

In Vitro Growth-Inhibitory Activity of Non Antibiotic Agents Toward Methicillin-Resistant *S. Aureus* and Esbl-Producing *Escherichia Coli*

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Abstract

Antibiotic resistance has become a major global health concern, particularly due to the increasing prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*. The exploration of non-antibiotic agents with antibacterial properties offers a potential alternative approach for addressing resistant bacterial infections. This study aimed to evaluate the antibacterial activity of N-acetylcysteine (NAC), ascorbic acid (vitamin C), and diclofenac against MRSA and ESBL-producing *E. coli*. This experimental laboratory study employed a randomized post-test-only control group design. Clinical isolates of MRSA and ESBL-producing *E. coli* were evaluated. The bacterial strains were treated with various concentrations (100%, 50%, 25%, 12.5%, and 6.25%) of NAC (initial concentration: 200 mg/mL), vitamin C (initial concentration: 200 mg/mL), and sodium diclofenac (initial concentration: 25 mg/mL). Antibacterial activity was assessed using the microdilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Diclofenac demonstrated the strongest antibacterial activity, with MIC and MBC values of 3.125 mg/dL against MRSA and 6.25 mg/dL against ESBL-producing *E. coli*. NAC showed moderate antibacterial activity, exhibiting bactericidal effects against *E. coli* (MIC/MBC: 100 mg/dL) but limited activity against MRSA (MIC: 200 mg/dL; MBC: >200 mg/dL). Vitamin C demonstrated the weakest antibacterial activity, showing bactericidal effects only against *E. coli* at 200 mg/dL and no bactericidal effect against MRSA. NAC, vitamin C, and sodium diclofenac demonstrated antibacterial activity against MRSA and ESBL-producing *E. coli*. Among the three tested agents, sodium diclofenac exhibited the strongest antibacterial activity, suggesting its potential role as an adjunctive agent in future investigations of strategies for managing resistant bacterial infections.



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INTRODUCTION

Antibiotic resistance is one of the major global public health challenges. In 2013, the CDC published *Antibiotic Resistance Threats in the United States*, reporting that at least 2 million people in the United States are infected with antibiotic-resistant bacteria

each year, resulting in at least 23,000 deaths (CDC, 2019). Infections caused by antibiotic-resistant bacteria have been associated with approximately 700,000 deaths annually worldwide and were projected to exceed 10 million deaths per year by 2020 (Garbacz et al., 2023). More recent estimates indicate that bacterial antimicrobial resistance was directly associated with more than one million deaths globally in 2021, with *Escherichia coli* and *Staphylococcus aureus* among the leading contributing pathogens. Community carriage of ESBL-producing *E. coli* has also increased worldwide, with a pooled global prevalence exceeding 20% (Beaumont et al., 2026; Bezabih et al., 2022; Ling, 2024; Mandujano-Hernández et al., 2024; Ng et al., 2025; Olaitan et al., 2025; Raffelsberger et al., 2023; Ramatla et al., 2023).

Antibiotic resistance enables bacteria to survive exposure to antimicrobial agents despite their susceptibility profiles under standard microbiological testing conditions (Abbas et al., 2024; Chiş et al., 2022; Gajic et al., 2022; Garcia-Maset et al., 2025; Urban-Chmiel et al., 2022; Zhu et al., 2022). To address antibiotic resistance, understanding the mechanisms underlying bacterial tolerance and resistance is essential. Antibiotic resistance is commonly assessed through the determination of the minimum inhibitory concentration (MIC), which represents the lowest concentration of an antimicrobial agent required to inhibit visible bacterial growth. Resistance mechanisms may occur through several pathways. Intrinsic resistance may arise from naturally occurring bacterial characteristics, including the absence or modification of antibiotic targets. Meanwhile, acquired resistance may develop through chromosomal mutations or horizontal gene transfer mediated by mobile genetic elements, including plasmids. These molecular mechanisms are generally categorized into three major groups: reduced intracellular antibiotic concentration, modification of antibiotic targets, and antibiotic inactivation (Ahmed et al., 2023; Bai et al., 2024; Darby et al., 2023; Haddad et al., 2024).

Recently, increasing resistance among bacterial pathogens to commonly used antibacterial agents has encouraged interest in non-antibiotic drugs with pharmacological properties that may exhibit antibacterial activity. Drug-repurposing approaches have identified antibacterial potential in several non-antibiotic compounds, including the antihypertensive agent hydralazine, which has been evaluated through *in vitro* and *in silico* approaches. This growing body of evidence supports the evaluation of widely available non-antibiotic compounds, such as *N*-acetylcysteine, ascorbic acid (vitamin C), and sodium diclofenac, for their potential growth-inhibitory activity against clinically important antibiotic-resistant pathogens.

N-Acetylcysteine (NAC) is a mucolytic agent that has also been reported to exhibit direct antibacterial activity against several bacterial species. Its bactericidal mechanisms may involve interference with bacterial cysteine metabolism or interactions with bacterial proteins through its sulfhydryl group.

Ascorbic acid (vitamin C) has also been investigated as a potential antibacterial agent, with several studies demonstrating its inhibitory effects against various microorganisms. Previous studies have reported antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* (6), *Helicobacter pylori* and *Campylobacter jejuni* (Zhang et al., 1997), *Mycobacterium tuberculosis* (8), and fungi such as *Aspergillus* species (Gupta & Guha, 1941). The antimicrobial effects of vitamin C were initially hypothesized to result from its ability to reduce environmental pH. However, subsequent studies have demonstrated antimicrobial activity against *Streptococcus pyogenes* even under neutral pH conditions.

Another important finding is that several nonsteroidal anti-inflammatory drugs (NSAIDs) have demonstrated antibacterial activity independent of their conventional anti-inflammatory mechanisms. NSAIDs are widely used for pain management and inflammation control, and several studies have reported their antibacterial effects. Among these agents, sodium diclofenac has received considerable attention due to its reported antimicrobial properties. Sodium diclofenac is primarily recognized as an anti-inflammatory, analgesic, and antipyretic agent. Beyond these established pharmacological effects, other NSAIDs have also demonstrated direct antibacterial properties; for example, aspirin has been reported to inhibit the growth of *Helicobacter pylori* and increase its susceptibility to antimicrobial agents. These findings suggest that antibacterial activity may represent a potential class effect among certain NSAIDs, including sodium diclofenac.

The urgency of this research is highlighted by the increasing global burden of antibiotic resistance and the limited therapeutic options available for infections caused by MRSA and ESBL-producing *E. coli*. The development of new antibiotics requires substantial time and financial resources, while resistance may emerge rapidly following their introduction. Drug repurposing of non-antibiotic agents offers a potential alternative strategy that may be explored more efficiently. This study provides novelty by directly comparing three widely available non-antibiotic agents—NAC, vitamin C, and sodium diclofenac—against clinical isolates of MRSA and ESBL-producing *E. coli* and by evaluating their relative antibacterial activity. The findings are expected to contribute to the expanding evidence base on drug repurposing and support further investigation into the potential clinical applications of these agents.

In this study, the *in vitro* growth-inhibitory activity of three non-antibiotic agents—*N*-acetylcysteine, ascorbic acid (vitamin C), and sodium diclofenac—was evaluated against methicillin-resistant *Staphylococcus aureus* (MRSA) and ESBL-producing *Escherichia coli*. This study aimed to identify potential non-antibiotic candidates for further investigation as adjunctive antibacterial agents in the management of resistant bacterial infections.

METHOD

This laboratory experimental study evaluated the antibacterial potential of *N*-acetylcysteine (NAC), sodium diclofenac, and vitamin C against methicillin-resistant *Staphylococcus aureus* (MRSA) and ESBL-producing *Escherichia coli*. The study was conducted from December 2024 to March 2025 at the Clinical Microbiology Laboratory of Universitas Airlangga.

The study employed a randomized post-test-only control group design. The samples were randomly assigned into treatment and control groups. The treatment group received NAC, sodium diclofenac, and vitamin C at various concentrations, while the control group received no treatment and served as a comparison reference. The antibacterial effects of the tested agents against MRSA and ESBL-producing *E. coli* were evaluated. Clinical isolates were obtained from the Microbiology Laboratory of Universitas Airlangga. The primary outcomes measured in this study were the minimum inhibitory concentration (MIC) and antibacterial activity determined through testing using a 96-well microplate reader.

The treatment design for antibacterial testing was as follows:

- a) A1: Bacterial suspension without addition of NAC, vitamin C, or diclofenac (positive control)

- b) A2: Solution of NAC / vitamin C / diclofenac at 100% concentration (negative control)
- c) A3: Bacterial suspension + NAC / vitamin C / diclofenac at 100% concentration
- d) A4: Bacterial suspension + NAC / vitamin C / diclofenac at 50% concentration
- e) A5: Bacterial suspension + NAC / vitamin C / diclofenac at 25% concentration
- f) A6: Bacterial suspension + NAC / vitamin C / diclofenac at 12.5% concentration
- g) A7: Bacterial suspension + NAC / vitamin C / diclofenac at 6.25% concentration

The 100% concentrations used in this study consisted of NAC (200 mg/mL), ascorbic acid (200 mg/mL), and sodium diclofenac (25 mg/mL). Each experimental group was performed with at least four replications. Bacterial isolates were cultured, and a suspension was prepared using Mueller-Hinton Broth (MHB). Colonies from the isolates were inoculated into MHB and homogenized using a vortex mixer. The bacterial suspension was adjusted to a 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL, to ensure a standardized bacterial concentration before testing.

The antibacterial activity test was conducted using the broth dilution method to determine the minimum inhibitory concentration (MIC) based on the procedure described by Schwalbe et al. (2007), with modifications according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS, 2003). Serial dilutions were prepared from the stock solutions in Mueller-Hinton Broth (MHB), with each tube adjusted to a final volume of 2 mL. Bacterial suspensions were added to each tube and incubated at 35°C for 24 hours. A tube without bacterial inoculation was used as a negative control.

The MIC was determined as the lowest concentration that inhibited visible bacterial growth after incubation, based on visual observation by three independent observers. The minimum bactericidal concentration (MBC) was determined by subculturing samples from tubes without visible growth onto Mueller-Hinton Agar (MHA). The MBC was defined as the lowest concentration that resulted in no bacterial colony growth after 24 hours of incubation.

RESULTS AND DISCUSSION

Results

Antibacterial activity was evaluated in two bacterial species, ESBL-producing *Escherichia coli* and MRSA, following treatment with N-acetylcysteine, sodium diclofenac, and vitamin C. In ESBL-producing *Escherichia coli*, NAC solution showed clear results at concentrations of 100% and 50%, but became turbid starting from 25% to 6.25%. In contrast, in MRSA, clarity was only observed at 100% concentration, while concentrations from 50% to 6.25% showed turbidity. Based on MIC results, the effective concentration of NAC against ESBL-producing *Escherichia coli* was 100 mg/dL, which also represented its MBC value. In contrast, for MRSA, the MIC was 200 mg/dL, but bactericidal activity was not achieved at this concentration, with MBC >200 mg/dL (Table 1).

Table 1. Antibacterial activity of N-acetylcysteine

Bacteria	Findings					MIC (mg/dL)	MBC (mg/dL)
	100% (200 mg/dL)	50% (100 mg/dL)	25% (50 mg/dL)	12.5% (25 mg/dL)	6.25% (12.5 mg/dL)		
E. coli ESBL	Clear	Turbid	Turbid	Turbid	Turbid	200	200
MRSA	Clear	Turbid	Turbid	Turbid	Turbid	200	>200

In the sodium diclofenac treatment, the solution showed clarity at almost all concentrations against ESBL-producing *Escherichia coli*, except at concentrations of 12.5% and 6.25%, which showed turbidity. In MRSA, clarity was observed at concentrations of 100%, 50%, and 25%, while at 12.5% it remained clear and at 6.25% it became turbid. The MIC and MBC values against ESBL-producing *Escherichia coli* were both 6.25 mg/dL. Meanwhile, for MRSA, both MIC and MBC values were 3.125 mg/dL (Table 2).

Table 2. Antibacterial activity of sodium diclofenac

Bacteria	Findings					MIC (mg/dL)	MBC (mg/dL)
	100% (25 mg/dL)	50% (12.5 mg/dL)	25% (6.25 mg/dL)	12.5% (3.125 mg/dL)	6.25% (1.5625 mg/dL)		
E. coli ESBL	Clear	Clear	Clear	Turbid	Turbid	6.25	6.25
MRSA	Clear	Clear	Clear	Clear	Turbid	3.125	3.125

In ESBL-producing *Escherichia coli*, clarity was only observed at the 100% concentration, while other concentrations showed turbidity. A similar pattern was observed in MRSA, where only the highest concentration (100%) showed clarity, while all other concentrations were turbid. The MIC and MBC results showed that for ESBL-producing *Escherichia coli*, vitamin C exhibited bactericidal activity at 200 mg/dL. However, for MRSA, although the MIC value was the same (200 mg/dL), bactericidal activity was not achieved at this concentration (MBC >200 mg/dL) (Table 3).

Table 3. Antibacterial activity of vitamin C

Bacteria	Findings					MIC (mg/dL)	MBC (mg/dL)
	100% (200 mg/dL)	50% (100 mg/dL)	25% (50 mg/dL)	12.5% (25 mg/dL)	6.25% (12.5 mg/dL)		
E. coli ESBL	Clear	Turbid	Turbid	Turbid	Turbid	200	200
MRSA	Clear	Turbid	Turbid	Turbid	Turbid	200	>200

Taken together, sodium diclofenac consistently showed the lowest MIC and MBC values against both organisms (MRSA: 3.125 mg/dL; ESBL-producing *E. coli*: 6.25 mg/dL), followed by N-acetylcysteine, which was bactericidal against ESBL-producing *E. coli* (100 mg/dL) but only bacteriostatic against MRSA (MIC 200 mg/dL; MBC >200 mg/dL), while vitamin C showed the weakest activity, exhibiting bactericidal effect against ESBL-producing *E. coli* only at the highest tested concentration (200 mg/dL) and no bactericidal effect against MRSA. Overall, this indicates a rank order of antibacterial potency of sodium diclofenac > N-acetylcysteine > vitamin C, with MRSA generally requiring higher concentrations than ESBL-producing *E. coli* to achieve an equivalent bactericidal effect.

Discussion

This study demonstrated that sodium diclofenac, N-acetylcysteine (NAC), and vitamin C each possess antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) and ESBL-producing *Escherichia coli*, albeit with markedly different potency. Sodium diclofenac exhibited the strongest and most consistent activity, with MIC and MBC values of 3.125 mg/dL against MRSA and 6.25 mg/dL against ESBL-producing *E. coli*. NAC showed intermediate, organism-dependent activity, being bactericidal against *E. coli* at 100 mg/dL but only bacteriostatic against MRSA even at the highest tested concentration (200 mg/dL). Vitamin C demonstrated the weakest activity among the three agents, with a bactericidal effect against *E. coli* observed only at 200 mg/dL and no bactericidal effect against MRSA within the tested concentration range.

The potent and consistent antibacterial activity of sodium diclofenac observed in this study is in line with a growing body of evidence that several NSAIDs possess intrinsic antibacterial properties independent of their classical anti-inflammatory, cyclooxygenase-inhibitory action. A comparable phenomenon has been reported for aspirin, which inhibits the growth of *Helicobacter pylori* and enhances its susceptibility to antimicrobial agents.¹⁰ Mechanistic studies have shown that the antibacterial action of diclofenac is attributable, at least in part, to inhibition of bacterial DNA synthesis, a mechanism distinct from its cyclooxygenase-inhibitory anti-inflammatory activity.¹³ Notably, diclofenac was the only agent in this study that achieved a lower MIC/MBC against MRSA than against ESBL-producing *E. coli*, in contrast to NAC and vitamin C, which were both less effective against MRSA. This finding suggests that diclofenac's mechanism of action may differ fundamentally from that of NAC and vitamin C, and may be less dependent on the structural differences between the gram-positive cell wall of *S. aureus* and the outer membrane of gram-negative *E. coli*.

In contrast, NAC showed an organism-dependent pattern, with a favorable bactericidal effect against ESBL-producing *E. coli* but only limited, bacteriostatic activity against MRSA. This is consistent with the proposed mechanism of NAC, which acts through its sulfhydryl group via competitive inhibition of cysteine utilization or direct interaction with bacterial cell proteins.⁴ The relatively thick peptidoglycan layer and lower outer membrane permeability of gram-positive *S. aureus*, compared with the thinner peptidoglycan and additional outer membrane of gram-negative *E. coli*, may partly explain the reduced susceptibility of MRSA to NAC observed in this study. This organism-dependent pattern suggests that NAC may be more suitable as an adjunct agent against gram-negative resistant pathogens such as ESBL-producing *E. coli* than as a standalone agent against MRSA. Comparable organism-dependent potentiating effects of NAC have also been reported when combined with beta-lactam antibiotics against carbapenem-resistant gram-negative pathogens such as *Klebsiella pneumoniae* and *Acinetobacter baumannii*.¹⁴

Vitamin C exhibited the weakest antibacterial activity among the three agents tested, requiring the highest concentration (200 mg/dL) to achieve a bactericidal effect against ESBL-producing *E. coli*, and showing no bactericidal effect against MRSA at any tested concentration. This finding is consistent with previous reports describing antibacterial effects of vitamin C against several bacterial species, including *Staphylococcus aureus* and *Enterococcus faecalis*,⁶ *Helicobacter pylori* and *Campylobacter jejuni*,⁷ and *Mycobacterium tuberculosis*,^{8,15} generally at relatively high concentrations. The comparatively modest activity of vitamin C observed in this study

may reflect differences in bacterial strain, inoculum size, and testing conditions compared with previous studies, and further supports its potential role as a complementary rather than primary antibacterial agent.

From a clinical perspective, these findings suggest that sodium diclofenac — a widely available, inexpensive, and already clinically approved drug — may hold particular promise as an adjunct or alternative antibacterial agent against both gram-positive and gram-negative antibiotic-resistant pathogens, warranting further investigation. NAC, given its favorable activity against ESBL-producing *E. coli*, may be more relevant for gram-negative resistant infections, whereas the role of vitamin C is likely best positioned as a supportive rather than a primary antibacterial agent.

This study has several strengths, including its use of clinical isolates of MRSA and ESBL-producing *E. coli* rather than reference laboratory strains, and its direct comparison of three non-antibiotic agents under standardized experimental conditions. However, several limitations should be acknowledged. First, this study was conducted using a single clinical isolate of each organism from one laboratory, which may limit the generalizability of the findings; testing across multiple clinical isolates would strengthen the external validity of these results. Second, the underlying molecular mechanisms of the observed antibacterial activity were not directly examined. Third, this study evaluated the agents individually; potential synergistic or antagonistic interactions between NAC, vitamin C, diclofenac, and conventional antibiotics were not assessed, despite their potential relevance for combination therapy. Finally, as an *in vitro* study, these findings require confirmation through *in vivo* and, ultimately, clinical studies before any of these agents can be considered for clinical application as adjunct or alternative antibacterial therapy.

CONCLUSION

N-Acetylcysteine (NAC), vitamin C, and sodium diclofenac demonstrated antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and ESBL-producing *Escherichia coli*, with sodium diclofenac exhibiting the strongest and most consistent activity against both bacterial species. These findings indicate the potential antibacterial properties of sodium diclofenac and suggest that it may be further investigated as an adjunctive agent in strategies for managing infections caused by resistant pathogens. NAC may have particular potential against ESBL-producing *E. coli*. Further studies involving multiple clinical isolates, mechanistic investigations, and evaluations of combination therapy with conventional antibiotics are required before these non-antibiotic agents can be considered for potential clinical application.

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