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Evaluating Neem (*Azadirachta indica*) Extracts as Alternative Bactericidal Agents Against Antibiotic-Resistant Pathogens

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Abstract

The rise of multidrug-resistant (MDR) pathogens threatens global health, prompting the search for alternative antimicrobials. This study evaluated the bactericidal activity of *Azadirachta indica* (neem) leaf extracts against clinical strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The leaves were authenticated, air-dried, and extracted using ethanol and water via cold maceration and Soxhlet extraction methods. Bacterial identity was confirmed with standard microbiological tests and subjected to antibiotic susceptibility testing using the disk diffusion method. The neem extract was analysed using a Shimadzu GC-MS-QP 2010 Plus system, and its antimicrobial activity was evaluated using the agar diffusion and broth microdilution methods. Antibiotic susceptibility showed high resistance to amoxicillin and ciprofloxacin, while meropenem was most effective. Hot ethanol extracts exhibited the strongest antibacterial activity, with inhibition zones up to 22.0 ± 1.4 mm for *E. coli*, and MIC/MBC assays confirmed bactericidal effects. Time–kill studies demonstrated complete elimination within 12–24 hours. GC-MS profiling revealed dominant compounds including n-hexadecanoic acid (19.8%), phytol (9.2%), and 9,12,15-octadecatriene-1-ol (8.1%). These results underscore the effectiveness of neem as a natural antibacterial agent and highlight its potential as a complementary or alternative therapy against antibiotic resistant pathogens.

Keywords: Antibiotic resistance, Neem (*Azadirachta indica*), Bactericidal activity, Time-kill Assay

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Introduction

Antibiotic resistance occurs when bacteria evolve mechanisms that protect them from the effects of antibiotics designed to kill or inhibit them. As a result, standard treatments become less effective or fail entirely, allowing infections to persist, spread more easily, and increase the risk of severe illness. Antibiotic resistance was estimated by the Centers for Disease Control and Prevention (CDC) to be responsible for approximately 23,000 deaths annually in the United States (Yu-Xuan *et al*., 2020). Multidrug-resistant (MDR) bacteria, defined as those resistant to at least one antimicrobial agent in three or more antibiotic classes, pose a serious public health threat due to their rapid emergence and spread (Magiorakos *et al*., 2012; Macesic *et al*., 2025). Of particular concern are the "ESKAPE" pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species which account for a large proportion of hospital-acquired as well as community infections worldwide (Alvarez-Ainza *et al*., 2024). Infections associated with these organisms are linked to prolonged illness, higher mortality, longer hospital stays, and increased treatment costs, particularly in low- and middle-income countries with resource-limited healthcare systems and high out-of-pocket expenses (WHO, 2023).

In Nigeria, multidrug-resistant (MDR) bacteria such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa* are commonly linked to urinary tract infections, pneumonia, and bloodstream infections (Makanjuola *et al*., 2018; Olowo-Okere *et al*., 2020; Christopher *et al*., 2025). For example, Adesina *et al*. (2025) reported that about 22.5% of *E. coli* isolates were resistant to multiple drugs, such as fluoroquinolones, penicillins, and several generations of cephalosporins. The burden of infections is exacerbated by limited diagnostic capacity, widespread empirical antibiotic use, and weak regulatory control of antimicrobial distribution. Generally, resistance development is driven by various mechanisms such as β-lactamase production e.g., ESBLs, *Amp*C, and carbapenemases), target modification and efflux pumps (Devi *et al*., 2024). These traits are often plasmid-mediated, enabling rapid horizontal gene transfer. The limited introduction of new antibiotics, driven in part by high research and development costs and unattractive economic incentives for pharmaceutical companies, has widened the gap between the rise in resistance and available effective treatment options (Dutescu and Hillier, 2021).

Medicinal plants, which constitute a central component of traditional, complementary, and integrative medicine, are widely utilized and accepted across many low- and middle-income countries. Their extensive use is driven by accessibility, affordability, cultural relevance, and generally low reported toxicity, making them an important resource for healthcare where conventional services may be limited (WHO, 2019). Plant-derived secondary metabolites such as alkaloids, flavonoids, tannins and phenolics, act through multiple antimicrobial mechanisms such as, potentially reducing the development of resistance. Neem (*Azadirachta indica*), popularly called "dogonyaro" in Nigeria belongs to the Meliaceae family and is widely used in traditional medicine for infections, inflammation, fever, and dental problems. Its leaves, bark, seeds, fruits, and oil contain bioactive compounds responsible for antibacterial, antifungal, antiviral, anti-inflammatory, and antioxidant activities (Wylie and Merrell, 2022)

Experimental studies show that *Azadirachta indica* (neem) extracts have broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, notably methicillin-resistant *S. aureus* (MRSA), *E. coli*, *K. pneumoniae* and *P. aeruginosa in-vitro*

(Wylie and Merrell, 2022; Altayb *et al*., 2022). Its mechanisms involve disruption of membrane integrity, inhibition of key enzymes, and suppression of biofilm formation. Furthermore, neem has demonstrated synergistic effects with conventional antibiotics and enhanced antimicrobial activity when incorporated into nanoparticle-based formulations (Bhinge *et al*., 2022; Almowallad and Alqahtani, 2024).

Although neem trees are abundant in Nigeria, few studies have systematically examined their antimicrobial potential. There is currently limited scientific validation of their activity against multidrug-resistant clinical isolates. Moreover, variations in phytochemical composition due to geographic location, extraction methods, and the specific plant parts used underscore the need for further evaluation of neem as a potential source of antimicrobial agents. Therefore, this study seeks to investigate the bactericidal effects of *Azadirachta indica* extracts against selected multidrug-resistant bacterial pathogens implicated in clinical infections, with the aim of informing evidence-based antimicrobial strategies.

MATERIALS AND METHODS

2.1 Ethical Approval

Ethical approval for this study was obtained from the Health Research Ethics Committee (HREC) at the College of Medicine, University of Lagos (Ref. CMUL/HREC/06/25/1967). The approval covered the use of clinical bacterial isolates obtained from hospital sources and ensured that all procedures complied with established ethical standards for research involving human-derived materials. Patient anonymity was strictly maintained, and no personal identifiers were linked to the isolates used in this study.

2.2 Collection and Identification of Bacterial Isolates

Previously identified isolates of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* from routine analyses were obtained from Lagos University Teaching Hospital (LUTH) and Randle General Hospital, Lagos. Isolates were confirmed by subculturing on selective media: Cetrimide Agar for *P. aeruginosa*, Mannitol Salt Agar for *S. aureus*, and Eosin Methylene Blue Agar for *E. coli* and *K. pneumoniae*. They were further subjected to Gram staining and biochemical tests, including catalase, coagulase, citrate utilization, oxidase, and Methyl Red-Voges Proskauer assays (Cheesebrough, 1998).

2.5 Collection and Preparation of Neem (*Azadirachta indica*) Extract

Fresh leaves were collected from CITS Garden, University of Lagos, authenticated at the Department of Botany herbarium (Voucher LUH 100419), washed, air-dried for 15 days, and powdered. Extraction was performed using cold maceration and Soxhlet (hot ethanol) methods. Extracts were concentrated, weighed, and stored in sterile bottles at cool temperature for subsequent analysis (Osuala *et al*., 2024). The percentage yield was calculated as:

$$\text{Percentage Yield of the Crude Extract (\%)} = \frac{\text{Weight of Crude Extract (g)}}{\text{Weight of Dried Pulverised Sample (g)}} \times 100$$

2.6 Antibiotic Susceptibility Testing (Disk Diffusion Method)

Antibiotic susceptibility testing was conducted using the Kirby–Bauer disk diffusion method on Mueller-Hinton agar, following CLSI guidelines (CLSI, 2023). For Gram-negative bacteria, the antibiotics tested included gentamicin (10 µg), amoxicillin-clavulanate (20/10 µg), ciprofloxacin (5 µg), and meropenem (10 µg), while Gram-positive bacteria were tested against gentamicin (10 µg), erythromycin (15 µg), vancomycin (30 µg), and

amoxicillin-clavulanate (20/10 µg). These antibiotics were selected to provide a representative agent from each major antimicrobial class, allowing assessment of multidrug resistance according to established definitions (Magiorakos *et al.*, 2012).

Antimicrobial Activity of Plant Extracts (Agar Well Diffusion Method)

The antimicrobial activity of aqueous, cold ethanol, and hot ethanol extracts was evaluated using the agar well diffusion method on Mueller-Hinton agar (Osuala *et al.*, 2024). Extracts were prepared at 100 mg/mL and tested against standardized bacterial inocula (0.5 McFarland). Wells (8 mm) were filled with 100 µL of each extract, allowed to diffuse, and incubated at 37°C for 24 hours. Antimicrobial activity was assessed by measuring zones of inhibition.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the extract was determined using the broth microdilution method in sterile 96-well microplates, with minor modifications of the procedure described by Nourmohammadi *et al.* (2025). Extracts were diluted in Mueller-Hinton broth to final concentrations of 100, 75, 50, and 25 mg/mL. Each well contained the extract, broth, and a standardized bacterial inoculum equivalent to 0.5 McFarland. Chloramphenicol served as a positive control, while wells containing only broth with inoculum or broth alone were used as negative and sterility controls, respectively. Plates were incubated at 37°C for 24 hours, and the MIC was recorded as the lowest extract concentration that showed no visible bacterial growth.

The minimum bactericidal concentration (MBC) was determined by subculturing aliquots from wells with no visible growth onto Mueller-Hinton agar and incubating at 37°C for 24 hours; the absence of growth indicated bactericidal activity, while visible growth indicated a bacteriostatic effect (Wiegand *et al.*, 2008, Osuala *et al.*, 2024).

Gas chromatography-mass spectrometry (GC-MS) analysis

Neem extract was analysed using a Shimadzu GC-MS-QP 2010 Plus system with an Elite-1 capillary column, helium as carrier gas, and electron ionization at 70 eV. Compound identification was based on the National Institute of Standards and Technology (NIST) database comparison (Osuala *et al.*, 2024).

Time-Kill Assay

A time-kill assay was conducted following the approach described by Techaoei (2022), in which plant extracts were evaluated over time at multiple MIC levels and surviving bacterial counts were quantified at defined intervals. Confirmed multidrug-resistant (MDR) isolates were adjusted to a 0.5 McFarland standard (~1×10^{8} CFU/mL) and further diluted to ~1×10^{6} CFU/mL in Mueller-Hinton broth. Cultures were exposed to neem extract at the minimum inhibitory concentration (MIC) and 2× MIC. Aliquots were taken at 0, 2, 4, 6, 8, 12, and 24 hours, serially diluted, plated on Mueller–Hinton agar, and incubated at 37 °C for 24 hr. Bactericidal activity was defined as a ≥3 $\log_{10}$ reduction in CFU/mL relative to the initial inoculum (Techaoei, 2022).

Data Analysis

The results were expressed as mean ± standard error of the mean (SEM). MIC and MBC values (µg/mL) and time-kill data (CFU/mL) were analysed using descriptive statistics and summarised in tables and figures.

RESULTS

Confirmatory Identification of Bacterial Isolates

Twelve bacterial isolates were confirmed using Gram staining and biochemical tests. Nine were Gram-negative rods (*Escherichia coli*,

Klebsiella pneumoniae, *Pseudomonas aeruginosa*), and three were Gram-positive

(*Staphylococcus aureus*), all exhibiting characteristic morphological and biochemical traits. *E. coli* (strains 567 and KS4) were obtained from wound, and strain 1101 from urine. *K. pneumoniae* (strain 405) was from blood, strain 406 from urine, and strain 1128 from sputum. *P. aeruginoa* (strain 1135) was isolated from wound, and strains 780 and 785 from sputum. *S. aureus* (strain 049) was recovered from a urethral swab, strain 060 from blood, and strain 0290 from a wound site. In all, four isolates were from wound (*E. coli* [567, KS4], *Pseudomonas* [1135], *Staphylococcus* [0290]), three from sputum (*Klebsiella* [1128], *Pseudomonas* [780, 785]), two from urine (*E. coli* [1101], *Klebsiella* [406]), two from blood (*Klebsiella* [405], *Staphylococcus* [060]), and one from urethral swab (*Staphylococcus* [049]).

Antibiotic Susceptibility Profiles of Bacterial Isolates

The antibiotic susceptibility results are summarised in Table 1. Of the nine Gram-negative isolates, three (*Klebsiella pneumoniae* 405 and 406, and *E. coli* 1101) were multidrug-resistant, showing resistance to at least one agent in three or more antibiotic classes. Resistance was highest to ciprofloxacin (7/9, 77.8%) and amoxicillin/clavulanate (5/9, 55.6%), moderate to gentamicin (3/9, 33.3%), and lowest to meropenem (1/9, 11.1%).

Notably, *K. pneumoniae* 405 was resistant to all tested antibiotics, while most other Gram-negative isolates remained susceptible to meropenem. For the three *S. aureus* isolates, two (049 and 0290) qualified as multidrug-resistant. All were resistant to erythromycin and vancomycin, with variable susceptibility to amoxicillin/clavulanate and gentamicin, and strain 049 exhibited resistance to all tested antibiotics.

Table 1: Antibiotic Susceptibility Profiles of the Bacterial Isolates

Gram Negative Bacteria				
Bacterial Isolate	**Ciprofloxacin**	**Amoxicillin/ Clavulanate**	**Gentamicin**	**Meropenem**
Kleb 405	10(R)	10(R)	9(R)	17(R)
Kleb 406	12(R)	13(R)	12(R)	23(S)
Kleb 1128	23(S)	19(I)	20(S)	22(S)
Esch KS4	17(R)	20(S)	19(S)	23(S)
Esch 1101	6(R)	15(R)	12(R)	24(S)
Esch 567	0(R)	11(R)	19(S)	27(S)
Pseudo 1135	30(S)	0(R)	18(S)	25(S)
Pseudo 785	0(R)	6(R)	15(S)	27(S)
Pseudo 780	23(S)	22(S)	18(S)	24(S)

Gram Positive Bacteria				
Bacterial Isolate	**Erythromycin**	**Amoxicillin/ Clavulanate**	**Gentamicin**	**Vancomycin**
Staph 049	10(R)	10(R)	9(R)	17(R)
Staph 060	0 (R)	0 (R)	20(S)	0 (R)
Staph 0290	0 (R)	29(S)	13(I)	0 (R)

Kleb= Klebsiella. pneumoniae; Esch = Escherichia coli; Pseudo= Pseudomonas aeruginosa; Staph= Staphylococcus aureus R= Resistant; S= Sensitive; I= Intermediate

Extraction yields and Antibacterial Activity of Neem (*Azadirachta indica*) Extracts

The ethanol extract weighed 7.9 g, corresponding to a percentage yield of 15.8%, while the aqueous extract weighed 12.9 g, yielding 25.8%. The antibacterial activity of the extracts varied across isolates and solvent type, including multidrug-resistant strains. Hot ethanol extracts produced the largest inhibition zones, with Esch 567 (MDR) showing 22 mm, followed by Esch KS4 (17 mm) and Staph 060 (MDR, 17 mm). Cold ethanol was particularly effective against MDR *S. aureus*, with inhibition zones of 21 mm for strains 049 and 060, and 18 mm for 0290. Aqueous extracts were generally less active; Esch 567 showed 16 mm, while some MDR isolates (Esch KS4, Kleb 1128) were not inhibited (0 mm). All isolates were resistant to the chloramphenicol control disk, except Pseudo 780, indicating that the observed inhibition was due to the plant extracts rather than inherent bacterial susceptibility. These results are summarised in Table 2.

Table 2: Inhibition of clinical bacterial isolates by Neem (*Azadirachta indica*) extracts (mean inhibition diameter, mm ± SD)

Bacterial Isolate	Hot Ethanol (mm)	Cold Ethanol (mm)	Aqueous (mm)
Kleb 405	14.0 ± 1.4	10.0 ± 1.4	8.0 ± 1.4
Kleb 406	13.0 ± 1.4	12.0 ± 1.4	12.0 ± 1.4
Kleb 1128	15.0 ± 1.4	15.0 ± 1.4	0.0 ± 0.0
Esch KS4	17.0 ± 1.4	15.0 ± 0.0	0.0 ± 0.0
Esch 1101	15.0 ± 1.4	14.0 ± 0.0	9.0 ± 0.0
Esch 567	22.0 ± 1.4	27.0 ± 1.4	16.0 ± 1.4
Pseudo 1135	12.0 ± 1.4	10.0 ± 1.4	9.0 ± 0.0
Psuedo 785	13.0 ± 1.4	13.0 ± 1.4	13.0 ± 1.4
Psuedo 780	16.0 ± 0.0	16.0 ± 1.4	12.0 ± 1.4
Staph 049	15.0 ± 1.4	21.0 ± 1.4	10.0 ± 1.4
Staph 060	17.0 ± 1.4	21.0 ± 0.0	13.0 ± 0.0
Staph 0290	11.0 ± 1.4	18.0 ± 1.4	15.0 ± 0.0

Kleb= *Klebsiella pneumoniae;* Esch = *Escherichia coli;* Pseudo= *Pseudomonas aeruginosa;* Staph= *Staphylococcus aureus*

MIC and MBC of Neem Extracts against Bacterial Isolates

The minimum inhibitory concentration (MIC) of neem extract was 25 mg/ml for all tested isolates (Table 3). All bacterial isolates had a minimum inhibitory concentration (MIC) of 25 mg/ml. The minimum bactericidal concentration (MBC) ranged from 75 mg/ml for *E. coli* KS4 and 567 to 100 mg/ml for all other isolates. The MBC/MIC ratios were predominantly ≤4, including ratios of 3 for *E. coli* KS4 and 567, indicating bactericidal activity across all tested isolates.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Neem Extracts Against Bacterial Isolates

Bacterial Isolate	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Kleb 405	25	100	4
Kleb 406	25	100	4
Kleb 1128	25	100	4
Esch KS4	25	75	3
Esch 1101	25	100	4
Esch 567	25	75	3
Pseudo 1135	25	100	4
Pseudo 785	25	100	4
Pseudo 780	25	100	4
Staph 049	25	100	4
Staph 060	25	100	4
Staph 0290	25	100	4

Kleb= *Klebsiella. pneumoniae;* Esch = *Escherichia coli;* Pseudo= *Pseudomonas aeruginosa;* Staph= *Staphylococcus aureus;* MIC = *Minimum Inhibitory Concentration;* MBC= *Minimum Bactericidal Concentration.* MBC/MIC ≤*4= Bactericidal; >4= Bacteriostatic*

GC-MS Analysis of Hot Ethanol Neem Extract

GC-MS analysis of the hot ethanol neem extract identified 18 compounds (Table 4). The most abundant compound was n-Hexadecanoic acid at 19.80%, while Quinoline, 2,3,4,4a,5,6-hexahydro-7-methoxy- was the least abundant at 1.18%. Other notable constituents included phytol and 9,12,15-octadecatriene-1-ol, with area percentages ranging between 1.18% and 19.80% (Figure 1).

Table 4: Compounds Identified from the Hot Ethanol Extract of Neem leaves

Peak	Retention time (s)	Area (%)	Compound ID	Molecular formula	Molecular weight (g/mol)	% Minimum quality
1	3.802	1.72	2-Cyanoethyl acrylate	$C_6H_7NO_2$	111.1	58
2	4.701	1.55	Benzoic acid, methyl ester	$C_8H_8O_2$	136.15	94
3	5.908	5.53	Octanoic acid, ethyl ester	$C_{10}H_{20}O_2$	172.26	95
4	7.121	1.38	Nonanoic acid, ethyl ester	$C_{11}H_{22}O_2$	172.26	91
5	8.260	3.01	Decanoic acid, ethyl ester	$C_{12}H_{24}O_2$	200.32	96
6	9.925	1.18	Quinoline, 2,3,4,4a,5,6-hexahydro-7-methoxy-	$C_{10}H_{11}NO$	165.23	52
7	12.586	2.90	Bicyclo[3.1.1]heptane,2,6,6—trimethyl-	$C_{10}H_{16}$	136.23	95
8	12.957	1.21	Neophytadiene	$C_{20}H_{38}$	278.52	94
9	13.341	4.77	Pentadecanoic acid, 14-methylmethyl ester	$C_{17}H_{34}O_2$	270.45	98
10	13.747	19.80	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	99
11	14.760	4.27	9-octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.49	93
12	14.874	9.20	Phytol	$C_{20}H_{40}O$	296.54	91
13	14.943	1.65	Methyl stearate	$C_{19}H_{38}O_2$	298.51	95
14	15.160	8.12	9,12,15-octadecatriene-1-ol(z,z,z)-	$C_{18}H_{32}O$	280.48	96
15	15.292	3.74	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	98
16	18.977	2.50	Oleic acid	$C_{18}H_{34}O_2$	282.47	83
17	19.091	1.63	Tricosanoic acid	$C_{23}H_{46}O_2$	354.60	59
18	19.492	2.41	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.56	83

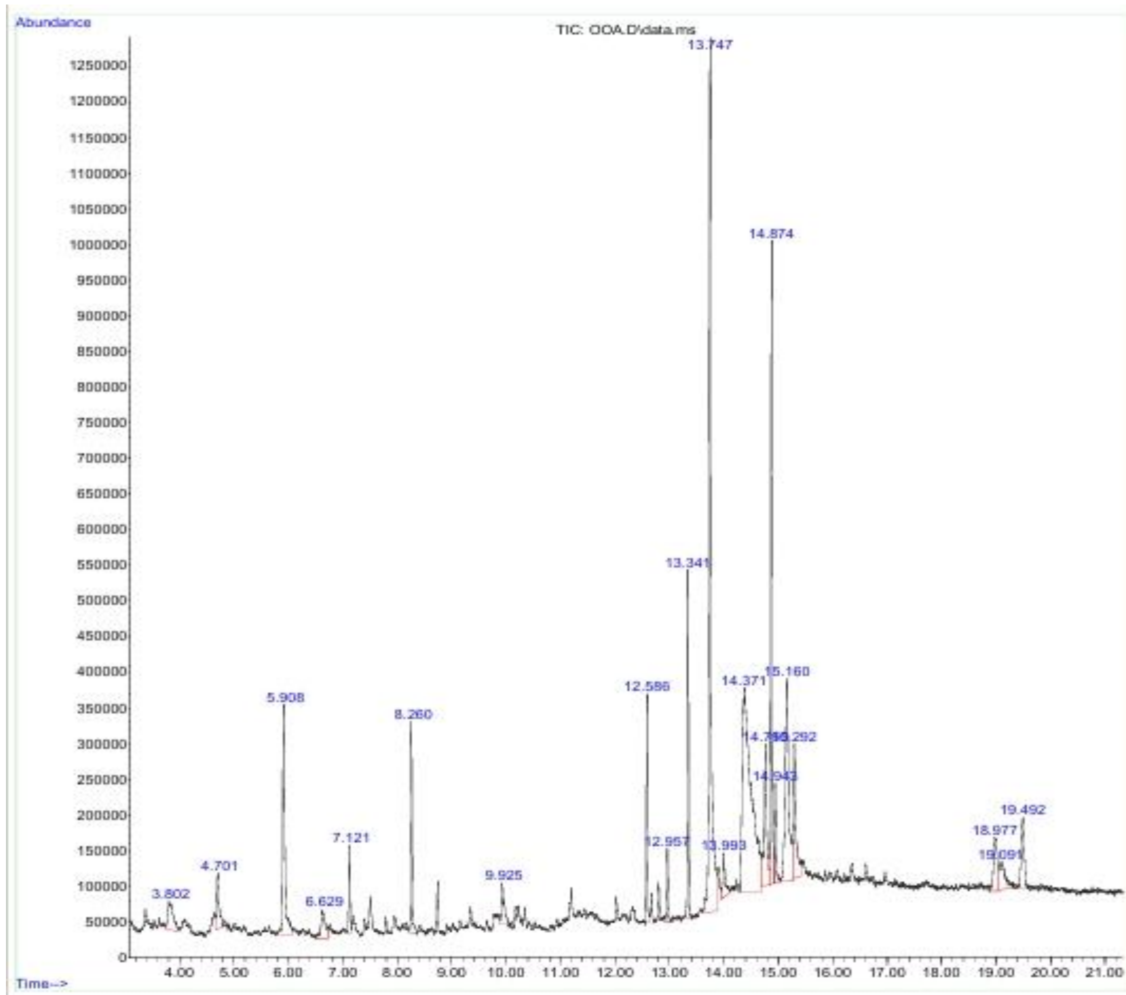

Figure 1: Chromatogram of Hot ethanol extract of Neem leaves

Time-Kill Kinetics of Hot Ethanol Neem Extract against the bacterial pathogens

The time-kill kinetics of hot ethanol neem extract demonstrated concentration- and time-dependent bactericidal activity against all tested resistant isolates (Figure 2). At 2× MIC, the extract completely inhibited *E. coli* 567, *K. pneumoniae* 406, *P. aeruginosa* 780, and *S. aureus* 049 within 12 hours. At MIC, total inhibition was achieved by 24 hours. Untreated controls showed steady bacterial growth over 24 hours, confirming the extract's potent bactericidal effect

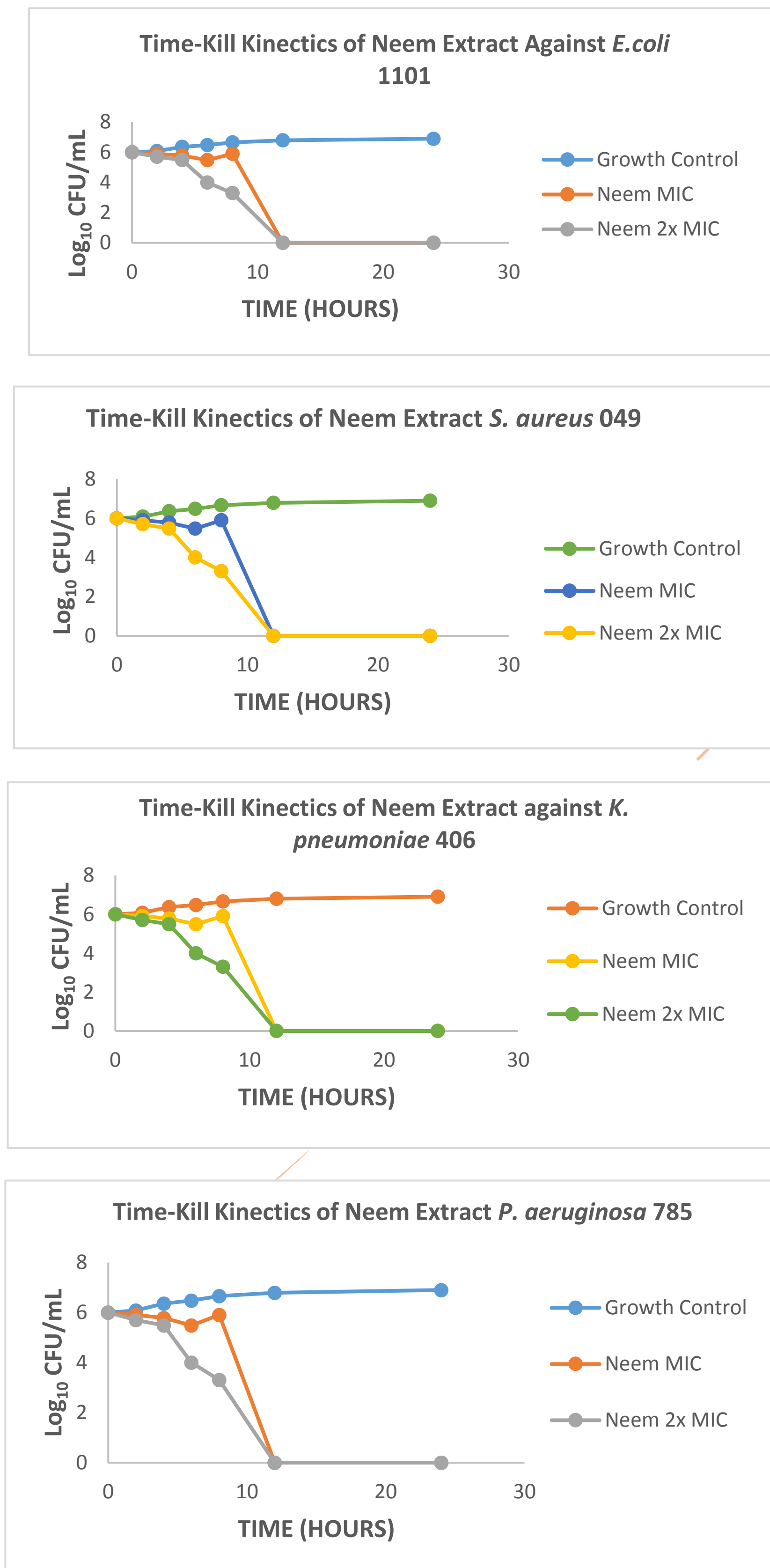

Figure 2: Time-kill kinetics of neem extract against bacterial Isolates at MIC and 2× MIC.

Discussion

Antimicrobial resistance among clinically significant bacterial pathogens remains a major challenge, with high levels of resistance observed against commonly used antibiotics. The bacterial species examined in this study, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*, are well recognized causes of clinical infections. The multidrug resistant strains identified, *Klebsiella pneumoniae* 405 and 406, *Escherichia coli* 1101, *Staphylococcus aureus* 049, and *Staphylococcus aureus* 0290, further highlight the growing burden of resistance in both Gram-negative and Gram-positive organisms. The resistance patterns observed in this study are consistent with findings from a study conducted in Lagos which reported that all clinical *Klebsiella* isolates showed resistance to multiple antibiotics including amoxicillin clavulanate and several cephalosporins, demonstrating widespread multidrug resistance (Akinpelu *et al*., 2020). Comparable trends have also been reported globally. Clinical studies have shown that fluoroquinolone resistant *Klebsiella pneumoniae* and *Escherichia coli* are increasingly associated with treatment failure in infections such as urinary tract and bloodstream infections, reflecting reduced efficacy of ciprofloxacin and related agents (Aditi *et al*., 2019). These observations reflect the clinical difficulty in treating infections caused by these pathogens and highlight the necessity for alternative therapies.

The antibacterial activity of *Azadirachta indica* extracts observed in this study was strongly solvent-dependent, with organic solvent preparations demonstrating greater efficacy than aqueous extracts. Ethanolic and methanolic extracts have been repeatedly shown to produce stronger antibacterial effects against clinical pathogens, likely due to enhanced solubility and extraction of bioactive phytochemicals such as flavonoids, tannins, and limonoids. Methanolic neem extract demonstrated measurable zones of inhibition against clinical isolates of bacterial pathogens, even at low concentrations, confirming the potency of organic solvent extracts (Altayb *et al*., 2022). Similarly, Al Akeel *et al.* (2015) reported that ethanolic neem leaf extracts exhibited superior antibacterial activity compared with aqueous preparations, emphasising the influence of solvent polarity on the recovery of active antimicrobial constituents. Also, a recent Nigerian study demonstrated that ethanolic neem leaf extracts produced significant inhibition of multidrug-resistant clinical isolates of *E. coli*, *K. pneumoniae*, and *S. aureus*, reinforcing the relevance of solvent-based extraction in enhancing antibacterial potency for locally prevalent multidrug-resistant pathogens (Adeluola *et al*., 2023). These studies indicate that ethanolic and methanolic neem extracts possess broad-spectrum antibacterial potential and may serve as promising complementary agents against multidrug-resistant clinical pathogens.

Whilst the MIC and MBC data confirm the bactericidal nature of the hot ethanol extract of *Azadirachta indica*, the time-kill assays further demonstrated that the extract exhibits a potent, time- and concentration-dependent bactericidal effect against multidrug-resistant strains. Complete eradication of bacterial populations was achieved within 12 hours at 2× MIC and by 24 hours at MIC, indicating that doubling the MIC markedly enhances bactericidal activity. Previous investigations using ethanolic neem extracts have also employed standardized MIC and MBC assays to demonstrate antibacterial efficacy against pathogenic bacteria (Ali *et al*., 2021). Another study reported MIC and MBC values for ethanolic neem leaf extract against *Escherichia coli* and other pathogens, with mean MIC values of 50 mg/ml for *E. coli* compared to 100 mg/ml for *S. aureus* (Adamu *et al*., 2019). These authors concluded that the MBC of *A. indica* leaf extract suggests a bacteriostatic effect against Gram-negative bacteria and limited activity against Gram-

positive bacteria. This, however, is inconsistent with our findings, whereby all isolates were completely inhibited by the hot ethanolic extract at MIC/MBC ratios ≤4, and time-kill assays showed total bacterial eradication at 2× MIC within 12 hours and at MIC by 24 hours. The observed discrepancies may be attributed to differences in bacterial strains, extraction procedures, solvent potency, or experimental conditions, highlighting variability in neem extract efficacy across studies.

GC-MS analysis of the hot ethanolic extract of *Azadirachta indica* leaves revealed a diverse profile of lipophilic phytochemicals, dominated by long-chain fatty acids and their derivatives. The most abundant constituent was n-hexadecanoic acid (19.80%), followed by the diterpene alcohol phytol (9.20%) and the unsaturated alcohol 9,12,15-octadecatriene-1-ol (8.12%). Other significant compounds included methyl and ethyl esters of medium- to long-chain fatty acids, such as pentadecanoic acid derivative (4.77%) and 9-octadecenoic acid methyl ester (4.27%). The dominance of fatty acids and their esters suggest notable antimicrobial potential. n-hexadecanoic acid (palmitic acid), oleic acid, and phytol have been reported to exhibit antimicrobial activity, primarily through membrane disruption and interference with microbial metabolism (Desbois and Smith, 2010, Silva *et al*., 2013).

However, this chemical profile contrasts with reports of ethanolic neem leaf extracts where Arabino-Hex-1-enitol, 1,5-anhydro-2-deoxy- was the dominant compound and a wider chemical diversity was observed (Ali *et al*., 2026b). However, a comparative GC-MS study of neem seed ethanol extracts showed n-hexadecanoic acid (~28.65%) as the major component, with fatty acids and their esters forming the bulk of the bioactive fraction (Ali *et al*., 2026a). Similarly, Osuala *et al*. (2024) identified n-hexadecanoic acid (palmitic acid) in ethanolic neem bark extracts, confirming the occurrence of long-chain fatty acids across neem tissues. Nevertheless, consistent with the present findings, other studies have reported palmitic acid, fatty acid esters, and related lipophilic metabolites as dominant constituents in ethanolic neem leaf extracts (Pandey *et al*., 2018; Altayb *et al*., 2022). The observed quantitative differences, including the relatively high palmitic acid content, likely reflect geographical variation and extraction conditions.

Conclusion

These findings demonstrate that the neem leaf extract exhibits broad-spectrum bactericidal activity against both Gram-positive and Gram-negative pathogens, including antibiotic-resistant strains, as evidence by agar diffusion, MIC/MBC, and time-kill assays. GC-MS profiling revealed key bioactive compounds, including n-hexadecanoic acid, phytol, octadecanoic acid methyl ester, and 9,12-octadecadienoic acid methyl ester, all of which have documented antibacterial activity. These results support the potential of neem leaf extract as a natural alternative or adjunct to conventional antibiotics, particularly in settings with high antimicrobial resistance (AMR).

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