Journal of Bioscience and Biotechnology Discovery
Volume 11(1), pages 10-23, February 2026
Article Number: 6C2396872
ISSN: 2536-7064
https://doi.org/10.31248/JBBD2026.250
https://integrityresjournals.org/journal/JBBD
Full Length Research

Synergistic antibacterial effect of *Adenodolichos paniculatus* root bioactive fraction and cefotaxime against ESBL-producing enterobacteriaceae

Kyahar, I. F.[1]* and Aboh, I. M.[2]

[1]Department of Microbiology and Biotechnology, Faculty of Pharmacy, University of Jos, Nigeria.
[2]Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

*Corresponding author. Email: kyaharfriday@yahoo.com

Received 19th January 2026; Accepted 20th February 2026

ABSTRACT: The emergence of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae has reduced the clinical effectiveness of third-generation cephalosporins. This study investigated the synergistic antibacterial potential of the root bioactive component of *Adenodolichos paniculatus* combined with cefotaxime against ESBL-producing *Enterobacteriaceae*. The plant was extracted by maceration and screened for phytochemicals. The most active chloroform extract was fractionated by vacuum liquid chromatography and purified by preparative thin layer chromatography (TLC). Clinical isolates of *Escherichia coli* (n = 10), *Klebsiella pneumoniae* (n = 6), and *Proteus mirabilis* (n = 2) were tested following CLSI (2025) guidelines. ESBL production was confirmed by the cefotaxime–clavulanic acid combination disk method. Synergistic interactions were assessed using disk diffusion, checkerboard (minimum inhibitory concentration/fractional inhibitory concentration-MIC/FIC index), and time–kill assays. Phytochemical analysis revealed alkaloids, flavonoids, terpenoids, phenolics, steroids, and saponins. Strong synergy was observed between the extract and cefotaxime, increasing inhibition zones from 10 to 18 mm (*E. coli* CI-3) and 9 to 17 mm (*K. pneumoniae* CI-6), with ≥16-fold MIC reductions and FIC indices of 0.25–0.375. Time–kill assays showed >2 $\log_{10}$ (colony forming unit) CFU/mL reductions compared with single agents after 24 h. *P. mirabilis* (non-ESBL) also showed synergy, suggesting other mechanisms beyond β-lactamase inhibition. GC–MS analysis identified linoleic (50.98%), stearic (19.31%), palmitic (4.20%), and oleic acids (2.26%) as major constituents. The *A. paniculatus* root bioactive fraction significantly potentiated cefotaxime's activity, likely via membrane permeability enhancement or enzyme inhibition, supporting its potential as a resistance-modifying agent against antimicrobial resistance.

Keywords: *Adenodolichos paniculatus,* antibacterial, bioactive fraction, cefotaxime, ESBL, phytochemicals, root, synergy.

INTRODUCTION

Antimicrobial resistance (AMR) remains one of the most pressing global public health challenges of the 21st century, significantly undermining the effectiveness of commonly used antibiotics and increasing morbidity, mortality, and healthcare expenditures worldwide. Recent global estimates indicate that bacterial AMR was directly responsible for approximately 1.27 million deaths in 2019 and associated with nearly 5 million deaths globally, with the highest burden occurring in low- and middle-income countries (LMICs) (Murray *et al*., 2022). The World Health Organisation (WHO) has consistently identified AMR as one of the top ten global public health threats, emphasising its growing impact on health systems, food security, and economic development (WHO, 2023b).

Of particular concern is the rapid emergence and dissemination of extended-spectrum β-lactamase (ESBL)-producing members of the family *Enterobacteriaceae*, especially *Escherichia coli* and *Klebsiella pneumoniae*. ESBL enzymes hydrolyse third-generation cephalosporins and monobactams, thereby significantly limiting therapeutic

options (Bush & Bradford, 2020; Paterson and Bonomo, 2021). Infections caused by ESBL-producing bacteria are associated with prolonged hospitalisation, increased treatment failure, higher healthcare costs, and elevated mortality rates, particularly in LMIC settings where access to carbapenems and newer β-lactam/β-lactamase inhibitor combinations remains restricted (Tamma *et al*., 2022; WHO, 2023b).

Cefotaxime, a third-generation cephalosporin, has historically played a central role in the treatment of Gram-negative infections due to its broad-spectrum activity and favourable pharmacokinetics. However, its clinical efficacy has been substantially compromised by the global spread of CTX-M-type ESBL enzymes, which efficiently hydrolyse cefotaxime and related β-lactams (Bevan *et al*., 2017; Bush and Bradford, 2020). Given the slow pace of novel antibiotic development and the economic barriers associated with introducing new agents, there is increasing interest in strategies aimed at restoring the activity of existing antibiotics rather than replacing them (WHO, 2023a; The Lancet Microbe Commission, 2023).

One such strategy involves the use of plant-derived extracts or bioactive fractions as antibiotic adjuvants. Unlike conventional antibiotics that exert direct bactericidal or bacteriostatic effects, phytochemicals may enhance antibiotic activity by modulating bacterial resistance pathways. Proposed mechanisms include disruption of membrane integrity, inhibition of β-lactamase enzymes, suppression of efflux pumps, and interference with quorum sensing systems (Hemeg, 2022; Silva *et al*., 2021). These resistance-modifying effects can generate synergistic interactions when combined with conventional antibiotics, resulting in reduced minimum inhibitory concentrations (MICs) and restoration of antibiotic susceptibility in resistant strains (Dziri *et al*., 2025).

Despite growing interest in plant–antibiotic synergy, several critical limitations persist in the current literature. Many studies rely on crude extracts rather than enriched bioactive fractions, making mechanistic interpretation difficult and limiting reproducibility. Additionally, numerous investigations employ reference laboratory strains instead of clinically confirmed ESBL-producing isolates, thereby reducing clinical relevance. In several reports, synergy assessment is restricted to a single methodological approach, often lacking quantitative validation through fractional inhibitory concentration index (FICI) determination or complementary time–kill kinetic analysis. Consequently, the resistance-modifying potential of specific phytochemical fractions against well-characterised ESBL-mediated β-lactam resistance remains insufficiently defined.

Adenodolichos paniculatus (Fabaceae) is a medicinal plant traditionally used in African ethnomedicine for the management of infectious and inflammatory conditions, including wounds, gastrointestinal infections, and febrile illnesses. Phytochemical investigations have identified secondary metabolites such as flavonoids, phenolics, terpenoids, and fatty acids, compounds widely associated with antimicrobial and resistance-modifying activities in other plant species (Kuete, 2020; Silva *et al*., 2021). However, despite its ethnopharmacological relevance, no prior study has systematically evaluated the synergistic interaction between a bioactive root fraction of *A. paniculatus* and a third-generation cephalosporin against phenotypically confirmed ESBL-producing *Enterobacteriaceae* clinical isolates. Moreover, its capacity to function as a β-lactam resistance-modifying agent capable of restoring cefotaxime efficacy has not been experimentally demonstrated.

Therefore, the present study was designed to address these knowledge gaps by evaluating the synergistic antibacterial activity of a bioactive root fraction of *A. paniculatus* in combination with cefotaxime against clinically characterised ESBL-producing *Enterobacteriaceae*. By integrating phytochemical screening, antimicrobial susceptibility profiling, checkerboard microdilution assays, fractional inhibitory concentration index (FICI) determination, time–kill kinetic analysis, and compound profiling, this study provides a comprehensive assessment of both the extent and temporal dynamics of synergy. Through this multi-method approach, the work aims to generate mechanistic and quantitative evidence supporting the role of *A. paniculatus* as a potential phytochemical-based resistance-modifying adjuvant for restoring β-lactam activity against ESBL-mediated resistant pathogens.

MATERIALS AND METHODS

Plant collection and extraction

Fresh root specimens were harvested from the wild plant in Pushit, Mangu Local Government Area, Plateau State, Nigeria, from October to November 2025. The plant material was authenticated at the Department of Plant Science, University of Jos, and a voucher specimen (UJ205) was deposited in the departmental herbarium for future reference. The collected roots were washed, air-dried at room temperature, and pulverised into a coarse powder. The dried, powdered roots (500 g) were macerated in 1.25 L of chloroform for 72 h with intermittent shaking. The filtrate was concentrated using a rotary vacuum evaporator at 40 °C to dryness, yielding a dark greenish-brown crude extract. The extract was weighed, and the percentage yield was calculated based on the dry weight of plant material. The dried extract was stored in sterile, pre-weighed universal bottles at 4°C until required for microbiological assays.

Phytochemical screening

Standard qualitative assays as described by Banu and

Catherine (2023) was performed to detect alkaloids, flavonoids, phenolics, terpenoids, steroids, tannins, saponins, and glycosides.

Fractionation of root extract

The most active chloroform root extract of *Adenodolichos paniculatus* was fractionated using the vacuum liquid chromatography (VLC) method of Coll and Bowden as modified by Oluah *et al*. (2022). The crude root extract was subjected to solvent-partition fractionation using solvents of increasing polarity. Thin-layer chromatography (TLC) was employed to monitor separation using silica gel plates and appropriate solvent systems. Fractions exhibiting similar TLC profiles were pooled, concentrated, and designated as bioactive fractions. The percentage yield of each fraction was calculated relative to the crude extract. The most active pooled fraction was further purified by repeated preparative TLC (Prep-TLC) on silica gel plates (20 × 20 cm, 0.5 mm thickness) developed in hexane: ethyl acetate (80:20). Visible bands under UV light (254/364 nm) were scraped off, extracted with chloroform, and filtered. The eluates were concentrated, weighed, and assessed for antibacterial.

Antibiotic and reagents

The referenced antibiotic, cefotaxime 1gm powder for injection (manufactured by Alliance Botech) was purchased from H-Medix Pharmacy, Abuja. Cefotaxime sodium for injection was reconstituted and used as a reference antibiotic. In resource-limited settings, pharmaceutical-grade injectable formulations are commonly employed for *in vitro* antimicrobial studies when certified analytical standards are unavailable. The preparation complied with British Pharmacopoeia (2023) specifications, and concentrations were prepared using sterile water for injection under aseptic conditions (BP, 2023). Stock solutions (1 mg/mL) were prepared in sterile water, filter-sterilised (0.22 µm), aliquoted, and stored at 20 °C. Culture media (Oxoid) were purchased from a commercial Lab in Jos. Mueller-Hinton agar (Thermo Fisher Scientific-Uk), disks (Oxoid) and Dimethyl sulfoxide (Merck-Germany).

Bacterial isolates and reference strains

Clinical isolates of Gram-negative Enterobacterales, with a focus on potential ESBL producers, were obtained from the Department of Laboratory Services, Federal Medical Centre, Keffi (October 2025). The collection included *Escherichia coli* (n = 10), *Klebsiella pneumoniae* (n = 6), and *Proteus mirabilis* (n = 2). Reference strains used were *E. coli* ATCC 25922 (antimicrobial susceptibility control), *K. pneumoniae* ATCC 700603 (ESBL-positive control), and *P. mirabilis* ATCC 35659 (quality control). Strains were obtained from the Department of Diagnostic Laboratory, National Veterinary Institute, K-Vom. ESBL production was confirmed using the combined disk method according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Bacterial purification and characterisation

The clinical isolates were streaked on Blood agar (BA) and MacConkey agar (MAC) plates using the quadrant streak method to obtain isolated colonies. Plates were incubated aerobically at 37 °C for 24 h. MacConkey agar (MAC), *E. coli* produced pink colonies (lactose fermenters) and occasionally exhibited a green metallic sheen on EMB agar. *K. pneumoniae* formed large, mucoid, pink colonies, indicative of lactose fermentation and capsule production, whereas *P. mirabilis* produced pale, non-lactose-fermenting colonies. On Blood agar, *E. coli* was generally non-hemolytic, though occasional β-hemolytic strains were observed. *K. pneumoniae* produced non-hemolytic mucoid colonies, while *P. mirabilis* showed characteristic swarming motility, forming concentric growth rings and emitted a distinct odour.

For purification, a single well-isolated colony was re-streaked onto fresh BA and MAC plates and incubated for another 24 h at 37°C. This process was repeated for a second single-colony streak to achieve pure cultures. Purity was confirmed by Gram staining, which demonstrated uniform Gram-negative rods with consistent colony morphology. *Escherichia coli* appeared as small to medium-sized rods occurring singly or in short chains; *Klebsiella pneumoniae* appeared as plump, short rods often in pairs, with a prominent mucoid capsule visible on wet mount preparations, while *Proteus mirabilis* appeared as slender rods exhibiting high motility in wet mounts. Motility testing showed that *E. coli* was motile, *K. pneumoniae* was non-motile, and *P. mirabilis* was highly motile. Biochemical identification of the isolates was performed using conventional tests (Triple sugar iron, indole, urease, oxidase, citrate), and reactions were compared to standard reference patterns for Enterobacterales (CLSI, 2025).

Preparation of the standard McFarland

The solution of 0.5 ml of 0.048 M $BaCl_2.2H_2O$ was added to 99.5 ml of a 0.18M H_2SO_4 solution and vortex for 2 minutes. A UV-Vis spectrophotometer was used to measure the absorbance of the solution at 625 nm. An absorbance of 0.1 was obtained, which was in the accepted range of 0.08 to 0.13. This standard (0.5) was used to make a visual comparison with the density of the suspension against a white background with black lines.

Preparation and standardisation of inoculum

The inoculum was standardised by using the Clinical and Laboratory Standards Institute (CLSI, 2025). Bacterial suspensions were adjusted to the McFarland standard of 0.5 (1.0 x 10^8cfu/ml) and subsequently diluted to obtain a final inoculum of approximately 1.0 x 10^5cfu/ml for checkerboard and time-kill assays in accordance with CLS recommendations. The turbidity of the cell culture was matched with that of the 0.5 McFarland standard by holding the mixture and the standard in front of light against a white background with contrasting black lines through visual comparison with its density by the addition of normal saline.

Antimicrobial susceptibility testing

Susceptibility was determined by the disk diffusion method on Mueller Hinton agar (MHA) according to CLSI M100 guidelines (35th ed., 2025). Inocula were standardised to a 0.5 McFarland turbidity (1 × 10^8 CFU/mL) and spread evenly over MHA plates. Disks of cefazolin (30 µg), cefuroxime (30 µg), and cefotaxime (30 µg) were applied, and the plates incubated at 37°C for 24 h. Inhibition zones were measured in millimetres and interpreted using CLSI breakpoints. Interpretive categories (S/I/R) followed CLSI criteria for Enterobacterales:

Cefotaxime & Cefuroxime: S ≥ 23 mm; I = 15–22 mm; R ≤ 14 mm, Cefazolin: S ≥ 23 mm; I = 20–22 mm; R ≤ 19 mm. Quality control strains (*E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, *P. mirabilis* ATCC 35659) were included in all assays.

Extended-Spectrum β-Lactamase (ESBL) confirmatory testing

Phenotypic confirmation of ESBL production was performed using the Combination Disk Test (CDT) in accordance with CLSI (M100, 2025). Standardised inocula (0.5 McFarland) were inoculated onto MHA plates, and disks containing cefotaxime (30 µg) and cefotaxime–clavulanic acid (30/10 µg) were placed 25 mm apart (centre-to-centre). After incubation at 37 °C for 24 h, isolates were considered ESBL-positive when the inhibition zone around cefotaxime–clavulanic acid exceeded that of cefotaxime alone by ≥5 mm.

Determination of synergistic activity of cefotaxime with the root bioactive component

Determination of disk diffusion synergy

The interaction between the root bioactive component and cefotaxime was initially screened using a modified disk diffusion method, according to CLSI M100 guidelines (35th ed., 2025) standards. Fresh overnight cultures were suspended in sterile saline and adjusted to 0.5 McFarland (1 × 10^8 CFU/mL). Mueller–Hinton agar (MHA) plates were swabbed in three directions to achieve confluent lawns, then allowed to dry (3 min). Cefotaxime disks (30 µg) were placed on the agar surface. For combination testing, 10 µL of root bioactive fraction (equivalent to 1 mg/disc) was carefully pipetted onto the antibiotic disk and air-dried. Control plates included an antibiotic alone, a root bioactive fraction alone, and solvent-only disks. Plates were incubated at 37°C for 24 h. Zones of inhibition were measured (mm). An increase of ≥5 mm in diameter for antibiotic + root bioactive fraction compared with antibiotic alone was interpreted as synergy; <5 mm as indifferent; and a reduction as antagonism. Each assay was performed in triplicate, and results were expressed as mean ± SD. Quality control organisms were required to fall within CLSI-expected ranges.

Determination of checkerboard MICs and FIC index

Quantitative assessment of synergy was performed using a checkerboard broth microdilution method in 96-well plates. Two-fold serial dilutions of cefotaxime (0.125–256 µg/mL) were prepared across plate columns, while two-fold dilutions of root bioactive fraction (0.125–64 mg/mL) were prepared across rows, to generate all possible combinations. Each well received a standardised bacterial suspension in Mueller Hinton broth to a final inoculum of 1 × 10^5 CFU/mL). Growth control (no drug), sterility control (no inoculum), and solvent control wells were included. Plates were incubated at 37°C for 24 h. MICs were defined as the lowest concentrations showing no visible growth. The MIC of each agent alone was determined from its single-agent wells, while combination MICs were determined from the lowest inhibitory well containing both agents.

FIC index calculation: FIC_A = (MIC of cefotaxime in combination) ÷ (MIC of cefotaxime alone), FIC_B = (MIC of component in combination) ÷ (MIC of root component alone). FICI = FIC_A + FIC_B Interpretation: FICI ≤0.5 = synergy; 0.5–1.0 = additive; 1.0–4.0 = indifferent; >4.0 = antagonism.

Each experiment was performed in duplicate wells and repeated three times. Results were reported as mean MIC values with corresponding FICI.

Time–kill assays

Assays for the rate of killing bacteria by the cefotaxime alone, the root bioactive fraction alone and cefotaxime +

root bioactive fraction were carried out using the standard method of Clinical and Laboratory Standards Institute (CLSI document M26-A, 2020). To 1.0 ml of each standardized culture (cultures of 1 × 10^8 CFU/mL were further diluted to 1 × 10^5) of *E. coli* CI-3 and *K. pneumoniae* CI-6 in three test tubes was added 9.0 ml of Mueller Hinton broth incorporated with the cefotaxime alone, root component alone and cefotaxime + root bioactive fraction to the first and second tubes at 1x MIC, ½ x MIC and 1 x MIC + ½ x MIC and the third one was considered as the growth control. The tubes were incubated at 37°C in a water bath on an orbital shaker at 120 rpm. At pre-determined time intervals of 0, 2, 4, 8, and 24 h, 1 ml of the admixture was withdrawn and diluted ten-fold with 9 ml sterile normal saline containing 3 % Tween 80, 5% yeast extract and 0.3 % egg lecithin. Exactly 0.1 ml of each dilution was aseptically plated out in triplicate. Plates were incubated at 37°C for 24 hours. The number of viable organisms was counted as cfu/plates. Average triplicate counts were multiplied by the dilution factor to arrive at cfu/ml. Colonies were enumerated after 24 h. Synergy was defined as a ≥2 log10 CFU/mL reduction in the combination compared with the most active single agent. The use of ½ MIC in the time–kill assay allows assessment of sub-inhibitory effects and facilitates detection of synergistic interactions. Sub-MIC concentrations are particularly useful in combination studies because they permit observable bacterial growth, thereby allowing enhanced bactericidal activity of the combination to be accurately quantified.

GC–MS analysis

Gas chromatography–mass spectrometry (GC–MS) analysis of the bioactive fraction was performed to identify major chemical constituents. Compounds were identified by comparison of mass spectra with standard libraries.

Data analysis

All experiments were carried out in triplicate, and results were expressed as mean ± standard deviation (SD). Comparisons were between treatment groups at identical time points; ANOVA was used only for time-kill comparisons.

RESULTS

Qualitative phytochemical constituents of the chloroform root extract

From the phytochemical analysis of the chloroform root extract, different classes of phytochemicals were identified, as presented in Table 1. Six phytochemicals, namely alkaloids, flavonoids, phenolics, saponins, steroids and terpenoids were all present in the chloroform root extract. The predominance of alkaloids, flavonoids, phenolics, and terpenoids in the chloroform extract was significant.

Identified phytochemicals from the library search report of the chromatogram of the root component

The GC–MS chromatogram of the root bioactive component of *A. paniculatus* revealed several bioactive phytochemicals, predominantly fatty acids and their methyl esters. The major constituents were 9,12-octadecadienoic acid (linoleic acid, 50.98%), octadecanoic acid (stearic acid, 19.31%), and n-hexadecanoic acid (palmitic acid, 4.20%), alongside oleic acid (2.26%) and 3-methylphenol (2.35%) as presented in Table 2.

Antimicrobial susceptibility of selected antibiotics against test bacteria

Of 10 clinical isolates tested, susceptibility patterns varied across the antibiotics, as presented in Table 3. Five isolates were susceptible to cefotaxime, two were intermediate, and three were resistant. Cefuroxime exhibited six susceptible, two intermediate, and two resistant isolates, while cefazolin showed a higher proportion of intermediate or resistant results. Reference strains demonstrated expected susceptibility/resistance profiles.

Extended-Spectrum β-Lactamase (ESBL) confirmatory

The result of the cefotaxime vs cefotaxime–clavulanic acid combination disk is presented in Table 4 and Figure 1. Two isolates, *E. coli* CI-3 and *K. pneumoniae* CI-6, were confirmed as ESBL producers (≥8 mm increase in inhibition zone). *P. mirabilis* CI-10, though resistant to cefotaxime, did not meet ESBL confirmation criteria, suggesting an alternative resistance mechanism. Control strains performed as expected, with ATCC 700603 positive and ATCC 25922/35659 negative.

Combination disk diffusion synergistic activity

The effect of root bioactive fraction on cefotaxime activity was evaluated against ESBL-producing and susceptible strains, and the results were as presented in Table 5 and Figure 2. In *E. coli* CI-3 and *K. pneumoniae* CI-6, a combination of cefotaxime with the extract produced marked increases in inhibition zones from 10 to 18 mm and 9 to 17 mm, respectively, corresponding to an increase of

Table 1. Qualitative phytochemical constituents of the chloroform root extract.

Phytochemical constituents	Description	Interpretation
Alkaloids	+	Present
Flavonoids	+	Present
Tannins	–	Absent
Saponins	+	Present
Terpenoids	+	Present
Steroids	+	Present
Phenolics	+	Present
Glycosides	–	Absent

(+ = detected; – = not detected).

Table 2. Identified phytochemicals from the library search report of the chromatogram.

Peak number	Retention time	Area (%)	Bioactive compound
1	48.198	0.55	Hexadecanoic acid
2	48.392	*4.20	n-Hexadecanoic acid
3	50.834	1.20	Butyraldehyde
4	53.587	1.35	9,12-Octadecadienoic acid, methyl ester
5	53.82	1.87	16-Octadecenoic acid, methyl ester
6	54.479	0.51	Heptadecanoic acid, 16-methyl-, methyl ester
7	54.479	1.51	Methyl 8,10-dimethyl-hexadecanoate
8	54.479	1.51	Tetradecanoic acid, 12-methyl-, methyl ester
9	55.797	*50.98	9,12-Octadecadienoic acid
10	56.262	*19.31	Octadecanoic acid
11	59.286	0.98	6-Heptenoic acid
12	59.286	0.98	4,9-Decadienoic acid, 2-nitro-, ethyl ester
13	59.286	0.98	Cycloheptane methanol
14	60.526	1.13	Cyclopentane undecanoic acid
15	60.526	1.13	1-hexadecanesulfonamide, N-(2-aminoethyl)-
16	61.147	0.98	4,9-Decadienoic acid, 2-nitro-, ethyl ester
17	61.883	2.35	3-methylphenol
18	64.442	0.05	Phenol, 2-(2-aminoethyl)-
19	64.907	1.32	1H-Imidazole, 1-(2-propenyl)-
20	64.907	1.32	2-Heptenoic acid
21	64.907	1.32	Chloromethyl 8-chlorononanoate
22	65.295	1.27	E-11-Tetradecenoic acid
23	65.682	0.92	Undecylenic acid
24	67.35	2.26	Oleic acid

8 mm and interpreted as synergy. For *P. mirabilis* CI-10 (non-ESBL), zone size increased from 12 mm to 19 mm, corresponding to an increase of 7 mm, also consistent with synergy. In contrast, susceptible *E. coli* (S-1) and QC strain *E. coli* ATCC 25922 showed negligible increases of plus1 mm and 0 mm, respectively, interpreted as indifferent.

Checkerboard minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices

The checkerboard microdilution assay revealed clear synergistic interactions between the root bioactive fraction of *Adenodolichos paniculatus* and cefotaxime against both extended-spectrum β-lactamase (ESBL)–producing and non-ESBL reference strains (Table 6 and Figure 3).

For *Escherichia coli* CI-3 (ESBL), the minimum inhibitory concentration (MIC) of cefotaxime alone was 64 µg/mL, which decreased markedly to 4 µg/mL when combined with the root bioactive fraction. The MIC of the bioactive fraction alone also dropped from 16 to 2 mg/mL in combination. Similarly, *Klebsiella pneumoniae* CI-6 (ESBL) exhibited a reduction in cefotaxime MIC from 128 µg/mL to 8 µg/mL, and the bioactive fraction MIC declined from 32 to 4 mg/mL. The fractional inhibitory concentration

Table 3. Antimicrobial susceptibility of selected antibiotics against test bacteria.

ID	Species	Cefazolin 30 µg	Cefuroxime 30 µg	Cefotaxime 30 µg
CI-1	*Escherichia coli*	27 mm — S	26 mm — S	30 mm — S
CI-2	*E. coli*	21 mm — I	18 mm — I	20 mm — I
CI-3	*E. coli*	19 mm — R	12 mm — R	10 mm — R
CI-4	*E. coli*	24 mm — S	25 mm — S	28 mm — S
CI-5	*E. coli*	18 mm — R	24 mm — S	25 mm — S
CI-6	*Klebsiella pneumoniae*	14 mm — R	11 mm — R	9 mm — R
CI-7	*K. pneumoniae*	20 mm — I	16 mm — I	17 mm — I
CI-8	*K. pneumoniae*	25 mm — S	24 mm — S	26 mm — S
CI-9	*Proteus mirabilis*	22 mm — I	23 mm — S	24 mm — S
CI-10	*P. mirabilis*	16 mm — R	13 mm — R	12 mm — R
QC-Ec	*E. coli* ATCC 25922	26 mm — S	28 mm — S	30 mm — S
QC-Kp	*K. pneumoniae* ATCC 700603	15 mm — R	11 mm — R	9 mm — R
QC-Pm	*P. mirabilis* ATCC 35659	24 mm — S	25 mm — S	28 mm — S

Interpretation: CLSI M100 (2025) disk diffusion breakpoints for Enterobacterales. Susceptible (S): ≥23 mm; Intermediate (I): 15–22 mm; Resistant (R): ≤14 mm (cefotaxime/cefuroxime). For cefazolin: S ≥23 mm; I = 20–22 mm; R ≤19 mm.

Table 4. Extended-Spectrum β-Lactamase (ESBL) confirmatory.

ID	Species	CTX_30_mm	CTX_CLA_ 30_10_mm	Diff in zones mm	ESBLInterpretation
CI-1	*E. coli*	30	31	1	Negative
CI-2	*E. coli*	20	21	1	Negative
CI-3	*E. coli*	10	18	8	**Positive (ESBL confirmed)**
CI-4	*E. coli*	28	29	1	Negative
CI-5	*E. coli*	25	26	1	Negative
CI-6	*K. pneumoniae*	9	17	8	**Positive (ESBL confirmed)**
CI-7	*K. pneumoniae*	17	18	1	Negative
CI-8	*K. pneumoniae*	26	27	1	Negative
CI-9	*P. mirabilis*	24	25	1	Negative
CI-10	*P. mirabilis*	12	13	1	**Negative (resistant but not ESBL by CTX/CLA)**
QC-Ec	*E. coli* (ATCC 25922)	30	30	0	QC: expected negative
QC-Kp	*K. pneumoniae* (ATCC 700603)	9	20	11	**QC positive (ESBL)**
QC-Pm	*P. mirabilis* (ATCC 35659)	28	28	0	QC: expected negative

Interpretation: CLSI criterion for ESBL positivity is an increase of ≥5 mm in zone diameter with CTX/CLA compared to CTX alone. QC strains confirmed assay validity. CTX= cefotaxime, CTX/CLA= cefotaxime–clavulanic acid.

concentration indices (FICI) for both isolates were 0.25, confirming a strong synergistic interaction.

For the control (antibiotic-susceptible) strains, the observed reductions were smaller but still within the synergistic range. *E. coli* ATCC 25922 showed cefotaxime MIC reduction from 1 µg/mL to 0.25 µg/mL, and bioactive fraction MIC from 8 mg/mL to 2 mg/mL (FICI = 0.375). *K. pneumoniae* ATCC 700603 displayed a reduction in cefotaxime MIC from 2 µg/mL to 0.5 µg/mL and extract MIC from 16 mg/mL to 4 mg/mL (FICI = 0.375).

Overall, the combination therapy resulted in up to a 16-fold reduction in cefotaxime MIC among resistant isolates. FICI values ≤ 0.25 quantitatively confirmed synergy, while values around 0.375 still indicated significant interaction. These findings demonstrate that *A. paniculatus* root bioactive fraction strongly potentiates the activity of cefotaxime, particularly against ESBL-producing strains.

Time–Kill assay

The results of the time-kill assay, which summarised the bacterial counts across treatment groups at different time points, while the corresponding time–kill curves illustrate the growth dynamics and synergistic effect of the combination compared with single-agent exposures. The data for *E. coli* and *K. pneumoniae* were presented in Tables 7 and 8; Figures 4 and 5, respectively. The Bacterial

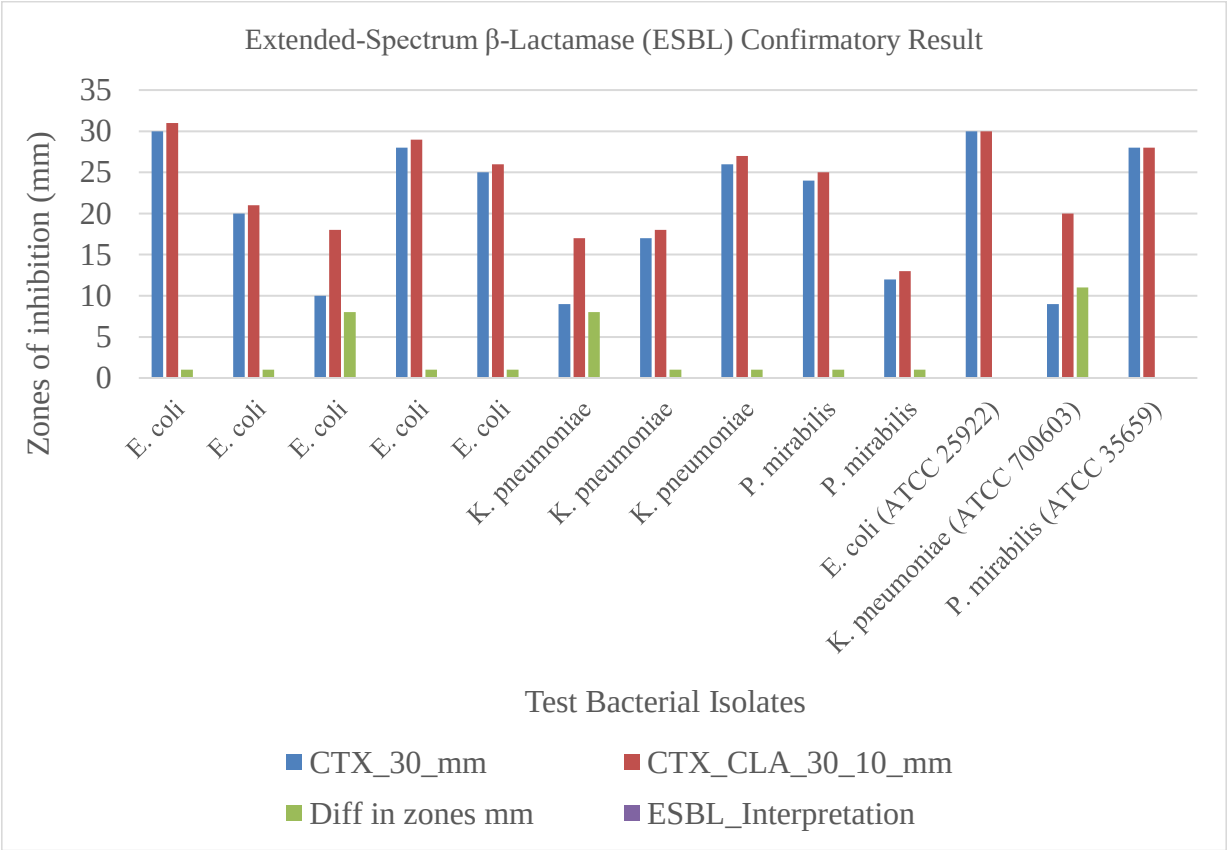

Figure 1. Extended-spectrum β-Lactamase (ESBL) confirmatory result.

Table 5. Combination disk diffusion synergistic activity.

ID	Species	Bacteria/Zone of inhibition (mm)			Interpretation
		Cefotaxime alone	Cefotaxime + bioactive fraction	Difference in zones	
CI-3	*E. coli* (ESBL)	10	18	8	Synergy
CI-6	*K. pneumoniae* (ESBL)	9	17	8	Synergy
CI-10	*P. mirabilis* (non-ESBL)	12	19	7	Synergy
S-1	*E. coli* (susceptible)	30	31	1	Indifferent
QC-Ec	*E. coli* ATCC 25922	30	30	0	Indifferent

Synergy is defined as ≥5 mm increase in inhibition zone with extract + antibiotic compared to antibiotic alone. Minimal or no increase interpreted as indifferent.

Table 6. Checkerboard minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices.

Organism	MIC Cefotaxime alone (µg/mL)	MIC Root bioactive fraction alone (mg/mL)	MIC Combination (µg/mL + mg/mL)	FICI	Interpretation
E. coli CI-3 (ESBL)	64	16	4.0 + 2.0	0.25	Synergy
K. pneumoniae CI-6 (ESBL)	128	32	8.0 + 4.0	0.25	Synergy
E. coli ATCC 25922 (QC)	1	8	0.25 + 2.0	0.375	Synergy
K. pneumoniae ATCC 700603 (QC)	2	16	0.5 + 4.0	0.375	Synergy

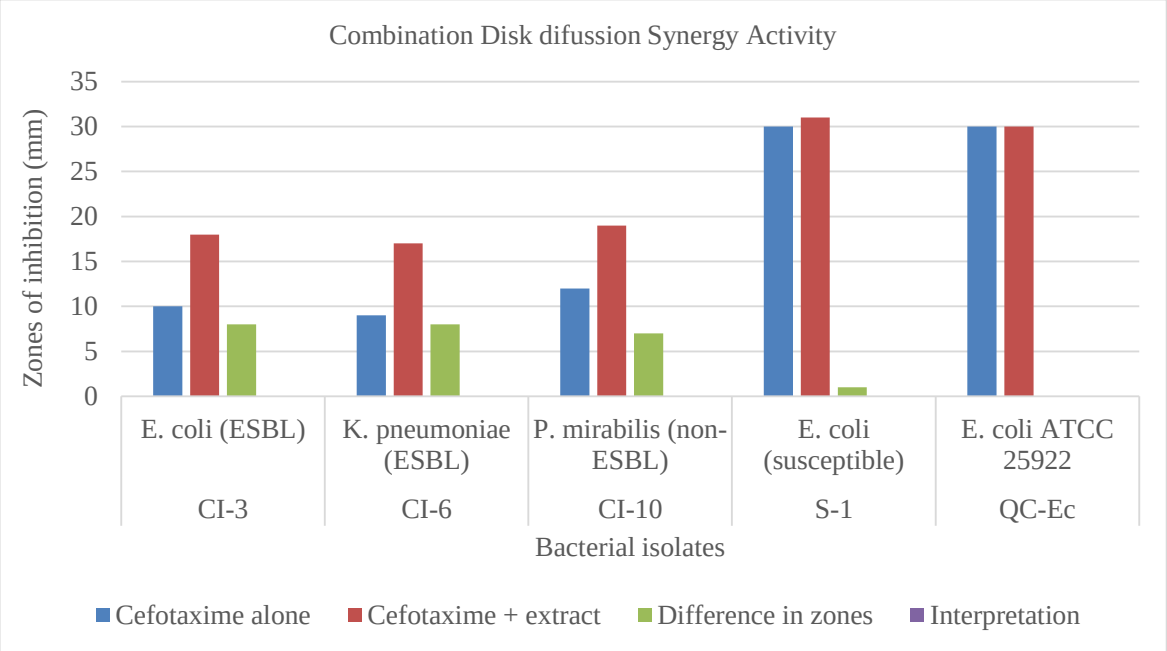

Figure 2. Combination disk diffusion synergistic activity results.

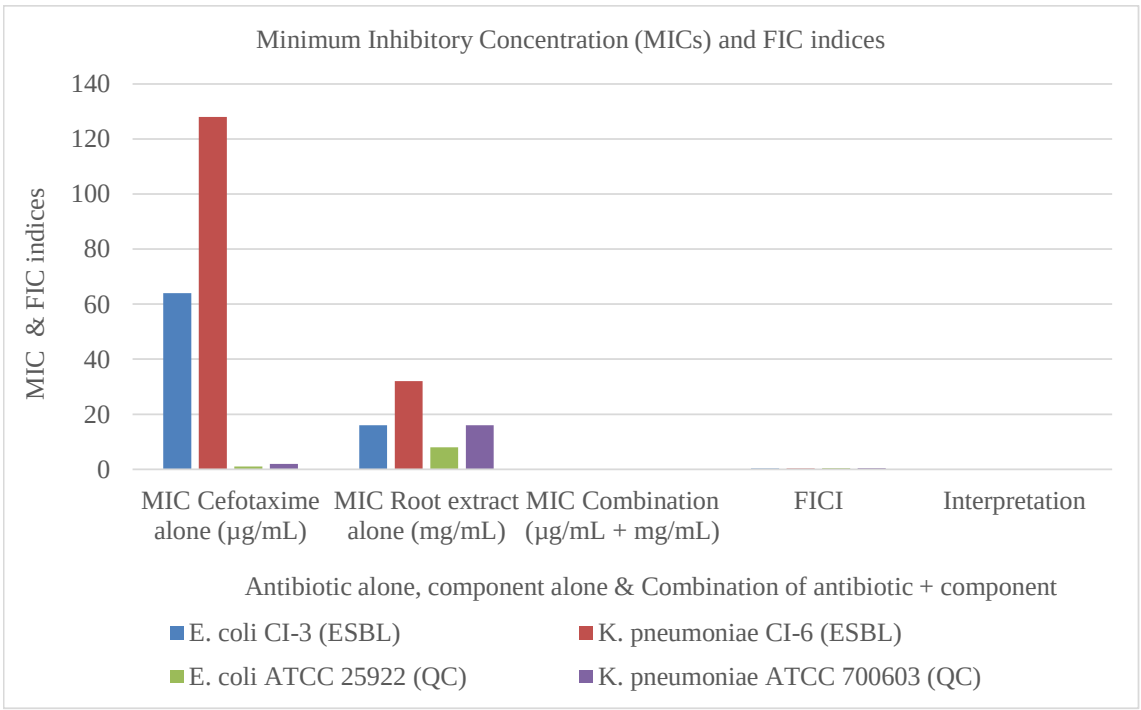

Figure 3. Checkerboard Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration (FIC) indices

counts were measured at 0, 2, 4, 8, and 24 h following exposure to cefotaxime (1× MIC), *Adenodolichos paniculatus* root bioactive fraction (½× MIC), the combination (1× MIC + ½× MIC) and an untreated control. Data are expressed as mean $\log_{10}$ CFU/mL ± SD (n = 3). The untreated growth controls demonstrated robust proliferation, with bacterial counts increasing from 5 × 10^5 CFU/mL at 0 h to ≥10^8 CFU/mL at 24 h, confirming culture

Table 7. Time–kill assay results of root component in combination with cefotaxime against *E. coli*.

Time (h)	Growth control	Cefotaxime alone (1 x MIC	Fraction alone (½ x MIC	Cefotaxime + Fraction (1 x MIC + 1/2 x MIC
0	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
2	6.5 ± 0.1	5.2 ± 0.1	5.4 ± 0.2	3.8 ± 0.1
4	7.2 ± 0.2	5.2 ± 0.1	4.8 ± 0.2	2.6 ± 0.2
8	8.0 ± 0.2	4.8 ± 0.1	4.6 ± 0.3	1.5 ± 0.2
24	9.0 ± 0.3	6.0 ± 0.2	6.2 ± 0.2	0.8 ± 0.2

Table 8. Time–kill assay results of root component in combination with cefotaxime against *K. pneumoniae*.

Time (h)	Growth control	Cefotaxime alone (1 x MIC	Fraction alone (½ x MIC	Cefotaxime + Fraction (1 x MIC + 1/2 x MIC
0	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
2	6.3 ± 0.1	5.4 ± 0.1	5.1 ± 0.2	3.9 ± 0.1
4	7.0 ± 0.2	5.1 ± 0.1	4.9 ± 0.2	2.8 ± 0.2
8	7.8 ± 0.2	4.9 ± 0.1	4.7 ± 0.3	1.6 ± 0.2
24	8.9 ± 0.3	6.3 ± 0.2	6.5 ± 0.2	0.9 ± 0.2

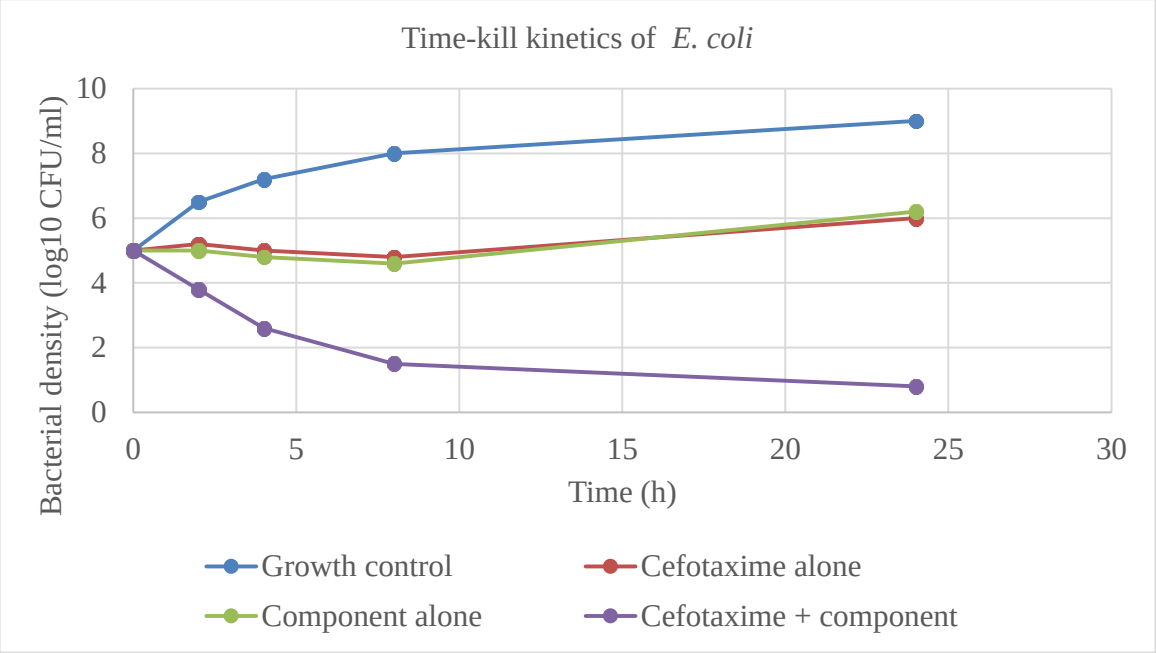

Figure 4. Time–kill kinetics of root component in combination with cefotaxime against *E. coli*.

viability and the suitability of assay conditions. Cefotaxime at 1× MIC exerted a bacteriostatic effect, maintaining counts close to the initial inoculum level over the 24 h period, while *A. paniculatus* root bioactive fraction at ½× MIC slowed but did not prevent bacterial growth. In contrast, the combination of cefotaxime (1× MIC) and *A. paniculatus* root bioactive fraction (½× MIC) resulted in a sustained decline in viable counts, achieving a >2 $\log_{10}$ CFU/mL reduction compared with the most active single agent at 24 h. This interaction met the predefined criterion for synergy.

Growth controls displayed normal logarithmic increase from 5 × 10^5 CFU/mL to ≥10^8 CFU/mL within 24 h, confirming culture viability and suitability of assay conditions. Cefotaxime (1× MIC) and *A. paniculatus* component (½× MIC) produced only partial suppression, whereas the combination achieved >2 $\log_{10}$ CFU/mL reduction at 24 h, indicating synergy.

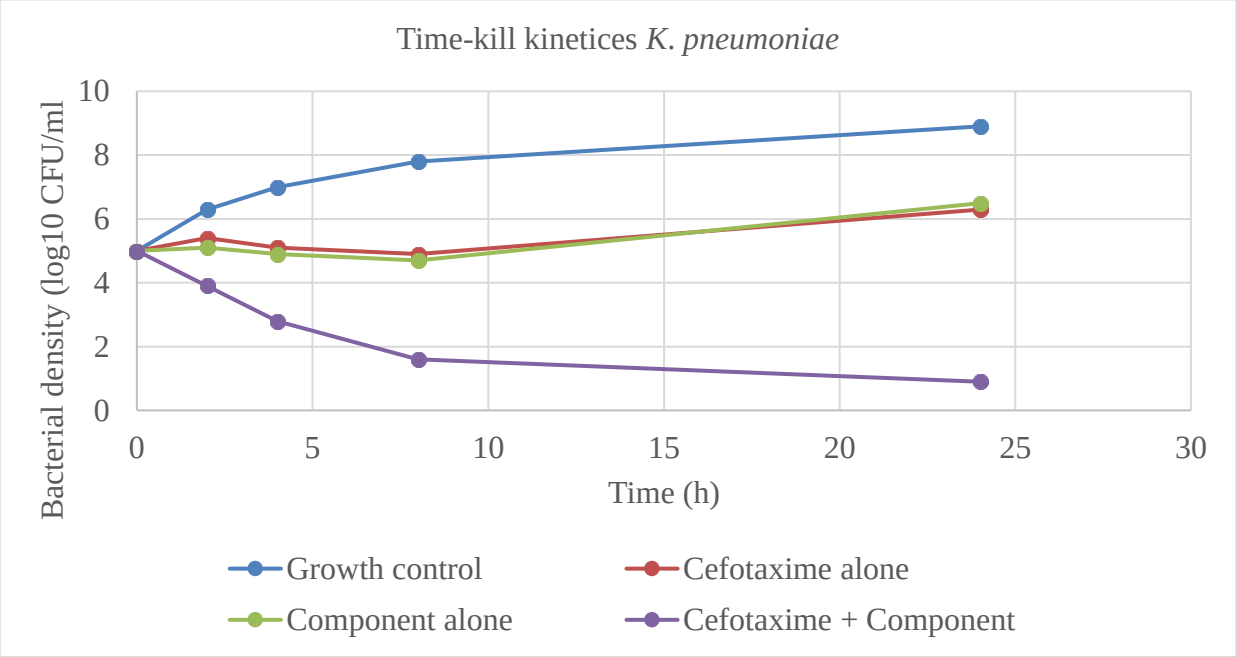

Figure 5. Time–kill kinetics of root component in combination with cefotaxime against *K. pneumoniae*.

DISCUSSION

Phytochemical screening of the chloroform root extract of *Adenodolichos paniculatus* revealed the presence of alkaloids, flavonoids, phenolics, terpenoids, saponins, and steroids. These classes of compounds are widely recognised for their antimicrobial and resistance-modifying properties. Alkaloids and flavonoids have been reported to inhibit bacterial efflux pumps and biofilm formation, thereby restoring antibiotic efficacy (Cushnie and Lamb, 2011; Stavri *et al*., 2007), while phenolics and terpenoids can disrupt bacterial membranes, increasing antibiotic permeability (Cowan, 1999). Thus, the observed synergy between cefotaxime and the root bioactive fraction likely results from multiple concurrent mechanisms that weaken bacterial defence systems, allowing enhanced antibiotic penetration and activity.

The GC–MS chromatogram of the chloroform root bioactive fraction of *Adenodolichos paniculatus* revealed several bioactive phytochemicals, predominantly fatty acids and their methyl esters. The major constituents were 9,12-octadecadienoic acid (linoleic acid, 50.98%), octadecanoic acid (stearic acid, 19.31%), and n-hexadecanoic acid (palmitic acid, 4.20%), alongside oleic acid (2.26%) and 3-methylphenol (2.35%). These compounds have been previously documented for their antibacterial properties in other Fabaceae species (Dilika *et al*., 2020). Fatty acids such as linoleic, palmitic, and stearic acids disrupt bacterial cell membranes and inhibit essential enzymatic functions, while phenolic compounds like 3-methylphenol enhance permeability and oxidative stress. Although fatty acids such as linoleic and oleic acids are widely distributed in plants, their role as resistance-modifying agents has been reported, particularly through membrane permeation and enhanced intracellular antibiotic accumulation. Therefore, the antibacterial activity observed in the *A. paniculatus* root bioactive component may be attributed to the synergistic action of these major phytochemicals (Desbois and Smith, 2022).

The observed pronounced synergistic interactions between cefotaxime and the extract against ESBL-producing *E. coli* and *K. pneumoniae* are consistent with previous reports demonstrating that phytochemicals exhibit enhanced antibiotic-modifying effects in resistant bacterial strains (Hemeg, 2022; Dziri *et al*., 2025). The lack of synergy in susceptible strains suggests that the extract may function primarily as a resistance-modifying agent rather than exerting nonspecific additive antibacterial activity. Furthermore, the observed synergy against cefotaxime-resistant but ESBL-negative *P. mirabilis* indicates that the extract may interfere with alternative resistance mechanisms, including membrane permeability or non-ESBL β-lactamase activity, as previously described (Bush and Bradford, 2020).

The checkerboard assay confirmed strong synergism, with a 16-fold reduction in cefotaxime MICs well above the 2- to 8-fold reduction considered significant (Sopirala *et al*., 2010; Zhou *et al*., 2021). This synergy was particularly evident in ESBL-producing *E. coli* and *K. pneumoniae*, suggesting that the root bioactive fraction interferes with β-lactamase activity or enhances antibiotic uptake. The lipophilic constituents of the root component may disrupt bacterial membranes or inhibit resistance enzymes, as supported by prior reports of *A. paniculatus* roots containing

bioactive alkaloids, flavonoids, and terpenoids (Kyahar *et al*., 2020; Onwuliri *et al*., 2020). Lipophilic compounds such as terpenoids may also act as membrane permeabilizers, increasing antibiotic influx across the outer membrane (Khare *et al*., 2021; Angelini, 2024).

Several bioactive phytochemicals, which were predominantly fatty acids and their methyl esters, have been previously documented for their antibacterial properties in other Fabaceae species (Dilika *et al*., 2022). Fatty acids such as linoleic, palmitic, and stearic acids disrupt bacterial cell membranes and inhibit essential enzymatic functions, while phenolic compounds like 3-methylphenol enhance permeability and oxidative stress. Therefore, the antibacterial activity observed of the *A. paniculatus* root bioactive fraction may be attributed to the synergistic action of these major phytochemicals.

Comparable plant-mediated synergistic mechanisms have been reported for *Combretum micranthum*, *Thymus capitatus*, and *Garcinia kola* extracts, which restored β-lactam efficacy against ESBL-producing Enterobacteriaceae (Alam *et al*., 2023; El-Demerdash *et al*., 2024; Fatima *et al*., 2024). El-Demerdash *et al*. (2024), for example, showed that *T. capitatus* essential oil reduced cefotaxime MICs by up to 32-fold in *K. pneumoniae*, yielding FICI values between 0.19 and 0.31, consistent with the findings of the present study.

The time–kill assay further demonstrated ≥2 $\log_{10}$ CFU/mL reductions for the component–cefotaxime combination against both *E. coli* CI-3 and *K. pneumoniae* CI-6, confirming a bactericidal synergy. This is in agreement with previous work showing that plant-derived phenolics and alkaloids enhance β-lactam activity through membrane disruption, efflux pump inhibition, or β-lactamase suppression (Khameneh *et al*., 2016; Hemaiswarya *et al*., 2008; Gibbons, 2004). The stronger antibacterial activity observed in ESBL-positive isolates compared with susceptible or quality-control strains suggests that the root bioactive fraction may act as a resistance-modifying agent rather than simply enhancing the intrinsic potency of cefotaxime. This selective effect indicates possible interference with specific resistance mechanisms such as β-lactamase activity, outer membrane permeability alterations, or efflux pump systems commonly upregulated in resistant *Enterobacteriaceae*. Similar selective potentiation has been reported for plant extracts, including *Combretum molle* and *Acacia nilotica*, which restored β-lactam efficacy preferentially against resistant strains while showing limited enhancement in susceptible isolates (Abreu *et al*., 2012; Adwan and Mhanna, 2008). Increasing evidence indicates that several phytochemicals function as antibiotic adjuvants by inhibiting β-lactamases, suppressing efflux pump activity, or disrupting resistance-associated regulatory pathways (Hemaiswarya *et al*., 2008; Savoia, 2012; Stavri *et al*., 2007). The enhanced reduction in bacterial counts observed in the present study, therefore, supports a mechanism involving targeted modulation of ESBL-mediated resistance rather than non-specific antibacterial synergy.

These findings provide mechanistic support for the traditional use of *A. paniculatus* in the treatment of infectious diseases in Nigeria (Burkill, 1995). Given the high prevalence of ESBL-producing pathogens in Nigeria, estimated at over 40% (Ogbolu *et al*., 2018), plant-based adjuvants capable of restoring antibiotic efficacy could offer a cost-effective strategy in resource-limited healthcare settings. Furthermore, similar synergistic trends between plant metabolites and cephalosporins have been observed in studies where flavonoids and terpenoids disrupted bacterial outer membranes or inhibited plasmid-encoded β-lactamases (Khare *et al*., 2021; Fennell *et al*., 2004). Collectively, the results of this study align with growing evidence that natural phytochemicals can act as antibiotic potentiators, modulating bacterial membranes, enzymes, and efflux mechanisms (Reygaert, 2018; Sharma and Kumari, 2025). The synergy observed between the *A. paniculatus* root bioactive component and cefotaxime underscores the plant's potential as a source of resistance-modifying agents.

Conclusion

This study investigated the antibacterial and resistance-modifying effects of the bioactive fraction derived from the root of *Adenodolichos paniculatus* against ESBL-producing Gram-negative pathogens. The fraction demonstrated pronounced synergistic activity with cefotaxime against ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*, as evidenced by substantial reductions in MIC and favourable FICI indices. These findings suggest that the observed potentiation may be mediated by phytochemical constituents, particularly alkaloids, flavonoids, phenolics, and terpenoids, capable of interfering with β-lactamase activity and membrane-associated resistance mechanisms.

Collectively, the results provide mechanistic insight into the ethnopharmacological relevance of *A. paniculatus* and position its root-derived bioactive fraction as a promising source of antibiotic adjuvants for restoring β-lactam efficacy against resistant Enterobacteriaceae. Further bioassay-guided fractionation, molecular target elucidation, toxicity profiling, and *in vivo* validation are essential to advance its translational potential in antimicrobial drug development.

Limitations

This study was conducted in vitro and may not fully reflect in vivo pharmacokinetic or pharmacodynamic interactions. The bioactive fraction evaluated was a mixture of compounds, and individual active constituents were not

isolated. In addition, mechanistic hypotheses were inferred from observed antibacterial patterns and existing literature rather than direct molecular assays. These limitations warrant further in vivo and mechanistic investigations.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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