activity of dibucaine and niflumic acid against *S. aureus* (MCC 2408) [14]. The bacteria ($\sim 5 \times 10^5$ CFU ml⁻¹), were grown with MHB supplemented with the drugs at concentrations of 1X, 2X, and 4X their respective MIC value. The culture without treatment was included as a control. Only DMSO at the same concentration which was used along with drugs was taken as vehicle control (V-control). The bacterial treatment sets (maintained at an equal volume of 1 ml for each time point) were incubated at 37 °C and their growth was observed during 24 h. At various time intervals (1, 2, 4, 8, 12, and 24 h) 100 µl of the sample was spread out on MHA plates. Colonies were counted and CFU ml⁻¹ was evaluated after overnight incubation. The Time-kill kinetics was plotted as log₁₀ CFU ml⁻¹ against time.

Bactericidal activity was defined as a ≥ 3 log₁₀ cycle decrease in CFU ml⁻¹ (99.9% kill) or no re-growth persisted throughout the duration of the experiment [15].

Scanning electron microscopy to assess potential structural damage caused by drugs to bacterial cells

Mid-logarithmic phase bacteria were washed twice with and re-suspended in PBS. The cells were incubated at room temperature 37 °C, overnight with MICs of dibucaine and niflumic acid to determine the structural disruption effect on *S. aureus*. Following incubation, bacterial cells were fixed with 2.5% glutaraldehyde and dehydrated using a succession of ethanol treatments before being studied by a Hitachi S-3400 electron microscope [16].

Checkerboard assay

Checkerboard synergy analysis was used to investigate the synergistic action of the drugs (dibucaine and niflumic acid) with antibiotics. Antibiotics were placed into the wells of 96-well polystyrene tissue culture plates in 2-fold serial dilutions along the assay plate, and varying concentrations of dibucaine and niflumic acid were loaded across the assay plates along with the bacteria. Fractional Inhibitory Concentration Index (FICI) values were determined according to the following formula: FICI = FICI A + FICI B. Where FICI A = MIC of compound A in combination/MIC of compound A alone, FICI B = MIC of compound B in combination / MIC of compound B alone. The FICI value of ≤ 0.5 represents “Synergy,” FICI > 0.5–4 indicates “no interaction” and FICI > 4.0 represents an “antagonism” [17].

Biofilm inhibition assay

The experiment investigated biofilm formation using 96-well polystyrene microplates [18]. Initially, a standardized suspension of *S. aureus* at a concentration of 10^6 CFU ml⁻¹ was added to the plate containing Tryptic Soy Broth (TSB) supplemented with 1% glucose and placed into wells of a 96-well flat-bottom plate. Under static circumstances, the plate was incubated at 37 °C overnight. After incubation, the non-adherent planktonic cells were washed with PBS. Anti-biofilm efficacy was evaluated by introducing individual antibiotics or drugs at their MICs, both alone and in combination. Kanamycin and tetracycline were combined for dibucaine, while streptomycin and ampicillin were paired for niflumic acid, using best synergistic doses determined previously. The control group consisted of untreated cells. DMSO served as a V-control, and uninoculated Tryptic Soy Broth (TSB) supplemented with 1% glucose acted as the negative control. The microplate was then incubated at 37 °C for 24 h. After incubation, the wells

were washed three times with 1X PBS, fixed with 99% methanol for 15 min, and allowed to dry in a laminar air-flow. The adherent biofilm cells were stained with crystal violet (0.5%) for 5 min, washed with 1X PBS until the negative control wells (without biofilms) appeared colorless, and air-dried for 15 min. The crystal violet was solubilized with 33% acetic acid, and the biofilm quantified by measuring absorbance at 630 nm using a microtiter plate reader [19].

EtBr efflux assay

The technique reported by Markham et al. [20], was used to perform EtBr efflux experiments with *S. aureus*. Mid-logarithmic-phase *S. aureus* cells were cultured in MHB media and loaded with EtBr ($10 \mu\text{g ml}^{-1}$) in the presence of reserpine ($25 \mu\text{g ml}^{-1}$) to prevent efflux during the loading process. Cells were incubated at 37°C for 20 min before being centrifuged and pelleted. The medium was decanted and the cell pellet was resuspended in fresh MHB medium, either with or without the drugs (dibucaine and niflumic acid) to an optical density at 600 nm of 0.2. Bacteria with no drug treatment was taken as negative control and reserpine treated cells were taken as positive control only for comparison. The fluorescence of ethidium bromide was examined under a fluorescence microscope, and the intensity of the light was evaluated using ImageJ software [21]. Briefly, the fluorescence intensity of randomly selected five cells was calculated for a time interval of 5 min for a period of 30 min. The fluorescent intensities of the individual cells were calculated according to the following formula: Corrected total cell fluorescence (CTCF) = Integrated Density – (Area of selected cell \times Mean fluorescence of background readings).

Determination of cytocompatibility of drugs with MTT assay

Primary cell lines, ATCC RAW 264.7 [22] and ATCC C3H/10T1/2 Clone 8 [23] were obtained from ATCC. These primary cell lines were cultured in a 96-well microplate with DMEM/F-12 medium with 5% CO_2 for 24 h at 37°C . 10^5 cells per well were seeded to test the cytotoxic effects of dibucaine and niflumic acid for a concentration range of niflumic acid ($7.05\text{--}225.77 \text{ mg l}^{-1}$) and dibucaine ($8.58\text{--}274.77 \text{ mg l}^{-1}$) $25 \mu\text{M}$ to $800 \mu\text{M}$. After the cellular confluency was completed, the serially double-diluted drug solutions were applied to the surface, and they were then incubated for 48 h at 37°C . Thereafter, freshly prepared 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent was applied to each well and incubated for 4 h. Then, the formazan was solubilized in DMSO and the absorbance was recorded at 570 nm using a SpectraMax

i3x Multi-Mode Microplate Reader. The survivability (%) of the cells was calculated by employing the following formula: $(\text{OD treated well} [-\text{blank}] / (\text{mean OD control well} [-\text{blank}])) \times 100$ [24].

Statistical tests and graphical plots

All experiments are done with minimum three replicates. The statistical test One way ANOVA followed by multiple comparison test were performed. Significant difference between the data was considered when P values are < 0.05 . Graphical plots were generated in GraphPad Prism 9.0.2 (GraphPad Software, Inc., San Diego, CA).

Results

Identification of dibucaine and niflumic acid and their effect on the growth of *S. aureus*

Twenty-three ion channel blockers from different prokaryotic and eukaryotic studies were initially selected according to various literature surveys, with ten of them showing homologous targets in *S. aureus*. The sequences of the known target of these blockers were obtained from the Drugbank (<https://go.drugbank.com/drugs/>) and UniProt [25] databases. Blastp was then employed to identify homologous proteins in *S. aureus* with an identity of $\geq 30\%$ with an E value cut-off of ≤ 0.001 [26]. Dibucaine, chlorpromazine, and niflumic acid emerged as the top hits (Table S1). Chlorpromazine is a potential anti-staphylococcal medication [27]. Based on these initial data, we focused our further studies on dibucaine and niflumic acid which could be potential antimicrobials.

The MIC values for two distinct drugs (dibucaine and niflumic acid) were determined by the Clinical & Laboratory Standard Institute (CLSI) standards using Broth-microdilution assay in Mueller Hinton Broth. A broad range of MICs for the two drugs were observed from 200 mg l^{-1} for dibucaine and 1600 mg l^{-1} for niflumic acid.

Time-kill kinetics assay of dibucaine and niflumic acid on *S. aureus*

To evaluate if the drugs (dibucaine and niflumic acid) were bactericidal or bacteriostatic in nature, Time-kill kinetics was carried out. In the case of dibucaine, it was observed that it maintains a minimum $3 \log_{10}$ cycle decrease in growth from 4 h at its MIC and 1 h in the case of 2X and 4X of its MICs (Fig. 1a), which contributes to the bactericidal action of dibucaine. In the case of niflumic acid, $3 \log_{10}$ cycles decrease was observed from for doses of 2X and 4X the MIC starting from 12 and 8 h, respectively (Fig. 1b).

Hence, it can be inferred that niflumic acid exhibits concentration dependent bactericidal activity. As demonstrated in the Time-kill curve (Fig. 1), the time of bacterial death decreases with increasing concentration for both drugs, which means the growth inhibition of bacteria is also concentration-dependent.

Effect of dibucaine and niflumic acid on bacterial cell membrane

The experimental objective was to discern and elucidate the potential ramifications these pharmaceutical agents dibucaine and niflumic acid might impose upon *S. aureus*. The ensuing micrographs, as delineated in Fig. 2, conspicuously revealed distinctive alterations on the surface of *S. aureus* following exposure to dibucaine and niflumic acid, in stark contrast to the non-treated (control) counterparts. Specifically, dibucaine manifested explicit evidence of direct damage to the cell wall and membrane, resulting in cellular lysis, as highlighted by

discernible red boxes in Fig. 2b. Conversely, niflumic acid treatment elicited marked bacterial clumping, amalgamated membranes, constricted bacterial cells, and conspicuous indentations on the cell walls leading to cellular lysis demarcated by discernible yellow boxes in Fig. 2c. The untreated or control cells were denoted by green box in Fig. 2a. The comprehensive analysis conducted confirms that dibucaine and niflumic acid directly interact with bacterial cell walls and membranes, leading to their destabilization.

Synergistic activity of dibucaine and niflumic acid in combination of antibiotics against *S. aureus*

In order to understand the effect on combination of drugs and antibiotics against bacteria, Firstly, the MIC of five different antibiotics were determined. Ampicillin had the lowest MIC of 0.5 mg l^{-1} for the antibiotics, while kanamycin and chloramphenicol had the highest MIC of 8 mg l^{-1} . Streptomycin and tetracycline had the MIC of 2 mg l^{-1} .

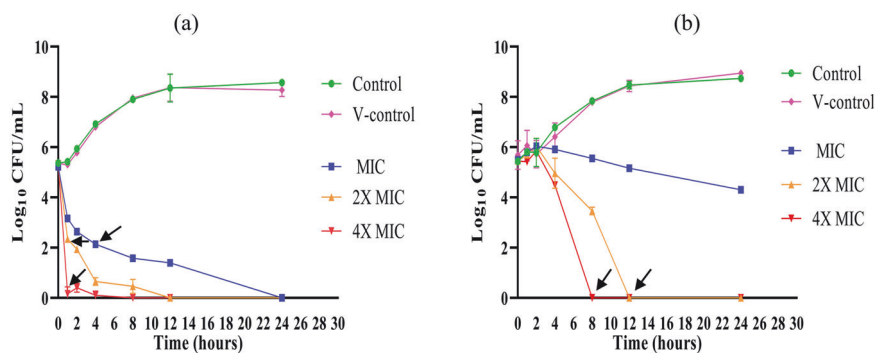


Fig. 1 Time-kill curves of *S. aureus* in the presence of dibucaine and niflumic acid. **a** Dibucaine exhibits a $3 \log_{10}$ decrease (black arrows) in growth compared to its minimum inhibitory concentration (MIC) after 4 h at the MIC, and within 1 h at 2X and 4X the MIC. **b** Niflumic acid demonstrates concentration dependent bactericidal activity, and

maintains a minimum $3 \log_{10}$ reduction in growth at 2X the MIC from 12 h, and at 4X the MIC from 8 h. The experiment included a growth control where no antimicrobial agents were added, as well as a control containing DMSO (V-control) without any antimicrobial agent to rule out any antibacterial activity associated with the solvent

Fig. 2 SEM images of *S. aureus* in the absence and presence of dibucaine and niflumic acid. **(a)** Untreated cells (Control) are indicated by the green box, **(b)** treated with dibucaine and damaged bacterial cells (cellular lysis) are indicated with red boxes, and, **(c)** treated with niflumic acid and bacterial cells (clumped cells with amalgamated membranes and indentations on the cell walls leading to cellular lysis) are indicated with yellow boxes

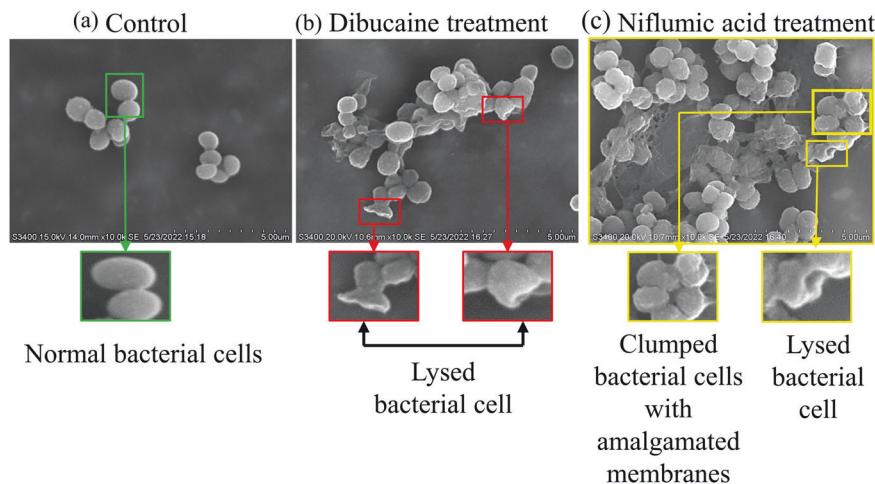


Table 1 MIC of different antibiotics in the presence of dibucaine and niflumic acid

Antibiotic (MIC mg/L)	Concentration of Drugs			MIC of antibiotics (mg l ⁻¹) in the presence of the drugs (FICI value)		Best combinations- Drugs (mg/L): Antibiotics (mg l ⁻¹) with fold reductions [X]	
	(μM)	(mg l ⁻¹)		Dibucaine	Niflumic acid	Dibucaine	Niflumic acid
		Dibucaine	Niflumic acid				
Ampicillin (0.5)	50	17.17	14.11	0.25 (0.58)	0.125 (0.26)	1.07:0.25 [2]	3.5:0.125 [4]
	25	8.58	7	0.25 (0.54)	0.125 (0.25)		
	12.5	4.29	3.5	0.25 (0.52)	0.125 (0.25)		
	6.25	2.14	1.75	0.25 (0.51)	0.25 (0.5)		
	3.125	1.07	0.88	0.25 (0.5)	0.25 (0.5)		
Streptomycin (2)	50	17.17	14.11	1 (0.58)	0.5 (0.26)	2.14:1 [2]	1.75:0.5 [4]
	25	8.58	7	1 (0.54)	0.5 (0.25)		
	12.5	4.29	3.5	1 (0.52)	0.5 (0.25)		
	6.25	2.14	1.75	1 (0.51)	0.5 (0.25)		
	3.125	1.07	0.88	2 (1)	1 (0.5)		
Kanamycin (8)	50	17.17	14.11	2 (0.33)	4 (0.5)	17.17:2 [4]	0.88:4 [2]
	25	8.58	7	4 (0.54)	4 (0.5)		
	12.5	4.29	3.5	4 (0.52)	4 (0.5)		
	6.25	2.14	1.75	4 (0.51)	4 (0.5)		
	3.125	1.07	0.88	4 (0.5)	4 (0.5)		
Tetracycline (2)	50	17.17	14.11	0.5 (0.33)	1 (0.5)	8.58:0.5 [4]	0.88:1 [2]
	25	8.58	7	0.5 (0.29)	1 (0.5)		
	12.5	4.29	3.5	1 (0.52)	1 (0.5)		
	6.25	2.14	1.75	1 (0.51)	1 (0.5)		
	3.125	1.07	0.88	1 (0.5)	1 (0.5)		
Chloramphenicol (8)	50	17.17	14.11	8 (1.08)	4 (0.5)	NA	0.88:4 [2]
	25	8.58	7	8 (1.04)	4 (0.5)		
	12.5	4.29	3.5	8 (1.02)	4 (0.5)		
	6.25	2.14	1.75	8 (1.01)	4 (0.5)		
	3.125	1.07	0.88	8 (1)	4 (0.5)		

NA not applicable

To investigate if there was any synergistic effect, different quantities (0.0325–8 mg l⁻¹) of antibiotics were mixed with various concentrations (3.125–50 μM) of the drugs (dibucaine and niflumic acid) but no more than 50 μM as higher concentrations were deemed as unreachable in the clinical setting owing to toxicity [28]. The FICI values indicated that tetracycline and kanamycin had the best synergism (FICI = 0.29 and 0.33) with dibucaine with a reduction in the MIC values of about 4 times. The most effective synergistic doses identified were tetracycline at 0.5 mg l⁻¹ combined with dibucaine at 8.58 mg l⁻¹ (25 μM), and kanamycin at 2 mg l⁻¹ paired with dibucaine at 17.17 mg l⁻¹ (50 μM). Dibucaine also showed considerable synergy with ampicillin with FICI values of 0.5 (Table 1)

and a reduction of MIC values by 2 times. No interactions were observed with streptomycin and chloramphenicol (Table 1).

Niflumic acid exhibited a synergistic interaction with each of the five antibiotics as detailed in Table 1. Particularly noteworthy were the most pronounced synergistic effects observed in conjunction with ampicillin and streptomycin, as evidenced by a fractional inhibitory concentration index (FICI) of 0.25. This synergy translated into a remarkable 4-fold reduction in the MIC values. The most effective synergistic doses identified were ampicillin at 0.125 mg l⁻¹ combined with niflumic acid at 3.5 mg l⁻¹ (12.5 μM), and streptomycin at 0.5 mg l⁻¹ paired with niflumic acid at 1.75 mg l⁻¹ (6.25 μM). Moreover, niflumic acid demonstrated a substantial

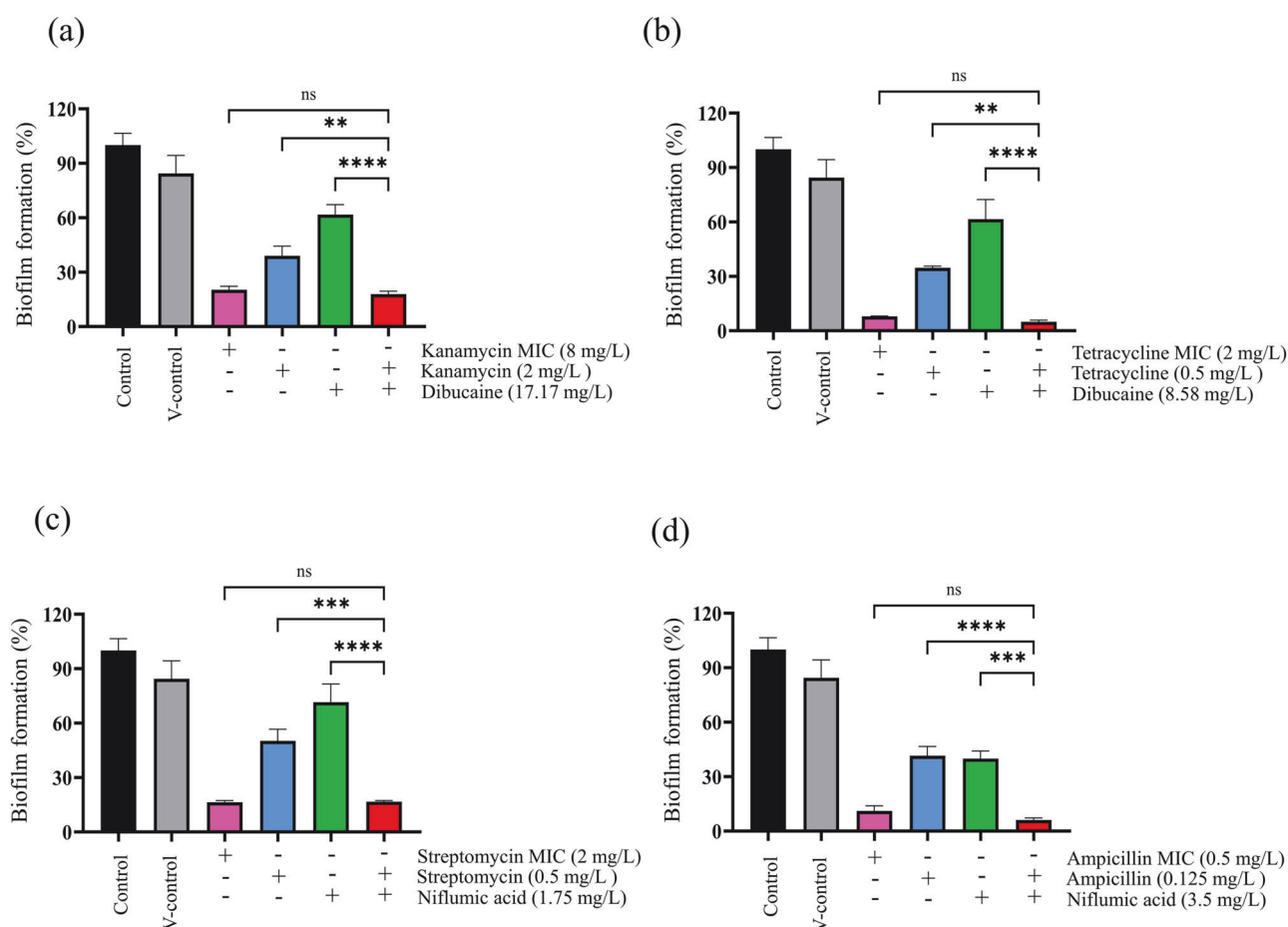


Fig. 3 Synergistic effect of *dibucaine* and *niflumic acid* in combination with antibiotics against formation of biofilm by *S. aureus*. Biofilms of *S. aureus* were treated with (a) kanamycin individually and in combination with dibucaine, (b) tetracycline individually and in combination with dibucaine (c) streptomycin individually and in combination with niflumic acid, and (d) ampicillin individually and in combination with niflumic acid. a–d the first bar graph (black) is control without treatment, the second bar (gray) is only DMSO (V-control) at the same concentration which was used along with

drugs, and the third bar (pink) is antibiotics at their respective MICs. The fourth (blue) and fifth (green) bar is antibiotics and drugs individually at their best synergistic doses. Sixth bar (red) is the combination of drugs along with respective antibiotics at their best synergistic doses. On biofilms, both the drugs were found to have synergistic effect in combination with respective antibiotics. The results were presented as the mean of SD. One way ANOVA was applied for statistical analysis. *P* values are as follows: ***P* value < 0.01, ****P* value < 0.001, *****P* value < 0.0001

degree of synergy with kanamycin, chloramphenicol, and tetracycline, as corroborated by FICI values of 0.5. This collaborative interaction resulted in a twofold reduction in the MIC values for each of these three antibiotics, thereby underscoring the potentiated antimicrobial effects achieved by the combined administration of niflumic acid with the aforementioned antibiotics.

Effect of the synergistic combination of antibiotics and drugs on biofilm formation

Bacterial biofilms are implicated in challenging-to-treat diseases, particularly *Staphylococcal* biofilms [29]. Therefore, we did investigate the impact of optimum synergistic combinations of drugs and antibiotics on biofilm formation by *S. aureus*. In order to generate the biofilm, induction was

performed followed crystal violet assay. Following a 24 h static incubation period, it was noticed that the formation of biofilm in the treatment groups was significantly decreased in comparison to the vehicle control (V-control). When biofilms were treated with combination of kanamycin (2 mg l^{-1}) and dibucaine (17.17 mg l^{-1}) at their best synergistic dosages (red bar, Fig. 3a), the formation of biofilms were significantly decreased compared to the treatment separately with identical concentration of kanamycin (2 mg l^{-1} , blue bar of Fig. 3a) and dibucaine (17.17 mg l^{-1} , green bar of Fig. 3a). Treatment with combination (red bar of Fig. 3a) found to have analogous effect with kanamycin at MIC (8 mg l^{-1} , pink bar of Fig. 3a). Similar trend of data was found in case of tetracycline and dibucaine (Fig. 3b), streptomycin and niflumic acid (Fig. 3c), and ampicillin and niflumic acid (Fig. 3d).

Respective antibiotics at their synergistic concentrations when in-combination with drugs, were found to be equally effective to their MICs, which are even four times of synergistic concentrations. Additionally, synergistic combinations yielded significantly higher reductions in biofilm formation compared to respective synergistic doses of antibiotics or drugs alone. These findings collectively indicate that dibucaine and niflumic acid acting synergistically with antibiotic pairs against biofilms.

Efflux activity of dibucaine and niflumic acid on *S. aureus*

In order to evaluate if dibucaine and niflumic acid could inhibit the efflux, we used EtBr efflux Assay. Bacteria with no treatment with drugs were taken as negative control (NC). To determine the drugs' efflux potency, a known efflux pump blocker, reserpine [30] was used, and treated cells with reserpine were taken as positive control (PC). Fluorescence microscopy images, of *S. aureus* treated with dibucaine and niflumic acid followed by EtBr efflux (frames captured and represented at a time interval of 5 min up to 30 min) under 1000X magnification was shown in Supplementary Figs. S1 and S2. In the investigation, the retention of EtBr from cells was tracked over time across treatments with drugs. Significant EtBr retention was decreased from 5 min for NC (Fig. S3 a), 15 min for PC (Fig. S3 b), 25 and 30 min for dibucaine at 50 and 100 μM (Fig. S3 c and d), and for 15 and 20 min for niflumic acid at 50 and 100 μM (Fig. S3 e and f).

Compounds if inhibit efflux, lead to more retention of EtBr and fluorescence in the treated cell compared to NC. Treatment with dibucaine (red and blue line, Fig. 4a) and niflumic acid (green and violet line, Fig. 4b) found to have efflux inhibition when compared to NC (gray line, Fig. 4a and b). Dibucaine seems found to have better efflux activity than reserpine, PC (black line, Fig. 4a). Whereas, niflumic acid found to have comparable efflux activity with PC (black line, Fig. 4b). As the fluorescence at 15 min (mid point of across the time) stayed steady [28], each treatment was compared (Fig. 4c and d) at this point for better understanding. Higher fluorescence means more dye retain in cells, indicating stronger efflux pump inhibition. Dibucaine (Fig. 4c) and niflumic acid (Fig. 4d) significantly blocked EtBr efflux compared to untreated cells (NC), while both showing similar inhibition capabilities compared to the PC, reserpine (Fig. 4c and d). At this 15 min midpoint there is no concentration dependent (50 and 100 μM) efflux inhibition for both the drugs (Fig. 4c and d). When observed up to 30 min, the significant retention of EtBr at 100 μM compared to 50 μM concentrations was found for both dibucaine (Fig. S3 c and d) and niflumic acid (Fig. S3 e and f). Dibucaine (Fig. S3 c and d) could able to retain the EtBr for more time

duration compared to niflumic acid (Fig. S3 e and f) in both the concentrations (50 and 100 μM). Thus, it can be inferred again that dibucaine might be better efflux inhibitor.

Cytocompatibility of dibucaine and niflumic acid

The effects of the dibucaine and niflumic acid on the cell viability of RAW 264.7 and C3H/10T1/2 Clone 8 cell lines were checked by MTT assay. MTT assay was conducted with a range of concentrations from 25 μM to 800 μM , niflumic acid (7.05–225.77 mg l^{-1}) and dibucaine (8.58–274.77 mg l^{-1}). In the study, dibucaine displayed notable cytotoxic effects starting from a concentration of 137.38 mg l^{-1} (400 μM), with a decrease in cell viability of 46.6% of C3H/10T1/2 Clone 8 at 274.77 mg l^{-1} (800 μM). On the other hand, niflumic acid did not exhibit a significant reduction in cell viability up to 225.77 mg l^{-1} (800 μM) when tested on the C3H/10T1/2 Clone 8 cell line compared to vehicle control (Fig. 5a and b). However, both drugs showed cytotoxicity from a concentration of 400 μM when tested on the RAW 264.7 cell line. At 800 μM , cell viability decreased by 71.5% for dibucaine and 66.5% for niflumic acid (Fig. 5c and d) respectively as compared to vehicle control. Within synergistic levels (not exceeding 50 μM), both the drugs were determined to be cyto-compatible or demonstrated no observable toxicity to the cell lines, indicating their safety profile. Our examination into cyto-compatibility revealed that both drugs are cyto-compatible up to 200 μM at the levels examined across murine cell lines which is in agreement with their safety profiles and thus can be employed in treatment practices with immediate effect.

Discussion

Staphylococcus aureus is a prominent pathogen with a demonstrated propensity to evolve resistance [31]. To overcome the occurrence of resistance in *S. aureus*, the development of novel anti-staphylococcal therapeutic strategies are urgently required [32]. The hunt for novel antimicrobials against *S. aureus* has yet to provide promising treatments [33]. When it comes to new antimicrobials with unique modes of action, our study uncovers two prospective antibacterial agents, dibucaine and niflumic acid, against *S. aureus*.

We found the MIC of compounds were 200 mg l^{-1} for dibucaine and 1600 mg l^{-1} for niflumic acid. Similar range of MIC values for other similar compounds were reported in various studies [34–36]. Although MIC of compounds are appeared high, our plan involves employing these drugs alongside antibiotics with a maximum intended dose of 50 μM , that is many times lesser than the MIC the compounds. Surpassing 100 μM were considered unachievable

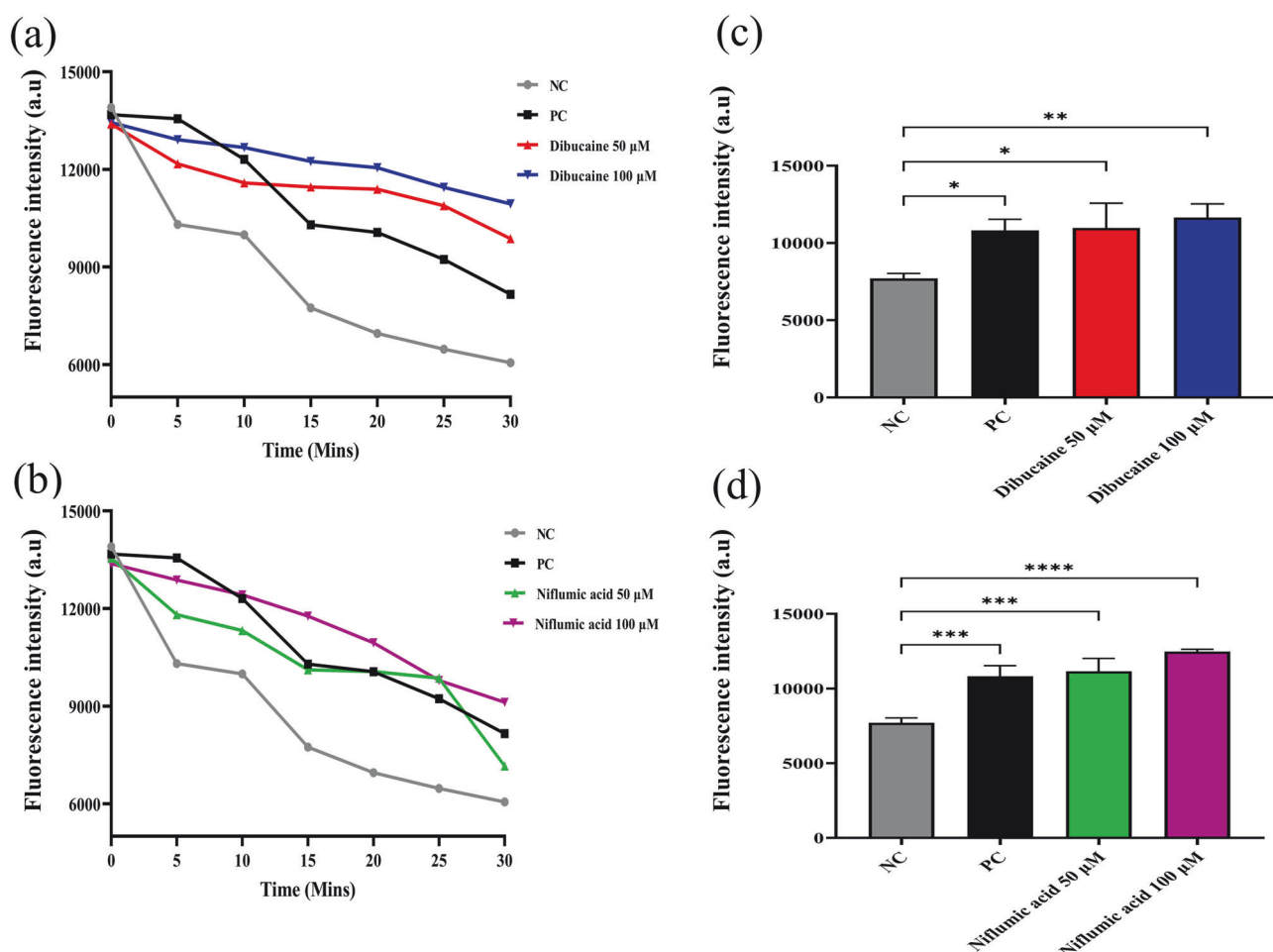


Fig. 4 Effect of dibucaine and niflumic acid on EtBr efflux potential on *S. aureus*. EtBr retention potentials in the cells due to efflux activity of (a) Dibucaine at concentration level of 17.17 mg l^{-1} or $50 \mu\text{M}$ (red line) and 34.34 mg l^{-1} or $100 \mu\text{M}$ (blue line); (b) Niflumic acid at 14.11 mg l^{-1} or $50 \mu\text{M}$ (green line) and 28.22 mg l^{-1} or $100 \mu\text{M}$ (violet line); untreated control (NC, gray line) and the reserpine as positive control (PC, black line). The data points are plotted at a time

interval of 5 min. The residual fluorescence of the drugs at mid-time point of 15 min is shown for (c) dibucaine (50 and $100 \mu\text{M}$) and (d) niflumic acid (50 and $100 \mu\text{M}$). Both the drug is showing significant efflux inhibition activity. The results were presented as the mean of the SD. One way ANOVA was applied for statistical analysis. P values are as follows: * P value < 0.05 , ** P value < 0.01 , *** P value < 0.001 , **** P value < 0.0001

in the clinic [28]. The Time-kill investigations have offered useful information on the possible action of antibacterial drugs in vitro and demonstrated that dibucaine and niflumic acids both rendered bactericidal activities (Fig. 1). Studies using SEM showed that both drugs do damage at cell wall and membrane (Fig. 2). Synergistic drug combinations are important for rapid pathogen clearance, enhancing the antimicrobial spectrum, and preventing the development of resistance, and dose-related toxicity in individuals [37]. Interestingly, each drug in combination with antibiotics, significantly enhanced the activity of antibiotics by lowering their MICs between 2–4 times (Table 1). For instance, the most effective combination observed is tetracycline and kanamycin with dibucaine, and ampicillin and streptomycin with niflumic acid, with concentrations with 4 times reduction of MIC of antibiotics. These results showed that these combinations have potentials to minimize the usage of

antibiotics which may be clinically beneficial for reducing the occurrence of antibiotic resistance.

Numerous illnesses that are challenging to cure are linked to bacterial biofilms. In therapeutic settings, *Staphylococcal* biofilms are extremely difficult to treat [29]. Both the drugs found to have acted synergistically with respective antibiotics (Fig. 3). The synergistic combinations of antibiotics and drugs demonstrated equal effectiveness to inhibit the formation of biofilms in comparison with the MICs of antibiotics (Fig. 3). This suggest that as the combination contains 4 times lesser quantities of antibiotics, it will hopefully be beneficial for therapy with possible reduction in occurrence of antibiotic resistance. This finding reinforces our assertion that the two drugs are highly effective in inhibiting the growth of *S. aureus* both in planktonic form and biofilms at synergistic combinations.

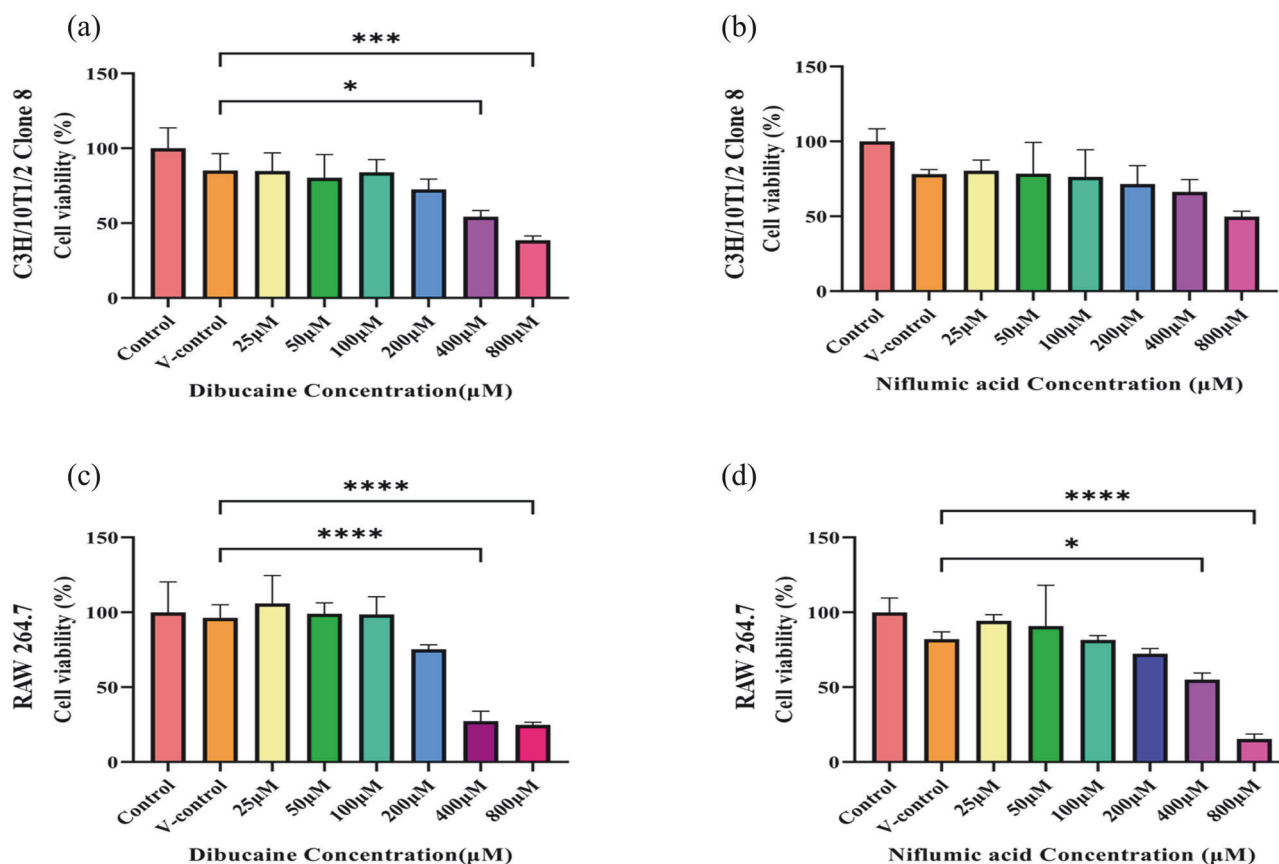


Fig. 5 In vitro cell cytotoxicity assay for dibucaine and niflumic acid in mice C3H/10T1/2 Clone 8 and RAW 264.7 cell lines. **a** Dibucaine induced cytotoxicity in C3H/10T1/2 Clone 8 cells from 400 μM, **(b)** Conversely, niflumic acid did not induce cytotoxicity in C3H/10T1/2 Clone 8 cells up to 800 μM, **(c)** RAW 264.7 cells exhibited cytotoxic effects starting from 400 μM with dibucaine treatment, **(d)** Similarly, RAW 264.7 cells displayed cytotoxic effects from 400 μM with

niflumic acid treatment. The experiment also included untreated Control samples and a control containing DMSO (V-control) at the same concentration used with the drugs. The results were presented as the mean of SD. One way ANOVA was applied for statistical analysis. *P* values are as follows: **P* value < 0.05, ****P* value < 0.001, *****P* value < 0.0001

Efflux pump blockers have been shown to dramatically diminish preformed biofilms in the past [38]. This encouraged us to hypothesize that the two drugs might have potentials to inhibit efflux pump in bacteria. Efflux mechanisms play a crucial role in bacteria developing antibiotic resistance. Thus, antibacterial drugs that possess anti-efflux properties offer a potential advantage in effectively managing infections. On experiment with EtBr, both drugs retained the EtBr in the bacterial cell significantly with the NC and comparable with the PC, reserpine (Fig. 4). The drugs were identified as potential efflux pump inhibitors in the bacteria at relevant effective concentration of 50 μM. This efflux activity of the drugs found to be concentration dependant. Then it can be possible that drugs are helping the antibiotics to remain in the cells for longer duration results into effective action. Consequently, this also possible that, the stress imposed could potentially prompt the bacteria to initiate a resistance response against these drugs via mutation of their efflux pumps to modify the target sites of these proposed drugs.

Dibucaine has previously received FDA approval, now mostly utilized for superficial sedation [39]. Niflumic acid is an investigational medication, not currently approved by FDA but approved in India (<https://www.apifirst.in/2023/02/22/complete-list-of-cdsc-approved-drugs-in-India/>) and many other countries. It is used for the treatment of both acute and chronic inflammatory conditions, including osteoarthritis and rheumatoid arthritis [40]. Dibucaine as a topical anesthetic in ointment form is currently in use [41]. While dibucaine may not be significant for systemic use, its combination with certain antimicrobial agents in topical formulations can enhance the overall effectiveness of treatment. For example, dibucaine-containing ointments can be used in conjunction with antibiotics to address skin infections or surgical wounds, providing both pain relief and aiding in infection control. In our study, both drugs are found to be cytocompatible at the synergistic doses tested in murine cell lines, which is in agreement with their safety (Fig. 5). Dibucaine, a local anesthetic, is primarily utilized for surface anesthesia [42]. While it's often employed for

spinal and lumbar anesthesia, clinical concentrations for this purpose typically range from 0.3 to 0.75% (7.9–19.7 mM) [43]. However, our obtained concentrations for antibacterial effects were much lower, peaking at 50 μM . In vitro studies have also indicated mild cytotoxicity of dibucaine, with concentrations above $\text{CC}_{50} > 229.7 \mu\text{M}$ in normal human skin keratinocytes (HEKa, HEKn) [43], consistent with our findings. Our study suggests that dibucaine could be potentially useful as a topical solution for antimicrobial purposes in clinical settings. On the other hand, patients administered niflumic acid at a safe dosage of 10 mg kg^{-1} reached an average plasma concentration of $59.5 \pm 13.3 \text{ mg l}^{-1}$ ($\sim 210 \mu\text{M}$) after 4 h [44]. Our study found that the maximum effective synergistic dose for antibacterial action of niflumic acid was 3.5 mg l^{-1} (12.5 μM) in combination of antibiotics, was roughly 17 times lower. In another in vitro study, niflumic acid displayed modest cytotoxicity, with an $\text{IC}_{50} > 250 \mu\text{M}$ in normal mouse fibroblast 3T3-L1 cell lines [45], consistent with our findings. These results suggest that concentrations of niflumic acid required for antibacterial effects are likely to be safely attainable both in vitro and in vivo.

Conclusion

The drift of multidrug resistance is predominantly alarming for *S. aureus* because of the severe and diverse infections posed by this adaptable pathogen. While potent anti-staphylococcal medicines and vaccines are being developed, innovative ways to therapy and prevention will become increasingly crucial in combating the current situation. In an effort to advance antibacterial drug discovery and development, our research newly identified two drugs, dibucaine and niflumic acid as promising adjuvant which act synergistically with antibiotics against *S. aureus*. The best synergy of dibucaine was found to have with kanamycin and tetracycline, whereas niflumic acid was with streptomycin and ampicillin. Further research is needed to validate their effectiveness against other pathogens and their role as antibiotic potentiators.

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Author contributions HS and JC conceived the idea and designed the workflow. JC performed the experiments. HS and JC analysed the data and prepared the draft manuscript. AM helps in data analysis and

drafting the manuscript. JS and SB performed the MTT assay. AM and RM helped to acquire the ELISA data and analysis. HS, JC, AM, and SB reviewed and edited the manuscript. HS supervised the work.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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