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Combined Antibacterial Effect of *Azadirachta indica* (Neem) Leaf Extract and Clindamycin Against *Staphylococcus aureus*

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Abstract

Antimicrobial resistance in *Staphylococcus aureus*, including resistance to commonly used agents such as clindamycin, continues to challenge infection management and has encouraged investigation into plant-derived antimicrobial adjuncts. This study evaluated the individual and combined antibacterial activity of 70% ethanolic *Azadirachta indica* (neem) leaf extract and clindamycin against *S. aureus* ATCC 25923. A laboratory-based experimental design was employed using qualitative phytochemical screening, broth microdilution, and checkerboard assay across nine independent trials conducted over three experimental days. Phytochemical screening confirmed the presence of saponins, flavonoids, tannins, and phenols in the neem leaf extract. The individual minimum inhibitory concentration of neem extract showed a modal value of 25 mg/mL, with observed values ranging from 12.5 to 25 mg/mL. The individual MIC of clindamycin was not determined within the tested range of 0.125-2 µg/mL, as bacterial growth persisted across all trials. In the checkerboard assay, the neem extract-clindamycin combination inhibited bacterial growth at sub-MIC concentrations of both agents. The fractional inhibitory concentration index ranged from 0.50 to 0.75, with a mean of 0.64 ± 0.14, indicating an additive antibacterial interaction, with some boundary-level evidence of potential synergy. These findings suggest that neem leaf extract may enhance clindamycin activity against *S. aureus* under in vitro conditions. Further studies using confirmed resistant and susceptible strains, expanded concentration ranges, and molecular validation are recommended.

Keywords: *Azadirachta indica; antimicrobial resistance; checkerboard assay; clindamycin; fractional inhibitory concentration index; Staphylococcus aureus*

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1. Introduction

Antimicrobial resistance remains a major public health concern, particularly in bacterial pathogens that have developed reduced susceptibility to commonly used therapeutic agents. Among these pathogens, Staphylococcus aureus is clinically important because of its capacity to cause a wide spectrum of infections, ranging from superficial skin and soft tissue infections to severe conditions such as pneumonia, bacteremia, septic shock, osteomyelitis, and endocarditis (Guo et al., 2020; Touaitia et al., 2025). Its ability to persist in human skin and nasal mucosa, combined with its virulence factors and adaptive resistance mechanisms, makes it a continuing challenge in clinical microbiology, infection control, and antimicrobial therapy (Bustos-Martínez et al., 2022).

The problem is intensified by the persistence of methicillin-resistant *Staphylococcus aureus* (MRSA), which remains a significant global and local health threat. Methicillin resistance is primarily associated with the staphylococcal cassette chromosome mec, which carries the *mecA* gene responsible for encoding penicillin-binding protein 2a, a protein with reduced affinity for beta-lactam antibiotics (Khasabuli et al., 2020; Kishk et al., 2019;

Neupane et al., 2025; Rezashateri et al., 2021). Because of these resistance mechanisms, infections caused by resistant *S. aureus* strains often require alternative therapeutic approaches. The World Health Organization has identified antibiotic misuse and overconsumption as major drivers of antimicrobial resistance, while surveillance data in the Philippines continue to show the relevance of MRSA as a public health concern (Department of Health, Research Institute for Tropical Medicine, 2024; World Health Organization, 2023).

Clindamycin, a lincosamide antibiotic under the macrolide-lincosamide-streptogramin B group, has been used as an alternative agent for *S. aureus* infections, particularly in cases involving penicillin allergy and skin, soft tissue, bone, joint, and respiratory infections (Goudarzi et al., 2020; Weingarten-Arams & Adam, 2002). Its antibacterial effect is primarily associated with inhibition of bacterial protein synthesis through binding to the 50S ribosomal subunit. However, clindamycin resistance has increasingly limited its therapeutic reliability. Resistance may occur through inducible or constitutive MLSB mechanisms, often linked to *erm* genes, which can reduce or prevent effective ribosomal binding and may lead to treatment failure if not properly detected (Khursheed et al., 2025; Nagarkoti et al., 2019; Sasirekha et al., 2013).

These limitations have encouraged renewed interest in plant-derived compounds as possible adjuncts to conventional antibiotics. Medicinal plants contain secondary metabolites that may contribute to antibacterial activity through mechanisms different from those of standard antibiotics. *Azadirachta indica*, commonly known as neem, has been widely studied for antimicrobial, anti-inflammatory, and bioactive properties (Yadav, 2024). Neem-derived phytochemicals, including flavonoids, terpenes, azadirachtin, nimbolide, and quercetin, have been associated with bacterial membrane disruption, efflux pump inhibition, interference with quorum sensing, and reduction of biofilm formation (Li et al., 2023; Mahmoud et al., 2024; Mudenda et al., 2023; Sarkar et al., 2016). These mechanisms suggest that neem may complement the antibacterial action of conventional antibiotics by weakening bacterial defense systems or improving antibiotic access to microbial targets.

Previous studies have reported antibacterial activity of *A. indica* against resistant bacterial strains, including MRSA, and have suggested that neem extracts may interact beneficially with selected antibiotics (Ali et al., 2021; Naeem et al., 2021; Neglo et al., 2022). Other studies have proposed that plant-derived compounds may enhance antibacterial activity when paired with conventional drugs, thereby offering a possible adjunct approach in antimicrobial resistance management (Chawla et al., 2022; El-Sakhawy, 2023). However, the specific interaction between ethanolic neem leaf extract and clindamycin against *S. aureus* remains insufficiently characterized, particularly when assessed through quantitative combination testing such as the checkerboard broth microdilution assay and fractional inhibitory concentration index.

Given this gap, the present study evaluated the combined antibacterial effect of 70% ethanolic *A. indica* leaf extract and clindamycin against *S. aureus*. Rather than presuming synergy, the study examined whether the combination produced synergistic, additive, indifferent, or antagonistic interaction based on minimum inhibitory concentration and FICI-based classification.

This study specifically aimed to:

1. identify the bioactive compound classes present in 70% ethanolic *A. indica* leaf extract;
2. determine the individual antibacterial activity of 70% ethanolic neem leaf extract and clindamycin against *S. aureus*;
3. evaluate the combined antibacterial effect of 70% ethanolic neem leaf extract and clindamycin against *S. aureus* using checkerboard broth microdilution and FICI-based interaction classification.

2. Review of Related Literature

2.1 Antibiotic Resistance and Clindamycin Resistance in Staphylococcus aureus

The increasing resistance of *Staphylococcus aureus* to commonly used antibiotics remains a persistent concern in clinical microbiology and infectious disease management. Rao et al. (2022) reported widespread resistance patterns

among *S. aureus* isolates obtained from humans, livestock, poultry, and swine, with consistently high penicillin resistance and clindamycin resistance reaching 64% in both swine and poultry isolates. These findings indicate that *S. aureus* resistance is not confined to hospital settings but may circulate across human, animal, and environmental interfaces.

Methicillin-resistant *S. aureus* is primarily associated with the staphylococcal cassette chromosome mec, which carries the *mecA* gene. This gene encodes penicillin-binding protein 2a, a modified target protein with reduced affinity for beta-lactam antibiotics (Lade & Kim, 2023; Taha et al., 2022). Beyond methicillin resistance, *S. aureus* can employ multiple mechanisms that reduce antimicrobial susceptibility, including cell wall modification, altered membrane permeability, efflux mechanisms, and enzymatic inactivation (Brdová et al., 2024; Nikolic & Mudgil, 2023). These mechanisms complicate treatment because resistance may affect both first-line and last-resort antibiotics.

Clindamycin remains clinically useful against selected *S. aureus* infections because it inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit and interfering with peptide bond formation (Bistas et al., 2024). It is also valued for its pharmacokinetic profile and its use in patients with penicillin allergy (Albrecht et al., 2024; Goyal & Singh, 2023). However, inducible and constitutive macrolide-lincosamide-streptogramin B resistance have reduced its reliability. The *erm*-mediated methylation of 23S rRNA can prevent effective clindamycin binding, while efflux and target-site modification may further contribute to resistance (Álvarez et al., 2022; Armillei et al., 2024; Jahanbakhshi et al., 2024). Regional studies have also emphasized the importance of D-testing in detecting inducible clindamycin resistance, since hidden resistance phenotypes may lead to treatment failure if not identified (Ambachew et al., 2022; Fateh Dizji et al., 2023; Thapa et al., 2021).

2.2 Antimicrobial Properties of Azadirachta indica Neem

The search for plant-derived antimicrobial agents has intensified as conventional antibiotics face declining effectiveness against resistant pathogens. *Azadirachta indica*, commonly known as neem, has received considerable attention because of its traditional medicinal uses and its reported antibacterial, antifungal, antioxidant, and anti-inflammatory properties. Neem contains several bioactive compounds, including azadirachtin, nimbolide, nimbin, salannin, quercetin, flavonoids, phenolics, tannins, saponins, terpenoids, and alkaloids, which have been associated with antimicrobial activity (Ahmed et al., 2023; Batra et al., 2022; Mudenda et al., 2023; Wylie & Merrell, 2022).

The antimicrobial effects of neem have been linked to several possible mechanisms. Mudenda et al. (2023) reported that neem-derived compounds may disrupt bacterial membranes, interfere with enzymatic functions, and affect nucleic acid-related processes. Kassé et al. (2017) observed that MIC values for neem-derived preparations may vary substantially depending on plant part, extraction method, and test organism, with values ranging from 10 to 500 mg/mL. Such variability is expected in crude plant extracts because their active chemical composition is affected by extraction solvent, concentration, drying conditions, and phytochemical stability.

Studies focusing on *S. aureus* further support neem's antibacterial potential. Ahmed et al. (2023) found that ethanolic and aqueous neem leaf extracts demonstrated activity against food-borne pathogenic bacteria, while neem-mediated silver nanoparticles showed stronger inhibitory effects than crude extracts. Ali et al. (2021) reported bacteriostatic and bactericidal effects of neem leaf extract against multidrug-resistant pathogens, with MIC values ranging from 12.5 to 100 mg/mL. Altayb et al. (2022), using both laboratory and computational approaches, identified phytochemical constituents in neem extract that showed inhibitory activity against *S. aureus* and *Escherichia coli*. Neem has also been associated with antibiofilm and anti-quorum sensing properties, which may be relevant for resistant pathogens that rely on biofilm formation and virulence regulation (Mahmoud et al., 2024; Mehrishi et al., 2022).

2.3 Plant Extract-Antibiotic Combinations as Adjunct Antimicrobial Strategies

The use of plant extracts in combination with antibiotics has been proposed as a possible strategy for improving antibacterial activity against resistant organisms. Plant-derived compounds may act as antibiotic potentiators by increasing cell membrane permeability, inhibiting efflux pumps, disrupting biofilm formation, reducing virulence

expression, or weakening bacterial defense mechanisms (Chawla et al., 2022; Eladl et al., 2024; Khare et al., 2021). These mechanisms are especially relevant when the plant extract and antibiotic have different sites or modes of action.

Neem has been identified as a candidate for such combination approaches. Emad et al. (2024) reviewed the combined effect of neem oil and gentamicin against *Pseudomonas aeruginosa*, highlighting the possible role of neem compounds in downregulating virulence-related pathways and improving antibiotic effectiveness. Wylie and Merrell (2022) similarly emphasized that neem-derived compounds may disrupt bacterial cell walls, inhibit biofilm formation, and interfere with metabolic processes associated with antimicrobial resistance. These mechanisms provide a conceptual basis for examining neem as an adjunct to conventional antibiotics.

Combination studies involving neem and antibiotics against *S. aureus* provide more direct support for the present investigation. Faujdar and Bisht (2024) reported that neem leaf extract combined with tetracycline produced larger zones of inhibition against *S. aureus* and other uropathogenic bacteria than antibiotic treatment alone. Bhinge et al. (2020) found that neem leaf extract, when combined with ciprofloxacin, gentamicin, tetracycline, and erythromycin, enhanced inhibitory activity against both Gram-positive and Gram-negative organisms, including *S. aureus*. In a later study, Bhinge et al. (2022) likewise reported reduced MICs when neem extract was combined with selected antibiotics. Windham et al. (2022) further suggested that nimbolide, a neem-derived compound, may enhance antibacterial activity when paired with standard antibiotics against *S. aureus*. These studies support the possibility that neem extract may improve antibiotic performance, although the extent of interaction may vary depending on the antibiotic class, bacterial strain, extract preparation, and testing method.

2.4 Methods for Evaluating Antibacterial Interactions

Antibacterial interaction studies commonly use agar diffusion, broth microdilution, checkerboard assay, MIC/MBC determination, and fractional inhibitory concentration index analysis. Agar diffusion methods are often used as preliminary screening tools because they provide visual evidence of inhibition through zone diameter measurements. Sivasamugham et al. (2021), for example, used agar-based methods to evaluate neem leaf extracts combined with amikacin and tetracycline against clinically relevant bacteria, including *S. aureus*. Arsene et al. (2021) likewise used diffusion and MIC-based methods to evaluate aqueous and ethanolic plant extracts in combination with standard antibiotics against multidrug-resistant uropathogenic bacteria.

While agar diffusion provides useful preliminary data, it has limitations in quantifying interaction strength, particularly when plant extracts differ in viscosity, solubility, molecular diffusion, or pigment intensity. For this reason, broth microdilution and checkerboard assays are often preferred for quantitative interaction testing. The checkerboard method allows two antimicrobial agents to be tested across serial concentration gradients, making it possible to determine whether their combined inhibitory effect is synergistic, additive, indifferent, or antagonistic based on the FICI (Gali, 2024).

Recent studies have increasingly adopted FICI-based interpretation to strengthen the reliability of antimicrobial combination testing. Boateng et al. (2025) used methanolic neem leaf extracts against resistant bacteria, including MRSA and ESBL-producing *E. coli*, and reported strong combined effects with selected antibiotics using FICI criteria. Gali (2024) similarly demonstrated the value of combining agar well diffusion and broth microdilution approaches when assessing plant extract interactions against *S. aureus*. These methods are relevant to the present study because they allow the antibacterial interaction between neem leaf extract and clindamycin to be quantified rather than inferred only from inhibition zones.

Related in vitro natural-product studies also show the value of assay-based pharmacological screening at the preliminary stage, particularly when crude extracts are being evaluated before fractionation, safety profiling, and translational validation (Diaz et al., 2025).

2.5 Synthesis and Gaps

The reviewed literature establishes four major points. First, *S. aureus* remains a clinically important pathogen because of its virulence, adaptability, and resistance mechanisms, including methicillin resistance and MLSB-related

clindamycin resistance (Brdová et al., 2024; Jahanbakhshi et al., 2024; Lade & Kim, 2023; Taha et al., 2022). Second, neem contains multiple bioactive compounds, including flavonoids, phenolics, tannins, saponins, azadirachtin, quercetin, and nimbolide, which have been associated with antibacterial, antibiofilm, and membrane-disruptive activity (Ahmed et al., 2023; Batra et al., 2022; Mahmoud et al., 2024; Mudenda et al., 2023). Third, plant extract-antibiotic combinations may enhance antibacterial activity by targeting bacterial structures and resistance mechanisms through complementary pathways (Chawla et al., 2022; Eladl et al., 2024; Khare et al., 2021). Fourth, checkerboard broth microdilution and FICI analysis provide a more rigorous approach for classifying antimicrobial interactions than inhibition-zone observations alone (Gali, 2024).

Despite these contributions, the specific interaction between 70% ethanolic neem leaf extract and clindamycin against *S. aureus* remains insufficiently characterized through quantitative FICI-based methods. Existing studies provide evidence of neem's antibacterial potential and its possible potentiating effect with other antibiotics, but fewer studies focus specifically on the neem-clindamycin combination. This study addresses that gap by evaluating the individual and combined inhibitory activity of neem leaf extract and clindamycin against *S. aureus* using broth microdilution, checkerboard assay, and FICI-based interaction classification.

3. Methodology

3.1 Research Design

This study employed a laboratory-based experimental quantitative design to evaluate the individual and combined antibacterial activity of 70% ethanolic *Azadirachta indica* neem leaf extract and clindamycin against *Staphylococcus aureus*. Antibacterial activity was assessed through broth microdilution and checkerboard assay, with minimum inhibitory concentration and fractional inhibitory concentration index used as the primary outcome measures. The original protocol included agar well diffusion as a preliminary screening method, but this component was not completed due to time and resource constraints. The study proceeded directly to broth microdilution checkerboard testing, which provided the quantitative MIC and FICI data required for interaction classification.

3.2 Test Organism, Plant Material, and Antibiotic

The test organism was *Staphylococcus aureus* ATCC 25923, procured from a certified microbiology laboratory to ensure species authenticity and standardization. The plant material consisted of authenticated *A. indica* neem leaves sourced from reputable suppliers and verified through the Jose Vera Santos Memorial Herbarium of the Institute of Biology, University of the Philippines Diliman. Only fresh, undamaged, and pest-free leaves were used. Pharmaceutical-grade clindamycin was obtained from a verified supplier and tested at concentrations of 2, 1, 0.5, 0.25, and 0.125 µg/mL. Control conditions included growth control, solvent control using 1% DMSO, and sterility control using uninoculated Mueller-Hinton broth.

3.3 Preparation and Extraction of Neem Leaves

Fresh neem leaves were washed with distilled water, shade-dried for approximately 7 to 10 days at a controlled temperature not exceeding 40°C, and ground into powder. Approximately 300 g of dried neem leaf powder was macerated in 70% ethanol for 72 hours, with frequent shaking. The mixture was filtered using Whatman No. 1 filter paper, concentrated using a rotary evaporator, and oven-dried at 55°C. The dried crude extract was stored in amber glass containers at 4°C to reduce light exposure and maintain stability. For antibacterial testing, the extract was reconstituted in dimethyl sulfoxide to prepare a 100 mg/mL stock solution, then serially diluted in Mueller-Hinton broth. The final DMSO concentration in test wells was maintained at not more than 1% v/v.

3.4 Qualitative Phytochemical Screening

The neem leaf extract was subjected to qualitative phytochemical screening to determine the presence of selected secondary metabolite classes. The froth test was used for saponins, the sodium hydroxide test for flavonoids, Braymer's test for tannins, and ferric chloride test for phenols. Reactions were interpreted based on the presence of

characteristic foam formation or color changes and recorded using an intensity scale of weak, moderate, or strong reaction.

3.5 Determination of Individual Antibacterial Activity

The individual antibacterial activity of neem leaf extract and clindamycin was determined using broth microdilution in sterile 96-well microtiter plates. Neem extract was tested at concentrations of 100, 50, 25, 12.5, and 6.25 mg/mL, while clindamycin was tested at 2, 1, 0.5, 0.25, and 0.125 µg/mL. The bacterial suspension was standardized to a 0.5 McFarland standard before inoculation. After incubation at 35-37°C for 18-20 hours, growth inhibition was assessed through visual observation, OD600 measurement, and resazurin color change. The MIC was defined as the lowest concentration that prevented visible bacterial growth and retained the blue resazurin indicator response.

3.6 Checkerboard Assay and FICI Determination

The combined antibacterial activity of neem leaf extract and clindamycin was evaluated using the checkerboard broth microdilution method. Serial dilutions of both agents were arranged in a 5 x 5 concentration matrix, allowing different neem and clindamycin concentrations to intersect in each well. Each well received the prepared antimicrobial combination, double-strength Mueller-Hinton broth, and standardized bacterial inoculum. The plates were incubated at 35-37°C for 18-20 hours. OD600 readings were obtained, followed by the addition of resazurin and further incubation for 3-4 hours at 37°C. A blue resazurin result indicated inhibition, while a pink result indicated viable bacterial growth. The assay was performed in three technical replicates across three experimental days, yielding nine independent trials.

The fractional inhibitory concentration of each agent was calculated by dividing the MIC of the agent in combination by its MIC when used alone. The FICI was obtained by adding the FIC value of neem extract and the FIC value of clindamycin. Interaction outcomes were interpreted using the following criteria: FICI <= 0.5 as synergistic, 0.5 < FICI <= 1.0 as additive, 1.0 < FICI <= 2.0 as indifferent, and FICI > 2.0 as antagonistic.

3.7 Data Analysis

The MIC results were summarized using modal values, ranges, means, and standard deviations where applicable. FICI values were calculated for each of the nine independent trials and interpreted according to standard antimicrobial interaction thresholds. Because OD600 readings in neem-containing wells may be affected by the intrinsic color and particulate properties of plant extracts, resazurin color change was treated as the primary indicator of bacterial viability in interpreting inhibition patterns. The analysis focused on determining whether the observed interaction between neem leaf extract and clindamycin was synergistic, additive, indifferent, or antagonistic.

3.8 Ethical and Biosafety Considerations

No human or animal subjects were involved in the study. Neem leaves were ethically sourced from authenticated plant material and used only for research purposes. All bacterial handling procedures followed biosafety level 2 protocols. Researchers used appropriate personal protective equipment, maintained aseptic technique, disinfected workspaces before and after experimentation, and sterilized biohazardous materials before disposal. Data were stored securely and reported with proper attribution and without fabrication or falsification.

4. Results and Discussion

4.1 Phytochemical Constituents of Azadirachta indica Leaf Extract

Qualitative phytochemical screening confirmed the presence of saponins, flavonoids, tannins, and phenols in the 70% ethanolic *Azadirachta indica* neem leaf extract. As shown in Table 1, flavonoids and phenols produced the strongest reactions, while tannins showed a moderate reaction and saponins showed a weak positive reaction. These findings indicate that the extract contained secondary metabolites commonly associated with antibacterial activity, including membrane disruption, enzyme inhibition, and possible enhancement of antimicrobial permeability.

Table 1. Qualitative Phytochemical Screening Results of 70% Ethanolic *Azadirachta indica* Leaf Extract

Phytochemical Test	Compound Detected	Positive/Indicative Reaction	Result/Intensity
Froth test	Saponins	Persistent foam/froth formation	+
NaOH test	Flavonoids	Intense yellow color turning colorless	+++
Braymer's test	Tannins	Deep green color formation	++
Ferric chloride test	Phenols	Greenish-blue or deep blue color formation	+++

The presence of these compounds supports the biological plausibility of neem's antibacterial activity. Previous studies have associated neem's antimicrobial properties with flavonoids, phenolics, tannins, saponins, terpenoids, azadirachtin, nimbolide, and related phytochemicals (Batra et al., 2022; Mudenda et al., 2023; Wylie & Merrell, 2022). In the present study, the confirmed phytochemical profile provided the basis for further testing of the extract's individual and combined antibacterial effects.

4.2 Individual Antibacterial Activity of Neem Leaf Extract

The individual antibacterial activity of the neem leaf extract was determined through broth microdilution across nine independent trials. As shown in Table 2, the modal MIC of the 70% ethanolic neem leaf extract against *S. aureus* ATCC 25923 was 25 mg/mL. This value was observed in seven of the nine trials. Two trials conducted on Day 2 produced a lower MIC of 12.5 mg/mL. The mean MIC was 22.2 ± 4.2 mg/mL.

Table 2. Individual MIC of 70% Ethanolic *Azadirachta indica* Leaf Extract Against *S. aureus* ATCC 25923

Day	Trial	Date	MIC Neem Alone (mg/mL)	Resazurin Color at MIC
1	1	May 5, 2026	25	Blue, inhibited
1	2	May 5, 2026	25	Blue, inhibited
1	3	May 5, 2026	25	Blue, inhibited
2	1	May 7, 2026	25	Blue, inhibited
2	2	May 7, 2026	12.5	Blue, inhibited
2	3	May 7, 2026	12.5	Blue, inhibited
3	1	May 8, 2026	25	Blue, inhibited
3	2	May 8, 2026	25	Blue, inhibited
3	3	May 8, 2026	25	Blue, inhibited
Modal MIC			25	
Mean ± SD			22.2 ± 4.2	

The findings show that neem extract inhibited *S. aureus* growth, although at a concentration higher than those typically expected for purified antimicrobial compounds. This is consistent with the nature of crude plant extracts, which contain complex mixtures of active and inactive constituents. The observed MIC range is also broadly consistent with reports that neem extracts can exhibit antibacterial activity across variable concentration ranges depending on

extraction method, phytochemical composition, and test organism (Ali et al., 2021; Altayb et al., 2022; Kassé et al., 2017).

4.3 Individual Antibacterial Activity of Clindamycin

The individual MIC of clindamycin could not be determined within the tested concentration range of 0.125 to 2 µg/mL. As summarized in Table 3, bacterial growth was observed across all tested clindamycin concentrations in all nine trials. Pink resazurin coloration confirmed metabolic activity, indicating that none of the tested concentrations produced complete inhibition.

Table 3. Individual MIC Result of Clindamycin Against *S. aureus* ATCC 25923

Experimental Days	Trials	Concentrations Tested (µg/mL)	Visual Growth	Resazurin Color	MIC Result
Days 1-3	Trials 1-9	2, 1, 0.5, 0.25, 0.125	Present in all wells	Pink in all wells	>2 µg/mL, not determined within tested range

This result requires cautious interpretation. *S. aureus* ATCC 25923 is normally used as a reference strain in susceptibility testing, and clindamycin inhibition would ordinarily be expected within a lower quality-control range. However, the persistence of growth across all tested clindamycin concentrations was reproduced across three separate experimental days. This pattern suggests that the laboratory-maintained stock used in the study may have exhibited a clindamycin-resistant phenotype, although this cannot be confirmed without D-zone testing or molecular screening for resistance markers such as *erm* genes. Therefore, the result should not be interpreted as definitive evidence that ATCC 25923 is intrinsically resistant to clindamycin. Rather, it indicates that the specific stock used in this experiment did not respond to clindamycin within the tested range.

For FICI computation, the manuscript used 4 µg/mL as the working MIC estimate for clindamycin, representing the next two-fold dilution above the highest tested concentration. This was necessary because the individual clindamycin MIC was reported as >2 µg/mL rather than directly measured. The resulting FICI values should therefore be interpreted with this limitation in mind.

4.4 Combined Antibacterial Activity of Neem Leaf Extract and Clindamycin

The checkerboard broth microdilution assay evaluated the combined activity of neem leaf extract and clindamycin using a 5 x 5 concentration matrix. Across all nine trials, the combination of neem extract and clindamycin inhibited bacterial growth at concentrations that were not inhibitory when tested individually. Specifically, the minimum neem concentration in combination was 6.25 mg/mL across all trials. The clindamycin concentration required in combination ranged from 1 to 2 µg/mL. The original manuscript reports that clindamycin 2 µg/mL combined with any tested neem concentration produced inhibition across all trials, while clindamycin 1 µg/mL combined with neem produced inhibition in six of nine trials.

Table 4. FICI Calculations for Neem Leaf Extract and Clindamycin Combination Across Nine Trials

Day-Trial	MIC Neem Combo (mg/mL)	MIC Clinda Combo (µg/mL)	MIC Neem Alone (mg/mL)	FIC Neem	FIC Clinda	FICI	Classification
1-1	6.25	2	25	0.25	0.50	0.75	Additive
1-2	6.25	2	25	0.25	0.50	0.75	Additive
1-3	6.25	1	25	0.25	0.25	0.50	Boundary-level

Day-Trial	MIC Neem Combo (mg/mL)	MIC Clinda Combo (µg/mL)	MIC Neem Alone (mg/mL)	FIC Neem	FIC Clinda	FICI	Classification
							potential synergy
2-1	6.25	1	25	0.25	0.25	0.50	Boundary-level potential synergy
2-2	6.25	1	12.5	0.50	0.25	0.75	Additive
2-3	6.25	1	12.5	0.50	0.25	0.75	Additive
3-1	6.25	1	25	0.25	0.25	0.50	Boundary-level potential synergy
3-2	6.25	2	25	0.25	0.50	0.75	Additive
3-3	6.25	1	25	0.25	0.25	0.50	Boundary-level potential synergy
Mean ± SD	6.25	1.22 ± 0.44	22.2 ± 4.2	0.31 ± 0.12	0.33 ± 0.12	0.64 ± 0.14	Additive overall

Note. Clindamycin-alone MIC was not directly determined within the tested range and was treated as 4 µg/mL for FICI computation, corresponding to the next two-fold dilution above the highest concentration tested.

The FICI values ranged from 0.50 to 0.75, with a mean of 0.64 ± 0.14. Based on the standard interpretive range where 0.5 < FICI <= 1.0 indicates an additive interaction and FICI <= 0.5 indicates synergy, the overall interaction is best described as additive, with four trials falling exactly at the synergy threshold. Because the clindamycin-alone MIC was estimated rather than directly measured, the exact 0.50 values are more cautiously interpreted as boundary-level potential synergy rather than definitive synergy. This conservative interpretation avoids overstating the strength of the interaction while still recognizing that the combination produced inhibition at sub-MIC concentrations of both agents.

4.5 Discussion

The findings indicate that 70% ethanolic *A. indica* leaf extract has measurable antibacterial activity against *S. aureus* ATCC 25923, with a modal MIC of 25 mg/mL. Although this concentration is higher than those typically associated with purified antibiotics, it is consistent with the expected behavior of crude plant extracts. Crude extracts contain multiple phytochemical constituents, only some of which may contribute directly to antimicrobial activity. Prior studies similarly reported variable neem MIC values depending on extraction procedures, bacterial strain, and test conditions (Ali et al., 2021; Altayb et al., 2022; Kassé et al., 2017).

The phytochemical profile provides a plausible explanation for the observed antibacterial activity. Flavonoids and phenolic compounds may affect membrane permeability, oxidative balance, and bacterial enzyme systems, while tannins may interfere with microbial proteins and saponins may increase membrane permeability. These mechanisms are consistent with prior reports that neem-derived compounds may disrupt bacterial structures, inhibit biofilm

formation, and interfere with processes related to antimicrobial resistance (Mahmoud et al., 2024; Mudenda et al., 2023; Wylie & Merrell, 2022).

The clindamycin result is methodologically important. The failure of clindamycin to inhibit the tested *S. aureus* stock within the 0.125-2 µg/mL range was reproduced across all trials. This may indicate phenotypic resistance in the laboratory-maintained strain, possibly associated with MLSB-related mechanisms, although the study did not perform D-zone testing or molecular confirmation. Previous studies have emphasized that inducible and constitutive clindamycin resistance in *S. aureus* may be missed without appropriate confirmatory testing (Ambachew et al., 2022; Fateh Dizji et al., 2023; Thapa et al., 2021). For this reason, the study's clindamycin finding should be treated as a limitation and a secondary observation requiring confirmatory susceptibility testing.

The combination results provide the main contribution of the study. Neem extract and clindamycin, when tested together, inhibited *S. aureus* at concentrations below their individual inhibitory levels. This pattern supports the presence of a meaningful antibacterial interaction. The overall mean FICI of 0.64 indicates an additive effect, while four trials at FICI = 0.50 suggest boundary-level potential synergy. This aligns with previous studies reporting enhanced antibacterial activity when neem extract or neem-derived compounds were combined with conventional antibiotics (Bhinge et al., 2020, 2022; Faujdar & Bisht, 2024; Windham et al., 2022).

Mechanistically, the additive interaction is plausible because neem and clindamycin act through different antibacterial pathways. Clindamycin inhibits protein synthesis through the 50S ribosomal subunit, whereas neem phytochemicals may affect membrane integrity, efflux activity, quorum sensing, and biofilm-related mechanisms (Bistas et al., 2024; Khare et al., 2021; Mahmoud et al., 2024; Sarkar et al., 2016). If neem compounds increase membrane permeability or weaken bacterial defense systems, clindamycin may act more effectively at lower concentrations. However, because the study did not directly test molecular mechanisms, this explanation should be treated as a plausible interpretation rather than a confirmed mechanism.

Overall, the study supports the conclusion that 70% ethanolic neem leaf extract may enhance the antibacterial activity of clindamycin against the *S. aureus* stock tested under in vitro conditions. The most defensible classification is additive interaction, with limited boundary-level evidence of potential synergy. The findings are promising but preliminary. They require confirmation using freshly certified *S. aureus* strains, verified clindamycin susceptibility profiles, expanded antibiotic concentration ranges, and molecular resistance testing before any therapeutic interpretation can be made.

5. Conclusions, Recommendations, and Implications

5.1 Conclusions

This study evaluated the individual and combined antibacterial activity of 70% ethanolic *Azadirachta indica* neem leaf extract and clindamycin against *Staphylococcus aureus* ATCC 25923 using qualitative phytochemical screening, broth microdilution, checkerboard assay, and FICI-based interpretation.

First, the qualitative phytochemical screening confirmed the presence of saponins, flavonoids, tannins, and phenols in the 70% ethanolic neem leaf extract. These compound classes provide a plausible biochemical basis for the observed antibacterial activity of the extract, particularly because such metabolites have been associated with membrane-disruptive, enzyme-inhibitory, and antimicrobial effects.

Second, the neem leaf extract demonstrated individual antibacterial activity against *S. aureus*. The modal MIC was 25 mg/mL, with values ranging from 12.5 to 25 mg/mL across the nine independent trials. This indicates that the crude ethanolic neem extract exerted measurable inhibitory activity, although at concentrations higher than those normally expected for purified antibiotic compounds.

Third, clindamycin did not produce complete inhibition within the tested concentration range of 0.125 to 2 µg/mL. Bacterial growth persisted across all nine trials, suggesting that the specific laboratory-maintained *S. aureus* ATCC 25923 stock used in the study may have exhibited reduced susceptibility or a resistant phenotype. However, this

interpretation remains provisional because confirmatory D-zone testing and molecular resistance profiling were not performed.

Fourth, the combination of neem leaf extract and clindamycin produced an enhanced antibacterial effect compared with either agent at the same sub-MIC levels. The FICI values ranged from 0.50 to 0.75, with a mean of 0.64 ± 0.14. Based on standard FICI criteria, the overall interaction is best classified as additive, with some boundary-level evidence of potential synergy in trials that reached FICI = 0.50. Therefore, the most defensible conclusion is that 70% ethanolic neem leaf extract may enhance the in vitro antibacterial activity of clindamycin against the tested *S. aureus* stock, but formal synergy was not uniformly established.

5.2 Recommendations

Future studies should first confirm the susceptibility profile of the *S. aureus* strain before conducting combination assays. The persistent growth observed across all clindamycin concentrations should be investigated using D-zone disk diffusion testing and molecular screening for resistance markers such as *ermA*, *ermB*, *ermC*, and *mecA*. If resistance is confirmed, subsequent experiments should either use a freshly certified ATCC stock or deliberately compare susceptible and resistant *S. aureus* strains.

The concentration range for clindamycin should also be expanded. Since inhibition was not achieved at 2 µg/mL, future assays should include higher concentrations, such as up to 4 or 8 µg/mL, while also including lower concentrations that cover the expected quality-control range for susceptible ATCC strains. This would allow the actual clindamycin MIC to be determined rather than estimated for FICI computation.

The neem extract should be further standardized before antibacterial testing. Crude plant extracts may vary due to leaf maturity, drying conditions, solvent extraction, storage, and phytochemical stability. Future research should quantify marker compounds such as azadirachtin, quercetin, or nimbolide through chromatographic methods before testing. This would improve reproducibility and allow stronger comparison across studies.

Further experiments should include additional bacterial strains, particularly clinical MRSA isolates and confirmed clindamycin-resistant *S. aureus* isolates. Testing only one reference strain limits generalizability. A broader bacterial panel would clarify whether the observed additive interaction is strain-specific or applicable to a wider range of clinically relevant isolates.

Future researchers should also consider complementary methods such as time-kill assays, minimum bactericidal concentration testing, biofilm inhibition assays, and molecular mechanism studies. These approaches would help determine whether the neem-clindamycin combination is merely inhibitory or whether it also improves bacterial killing, biofilm disruption, or resistance-modulating activity.

Finally, no clinical use should be inferred at this stage. The findings should be treated as preliminary in vitro evidence. Any possible therapeutic application would require toxicity testing, formulation studies, pharmacokinetic evaluation, in vivo validation, and eventual clinical investigation.

5.3 Implications of the Study

The study contributes to antimicrobial research by showing that neem leaf extract may function as a potential adjunctive agent that enhances the antibacterial activity of clindamycin under controlled in vitro conditions. This supports the broader scientific interest in plant-derived compounds as possible antibiotic potentiators, especially in the context of antimicrobial resistance.

For medical technology and clinical laboratory education, the study demonstrates the value of broth microdilution, checkerboard assay, resazurin-based viability assessment, and FICI interpretation in evaluating antimicrobial combinations. It also shows the importance of strain verification, proper susceptibility testing, and cautious interpretation when reference strains produce unexpected resistance patterns.

In health professions education, such laboratory-based studies also support curriculum-practice alignment by exposing students to assay design, biosafety, result interpretation, and the cautious translation of laboratory findings into professional judgment (Bermido et al., 2025).

For pharmacological and biomedical research, the results suggest that neem-derived phytochemicals may warrant further investigation as adjunct compounds. However, the evidence supports only additive interaction at this stage. The findings should therefore be framed as a basis for further experimental validation rather than as proof of a therapeutic alternative.

For public health, the study aligns with the need to explore accessible and locally available antimicrobial adjuncts, particularly in settings where antimicrobial resistance is a growing concern. Nevertheless, plant-based adjuncts must be evaluated through rigorous laboratory, toxicological, and clinical standards before they can be considered for practical use.

From a health analytics perspective, laboratory-generated antimicrobial evidence can also contribute to decision quality when integrated with clinical, operational, and public-health data systems (Atento et al., 2025).

Overall, the study provides useful preliminary evidence that 70% ethanolic *A. indica* leaf extract can enhance the in vitro inhibitory activity of clindamycin against the tested *S. aureus* stock. Its main contribution lies in generating a quantitative, FICI-based basis for further investigation of neem-antibiotic interactions.

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