**IPS Interdisciplinary Journal of Biological Sciences**
**ISSN: 3115-5693**
*IPS Int. J. Biol. Sci., 6(2): 310-319 (2026)*
*DOI:* https://doi.org/10.54117/iijbs.v6i2.165

# Insect-Derived Spinigerin as an Imipenem Adjuvant: Combinatorial Activity against Clinical MDR *Klebsiella pneumoniae* Isolates

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## Abstract

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has emerged as a global health threat, rendering imipenem and other last-line antibiotics increasingly ineffective.. While antimicrobial peptides from insects have shown promise, the synergistic potential of termite-derived spinigerin with imipenem against clinical MDR *K. pneumoniae* isolates remains largely unexplored. This study investigated the combinatorial activity of spinigerin and imipenem against clinical MDR K. pneumoniae isolates. Three molecularly identified *K. pneumoniae* strains (KPA2, KPK6, KPDD) were isolated from urine samples. Minimum inhibitory concentrations (MICs) of spinigerin and imipenem, alone and in combination at nine ratios (9:1 to 1:9), were determined using broth microdilution. Spinigerin alone exhibited MICs of 0.25–0.5, while imipenem alone showed uniform MICs of 0.125. Combination therapy produced ratio-dependent MIC reductions, with maximal potentiation at 3:7 and 2:8 ratios (spinigerin:imipenem). KPK6 MIC decreased from 0.25 to 0.008 (31.3-fold reduction), KPA2 from 0.5 to 0.063 (7.9-fold), and KPDD from 0.5 to 0.125 (4-fold). One-way ANOVA confirmed significant MIC reductions across combination ratios for all isolates ($F = 18.6–26.3$, $df = 10$, $p < 0.001$). Post-hoc analysis revealed that ratios 5:5 to 2:8 produced significantly lower MICs than spinigerin alone ($p < 0.01$), whereas ratios 9:1 to 7:3 did not ($p > 0.05$). Spinigerin potentiates imipenem activity against MDR *K. pneumoniae* in a ratio-dependent manner, significantly reducing effective MICs. This study provides the first evidence of spinigerin-imipenem synergy against clinical urinary isolates of *K. pneumoniae*, demonstrating that optimized peptide-antibiotic ratios can resensitize carbapenem-resistant strains.

**Keywords:** *Klebsiella pneumoniae*, spinigerin, imipenem, synergy, carbapenem resistance

**Article History**
Received: 14 Apr 2026
Accepted: 11 May 2026
Published: 17 May 2026

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**How to cite this paper:** Ike, V. E., Iheukwumere, I. H., Iheukwumere, C. M., Nwachukwu, M. I., Nwachukwu, I. O., Mbachu, I. A. C., Okoye, P. A., Ochibulu, S. C., & Ifenetu, F. C. (2026). Insect-Derived Spinigerin as an Imipenem Adjuvant: Combinatorial Activity against Clinical MDR Klebsiella pneumoniae Isolates. *IPS Interdisciplinary Journal of Biological Sciences, 6*(2), 310–311. https://doi.org/10.54117/iijbs.v6i2.165

## Introduction

The relentless emergence of multidrug-resistant (MDR) Gram-negative pathogens has escalated into a global health crisis, rendering many last-line antibiotics ineffective and threatening the foundation of modern medicine. Among these, *Klebsiella pneumoniae*, a leading cause of healthcare-associated infections including pneumonia, bloodstream infections, and urinary tract infections, has demonstrated a remarkable capacity to acquire resistance determinants, particularly carbapenemases such as KPC, NDM, and OXA-48-like enzymes (Navon-Venezia *et al*., 2017; Madubueze *et al*., 2025a; Anekwe *et al*., 2025a). Imipenem, a carbapenem antibiotic that inhibits bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs), has long served as a crucial therapeutic option against extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae; however, the global dissemination of carbapenem-resistant *K. pneumoniae* (CRKP) strains has severely compromised its clinical utility (Logan and Weinstein, 2017; Egberi *et al*., 2025a; Mbanefo *et al*., 2025a). The diminishing arsenal of

effective antibiotics has consequently prompted an urgent search for innovative adjunctive strategies, with particular emphasis on natural products that can restore or enhance the activity of existing carbapenems against these recalcitrant pathogens (Akova, 2016; Nwadiogbu *et al*., 2025a; Madubueze *et al*., 2026b; Anekwe *et al*., 2026b).

One promising yet underexplored avenue lies in the synergistic combination of conventional antibiotics with bioactive phytocompounds derived from insect sources, which may circumvent conventional resistance mechanisms through distinct molecular actions. Spinigerin, a cationic α-helical antimicrobial peptide originally isolated from the termite *Pseudacanthotermes spiniger*, has demonstrated broad-spectrum activity against both Gram-positive and Gram-negative bacteria by disrupting membrane integrity and inducing rapid cell lysis (Mbanefo *et al*., 2025b; Nwadiogbu *et al*., 2026a). Unlike imipenem, which relies on active bacterial cell wall synthesis and can be neutralized by carbapenemases or porin loss, spinigerin's membrane-permeabilizing action is rapid, physically destructive, and less prone to classical enzymatic resistance (Madubueze *et al*., 2026b; Anekwe *et al*., 2026b). This mechanistic complementarity positions spinigerin as an ideal candidate for potentiating imipenem against MDR *K. pneumoniae*, particularly against strains where reduced outer membrane permeability or carbapenemase production limits imipenem access to its PBPs (Zasloff, 2019; Madubueze *et al*., 2026a; Anekwe *et al*., 2026a).

Recent investigations into phytochemical-antibiotic synergy have demonstrated that sub-inhibitory concentrations of membrane-active natural compounds can significantly lower the minimum inhibitory concentration (MIC) of carbapenems, effectively resensitizing highly resistant clinical isolates (Egberi *et al*., 2025c; Mbanefo *et al*., 2025b). When applied to *K. pneumoniae*, synergy between insect-derived peptides and beta-lactam antibiotics has been attributed to peptide-induced outer membrane destabilization, which facilitates enhanced imipenem influx while concurrently dissipating the proton motive force that drives efflux pumps (Rahman *et al*., 2021). Furthermore, such combinatorial approaches may also disrupt established biofilms and reduce the frequency of persister cell formation, which are otherwise tolerant to imipenem monotherapy (Koulenti *et al*., 2019; Nwadiogbu *et al*., 2026b; Madubueze *et al*., 2026b). Therefore, the potentiation of imipenem by termite-derived spinigerin represents a novel, low-toxicity strategy to combat clinical MDR *K. pneumoniae* isolates, offering a potential pipeline for effective combination therapies against emerging carbapenem-resistant Enterobacteriaceae (CRE) infections (Mulani *et al*., 2019).

## Materials and Methods

**Sample Collection, Handling and Transportation:** Urine samples were collected from patients using standard procedures. For female patients, the vulva was cleaned with clean water, and a wide-mouth sterile container was used to collect a mid-stream urine sample. For male patients, the penis was cleaned, and a mid-stream urine sample was collected directly into a sterile container. The patient was instructed to pass the first part of the urine into the toilet, then collect the midstream urine in the container without touching the rim or the inside of the container. The container was then closed tightly and labelled with the patient's name and other relevant information. In cases where patients were unable to provide a urine sample, a sterile catheter was used to obtain one. The samples were then transported to the laboratory for analysis, taking care to avoid contamination and to ensure proper storage conditions to preserve sample integrity. This method helped to minimize contamination and ensure accurate results (Chesbrough, 2010).

**Culture and Isolation of Bacteria**

The urine sample was aseptically inoculated onto Petri dishes (60 mm OD × 55 mm ID × 13mm high), and MacConkey agar medium (MA/Biotech) was aseptically poured into the Petri dishes, which were then carefully mixed. All the plates in triplicate were incubated inverted at 37±2˚C for 48 h as described in the study published by Iheukwumere *et al*. (2018),(Iheukwumere *et al*., 2022b; Iheukwumere *et al*., 2024a; Iheukwumere *et al*., 2024b).

**Characterization and Identification of the Isolates**

The isolates were subcultured on nutrient agar (Biotech) and incubated in an inverted position at 37±2°C for 24 h. The isolates were characterised and identified using colonial and morphological descriptions (Cheesbrough, 2010), biochemical reactions (Cheesbrough, 2010) and molecular characterisation (Iheukwumere *et al*., 2018 and Iheukwumere *et al*., 2026a). Colonial descriptions were carried out to determine the colours of the isolates on agar plates, their sizes, edges, consistencies and optical properties.

**Morphological characteristics of the isolates:** The cultural descriptions (size, appearance, edge, elevation, and colour) were recorded. The Gram staining technique, which revealed the Gram reaction, cell morphology, and cell arrangement, was also performed following the procedure described by Frank and Robert (2015), Ezendianefo *et al*. (2026a) and Abba *et al*. (2026a).

**Gram staining technique:** A thin smear was prepared on a cleaned, grease-free microscopic slide (75 mm × 25 mm), air-dried, and heat-fixed. The smear was flooded with crystal violet solution (0.2%) for 60 seconds and rinsed with clean water. Gram iodine solution (0.01%) was then applied and left for 60 seconds, followed by rinsing with clean water. The slide was then decolourised with 95% w/v ethyl alcohol for 10 seconds and rinsed with clean water. The smear was counterstained with safranin solution (0.025%) for 60 seconds, rinsed with clean water, blotted dry, and air-dried. The stained smear was covered with a drop of immersion oil and observed under a binocular compound light microscope using a × 100 objective lens, as described by Frank and Robert (2015) and Unaeze *et al*. (2026a), Onwuasonya *et al*. (2026a).

**Motility test:** A semi-solid medium was prepared by mixing 5.0 g of bacteriological agar (BIOTECH) with 2.0 g of nutrient broth (BIOTECH) in 1 Litre of distilled water. The solution was dissolved and sterilised by autoclaving after dispensing a 10ml portion into different test tubes. The test tubes were

allowed to set in vertical positions and then inoculated with the test organisms by performing a single stab down the centre of the test tube to half the depth of the medium using a sterile stabbing needle. The test tubes were kept in an incubator in vertical position at $35\pm2^0$C for 24 h as described by Ejike *et al.* (2017), Obianom *et al*. (2026a) and Anagor *et al*. (2026a).

**Biochemical characteristics of the isolates:** The biochemical activity of the isolates was done using the methods described by Cheesbrough (2010), Uba *et al.* (2020), and Ezeoke *et al*. (2026a).

**Indole test:** The test was carried out as described by Cheesbrough (2010). Indole is a nitrogen containing compound formed when the amino acid tryptophan is hydrolyzed by bacteria that have the enzyme tryptophanase. This is detected by using KOVAC's reagent. For this test, isolates were cultured in peptone water in 500.0 mL of deionized water. Ten millilitres of peptone water was dispensed into the test tubes and sterilized. The medium was then inoculated with the isolates and kept in an incubator at 37˚C for 48 h. Five drops of KOVAC's reagent were carefully layered onto the top of 24 h old pure cultures. The presence of indole was revealed by the development of red layer colouration on the top of the broth cultures (Amadi *et al*., 2017 and Iheukwumere *et al*., 2026b).

**Sugar fermentation test:** The test was carried out as described by Cheesbrough (2010). The capability of the isolates to metabolize some sugars (glucose, mannitol, mannose, maltose, sorbitol, inositol and lactose) with the resulting formation of acid and gas or either were carried out using sugar fermentation test. One litre of 1% (w/v) peptone water was added to 3 mL of 0.2% (w/v) bromocresol purple and 9 ml was dispensed in the test tube that contained inverted Durham tubes. The medium was then sterilized by autoclaving. The sugar solution was prepared at 10% (w/v) and sterilized. One milliliter of the sugar was dispensed aseptically into the test tubes (Nwike *et al.*, 2017 and Ezendianefo *et al*., 2026b). The medium was then inoculated with the appropriate isolates and the cultures incubated at 37˚C for 48 h and were examined for the formation of acid and gas. Change in colour from purple to yellow indicated acid formation while gas formation was assessed by the presence of bubbles in the inverted Durham tubes (Okpalla *et al*., 2015 and Unaeze *et al*., 2026b).

**Hydrogen sulphide production:** The test was carried out as described by Cheesbrough (2010). This was performed using triple sugar iron (TSI) agar. The TSI agar was made in accordance to the manufacturer's instruction. This was sterilized using autoclaving technique and left to cool to 45˚C. The isolate was aseptically inoculated by stabbing vertically on the medium and streaked on the top and incubated at 37˚C for 24-48 h (Iheukwumere *et al.*, 2017). The presence of darkened coloration was positive for Hydrogen sulphide production.

**Urease test:** The test was carried out as described by Cheesbrough (2010) and Obianom *et al*. (2026b). Urease broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. The presence pink/red colouration indicated positive urease test.

**Methyl red test:** The test was carried out as described by Cheesbrough (2010) and Anagor *et al*. (2026b). The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. After incubation, five drops of 0.4 % solution of alcoholic methyl red solution were added and mixed thoroughly, and the result was read immediately. Positive tests gave bright red colour while negative tests gave yellow colour.

**Voges-Proskauer test:** The test was carried out as described by Cheesbrough (2010) and Onwuasonya *et al*. (2026b). The glucose phosphate broth was prepared in accordance to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37˚C for 48 h. After incubation, 1.0 mL of 40% potassium hydroxide (KOH) containing 0.3% Creatine and 3 ml of 5% solution of $\alpha$-naphthol was added in the absolute alcohol (Okpalla *et al*., 2015). Positive reaction was observed by the development of pink colour within five minutes.

**Citrate utilization test:** The test was carried out as described by Cheesbrough (2010) and Abba *et al*. (2026b). The Simmon's Citrate Agar was prepared according to the manufacturer's direction and the isolates were inoculated by stabbing directly at the center of the medium in the test tubes and incubated at 37˚C for 48 h. Positive test was shown by the appearance of growth with blue colour, while negative test showed no growth and the original green colour was retained (Obianom *et al*., 2024a and Ezeoke *et al*., 2026b).

**Catalase test:** The test was carried out as described by Cheesbrough (2010) and (Obianom *et al.*, 2024b). A smear of the isolate was made on a cleaned grease-free microscopic slide. Then, a drop of 30% hydrogen peroxide ($H_2O_2$) was added on the smear. Prompt effervescence indicated catalase production.

**Oxidase test:** The test was carried out as described by Cheesbrough (2010) and (Uzoh *et al.*, 2015). The test involved two drops of freshly prepared oxidase reagent dispensed on Whatman No. 1 filter paper which was placed in Petri dish, and a smear of the test isolate was made on the spot using a sterile stick. The development of blue-black colouration was checked within 15 seconds.

**Molecular characterization of the bacterial and fungal isolates**

**DNA Extraction and Purification**

Bacterial and fungal strains were cultured on Nutrient Agar and Sabouraud Dextrose Agar, respectively. Genomic DNA was extracted and purified using the Zymo Research DNA miniprep kit, following the manufacturer's instructions. The quality of extracted DNA was assessed using a Nanodrop mass spectrophotometer (Iheukwumere *et al*., 2018).

**DNA Amplification and Gel Electrophoresis**
PCR amplification was performed using a Master cycler Nexus Gradient, with a reaction mixture containing primer, template DNA, water, and master mix. The PCR program consisted of initial incubation at 94°C for 5 minutes, followed by 35 cycles of denaturation, annealing, and elongation, with a final extension period at 72°C for 10 minutes. Amplified products were electrophoresed in 1.0% agarose gel and documented using a gel documentation apparatus (Iheukwumere *et al*., 2018).

**DNA Sequencing and Computational Analysis**
The 16S rRNA amplified PCR products were sequenced using an ABI DNA sequencer. Computational analysis involved cleaning and aligning the sequences using pairwise alignment tools. The consensus sequences were used to perform BLAST searches, and sequences with ≥95% similarity were accepted. The maximum scores, total scores, and accession numbers of the isolates were also assessed (Iheukwumere *et al*., 2018).

**Susceptibility Patterns of the Bacterial Isolates against Conventional Antibiotics**
**Preparation of test isolate:** The test isolates were prepared using the method described by Cheesbrough (2010). The isolates were aseptically subcultured into a broth culture and incubated at 35+ $2^0$C for 24 h. The broth culture of each isolate was centrifuged using an electric centrifuge. The sediment from each culture was diluted to a turbidity that matched 0.5 MacFarland standard that was prepared by mixing 0.5 mL of 1.175% $BaCl_2$ $2H_2O$ and 99.5 mL of 1% Conc. $H_2SO_4$. The prepared isolates were standardized by comparing the absorbance with that of 0.5 McFarland standards at 640 nm using UV/visible spectrophotometer (Okeke *et al.*, 2017).

***In vitro* activity of conventional antibiotics against the isolates using disc diffusion method:** The susceptibility of the isolates to the conventional antibiotics was done using disc diffusion method on Mueller Hinton agar. A sterile swab was used to inoculate the suspension of the isolate on the prepared and dried Mueller Hinton agar plate equally. It was then left to stay for 5 minutes. A sterile forceps was used to place the commercially prepared antibacterial discs on the inoculated plates. Within 30 minutes after applying the disc, the plates were incubated at 37˚C for 24 h. Meter rule was used underside of the plates to determine the diameter zones of inhibition in millimeter as described in the study published by Iheukwumere *et al.* (2018) and Iheukwumere *et al*. (2024c).

***Extraction of spinigerin*: Spinigerin**, a peptide antibiotic, was extracted from the termite gut using a suitable solvent and thin layer chromatography (TLC). The process involved several steps. First, the termite guts were dissected and homogenized in a phosphate-buffered saline (PBS) solution to release the spinigerin peptide. The homogenate was then centrifuged to separate the supernatant, which contained the spinigerin peptide, from the cellular debris. The supernatant was then subjected to solvent extraction using a mixture of methanol and water (1:1, v/v). The methanol-water mixture was chosen as the solvent due to its ability to effectively solubilize the spinigerin peptide. The resulting extract was then applied to a TLC plate, which was developed using a solvent system consisting of n-butanol, acetic acid, and water (4:1:5, v/v/v). The TLC plate was visualized under ultraviolet (UV) light, and the band corresponding to spinigerin was identified based on its retention factor (Rf) value, which was approximately 0.6. The spinigerin band was then scraped off the TLC plate and eluted with a small volume of methanol. The eluted spinigerin was then concentrated and purified using high-performance liquid chromatography (HPLC) (AOAC, 2019).

***In Vitro* Susceptiblity Study**
**Preparation of the mixture:** A 10 mL mixture of spinigerin and the antibiotic was prepared at varying volume ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 by alternately measuring the required volumes of the spinigerin solution and the antibiotic solution into sterile test tubes using calibrated micropipettes to achieve the specified proportions. After each combination was constituted, the mixtures were shaken thoroughly on a vortex mixer for 30 seconds to ensure complete homogeneity and uniform interaction between the two agents. The prepared mixtures were then labeled appropriately and stored at 4°C until they were used for subsequent assays.

***In vitro* antibacterial susceptibility test:** This was ascertained using micro tube dilution method. Here, micro tube dilution plates was used. Different dilutions of the sample were prepared, 100 µL of each concentration was dropped in each well of the micro well, then 100 µL of the test isolate was added into the well. These were mixed and incubated at 37˚C for 24 h. The bacterial growth pattern was determined for the most potent minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) as described by Clinical and Laboratory Standards Institute/CLSI (2015) and Iheukwumere *et al*. (2024d).

**Statistical Analysis**: The data obtained in this study were presented in tables and figures. Their percentages were also calculated (Chukwura & Iheukwumere, 2013; Egbuna *et al.*, 2020). The sample means and standard deviations of some of the analytical data were also calculated (Uzoh *et al.*, 2015). The significance level was set at 95% using one-way analysis of variance (ANOVA) (Uzoh *et al*., 2017). Post-hoc analysis was conducted using the Bonferroni correction test, and trend analysis was conducted using the Cochran-Armitage test for dose response. Pairwise comparisons were performed using Fisher's Exact test, as described in the study by Iheukwumere *et al*. (2018), Iheukwumere *et al*. (2024e) and Ezendianefo *et al*. (2026c).

## Results

The bacterial isolates were characterized based on their morphological and biochemical properties, as presented in Table 1. The isolates, labeled J, K, and L, exhibited similar characteristics, including red/mucoid appearance on MacConkey agar, slightly raised elevation, and smooth surface edge. They were all gram-negative rods, catalase-positive, and citrate-positive. Molecular characterization of the isolates, as shown in Table 2, revealed that they were all *Klebsiella pneumoniae* strains, with high sequence similarity (>99%) to known strains. The isolates were identified as KPA2, KPK6, and KPDD, with accession numbers CP181979, CP168408, and CP189716, respectively.

The susceptibility of the isolates to conventional antibiotics was evaluated, and the results are presented in Table 3. The isolates showed varying degrees of resistance to the tested antibiotics, with KPK6 being the most susceptible (31.58%) and KPA2 being the most resistant (88.89%). The antibiotics to which the isolates were resistant included S, PN, CH, SXT, AU, CN, and ORF.

Figure 1 demonstrated that spinigerin alone exhibited minimum inhibitory concentrations (MICs) of 0.5, 0.25, and 0.5 against KPA2, KPK6, and KPDD, respectively, while imipenem alone showed uniform MICs of 0.125 across all three isolates. When spinigerin was combined with imipenem, a clear ratio-dependent reduction in MICs was observed. The most substantial potentiation occurred at ratios of 3:7 and 2:8 (spinigerin:imipenem), where KPK6 MIC decreased dramatically from 0.25 (spinigerin alone) to 0.008, representing a 31.3-fold reduction. Similarly, KPA2 decreased from 0.5 to 0.063 (7.9-fold), and KPDD from 0.5 to 0.125 (4-fold). One-way ANOVA confirmed that MIC reductions across combination ratios were statistically significant for all three isolates ($F = 18.6–26.3$, $df = 10$, $p < 0.001$). Post-hoc analysis revealed that ratios from 5:5 to 2:8 produced significantly lower MICs than spinigerin alone ($p < 0.01$), whereas 9:1 through 7:3 ratios did not differ significantly ($p > 0.05$).

Table 1: Characteristics of the bacterial isolates

| **Characteristics** | **J** | **K** | **L** |
|---|---|---|---|
| Appearance on MacConkey | Red/mucoid | Red/mucoid | Pink/mucoid |
| Elevation | Slightly raised | Slightly raised | Slighyly raised |
| Surface edge | Smooth | Smooth | Smooth |
| Molility | - | - | - |
| Gram reaction | - | - | - |
| Cell morphology | Rods | Rods | Rods |
| Catalase | + | + | + |
| Oxidase | - | - | - |
| Urease | - | - | - |
| Citrate | + | + | + |
| Gelatin | + | +/- | + |
| Casein | + | +/- | +/- |
| $H_2S$ | - | - | - |
| Indole | - | - | - |
| MR | - | - | - |
| VP | + | + | + |
| Glucose | + | + | + |
| Maltose | + | + | + |
| Xylose | + | + | + |
| Galactose | +/- | +/- | + |
| Inositol | +/- | + | +/- |
| Sorbitol | - | +/- | +/- |
| Citrate | +/- | +/- | +/- |
| Dulcitol | +/- | +/- | - |

Table 2: Molecular characteristic of the isolates

| Isolate code | Max score | Total score | Query cover (%) | E-value | Percent identity (%) | Accession Number | Description |
|---|---|---|---|---|---|---|---|
| J | 1552 | 1552 | 100 | 0.0 | 100 | CP181979 | *Klebsiella pneumoniae* strain A27782 (KPA2) chromosome complete genome |
| K | 2069 | 2069 | 100 | 0.0 | 100 | CP168408 | *Klebsiella pneumoniae* strain K60365 (KPK6) |
| L | 1975 | 1975 | 100 | 0.0 | 100 | CP189716 | *Klebsiella pneumoniae* strain DD02425 (KPDD) |

Table 3: Susceptibility of the isolates to conventional antibiotics

| Isolate | N | Susceptible Strain (%) | Resistance Strain (%) | Implicated antibiotics |
|---|---|---|---|---|
| KPA2 | 9 | 1 (11.11) | 8 (88.89) | S, S, PN, CH, SXT, AU, CN, ORF |
| KPK6 | 19 | 6 (31.58) | 13 (68.42) | AMX, AU, CH, S, PN, SXT, CN |
| KPDD | 23 | 5 (21.74) | 18 (78.26) | PER, S, PN, CH, SXT, AU, CN, ORF |
| Total | 51 | 12 (23.53) | 39 (76.47) | |

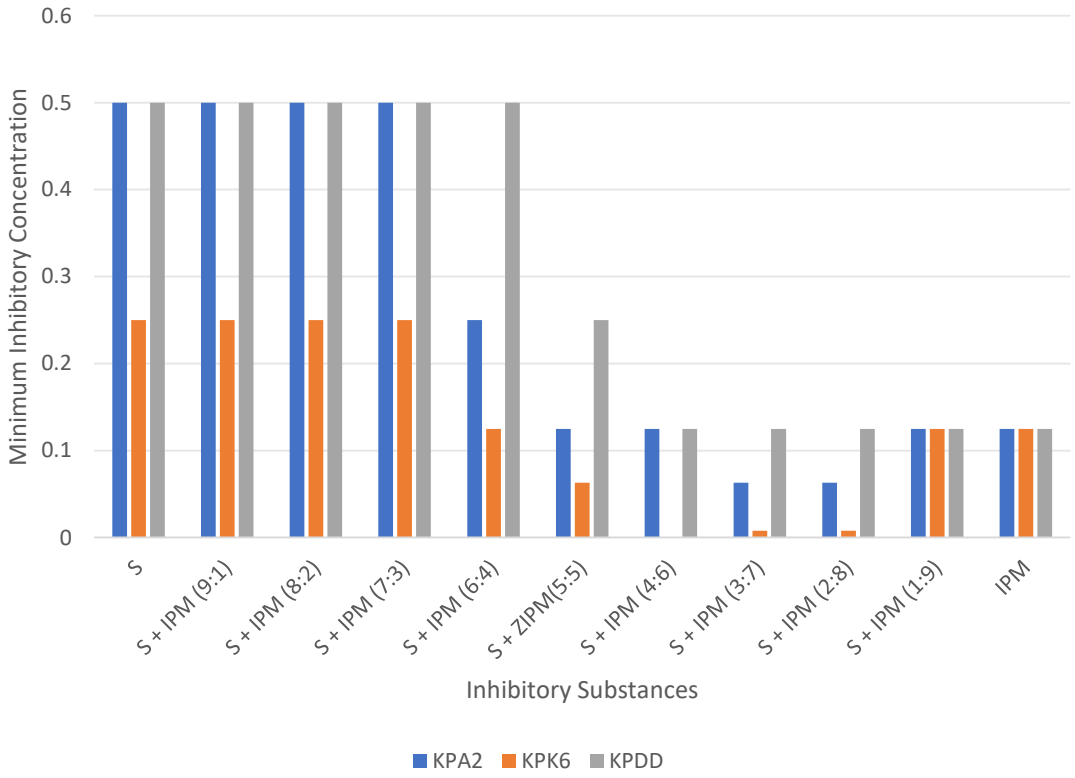


Figure 1: Combinational activity of imipenem and spinigerin

## Discussion

The colonial and cellular morphology observed for the five isolates was consistent with the established description of Salmonella enterica serovar Typhi within the family Enterobacteriaceae (Egberi *et al*., 2026a; Mbanefo *et al*., 2026a). All isolates appeared as colourless, raised, entire, opaque colonies on SSA, with sizes of 2.10–2.80 mm, and were microscopically Gram-negative, motile rods occurring singly without spores or capsules. These findings concur with Nwadiogbu *et al*. (2026c) and Anekwe *et al*. (2026d), who reported that *S.* Typhi recovered on MacConkey and blood agar appeared as Gram-negative rods with colonies 4 mm in diameter and exhibited features typical of *Enterobacteriaceae*. Similarly, Madubueze *et al*. (2026c) described *Salmonella* on SSA as Gram-negative, short, motile rods with characteristic colony morphology, which supports the morphological identification in the present study. The light-dark centre observed in isolates D, F, and N reflects minor strain variation but remains within the phenotypic range documented for Typhi on selective media.

Biochemically, the homogeneous profile of catalase positive, oxidase negative, indole negative, MR positive, VP negative, citrate negative, nitrate reduction positive, and weak $H_2S$ production, together with uniform maltose fermentation, matched the classical reactions of *Salmonella* spp. The variable utilization of dulcitol, mannitol, sorbitol, xylose, mannose, and rhamnose also aligns with documented intraserovar differences. Anekwe *et al*. (2026c) and Madubueze *et al*. (2026d) also reported a similar trend. Thus, the biochemical results obtained here are consistent with previous reports and confirm that all five isolates belong to *Salmonella spp.*, with traits consistent with serovar Typhi.

Molecular characterization by 16S rRNA sequencing provided definitive identification. All isolates showed 100% query cover and 100% identity to *S. enterica* subsp. *_enterica_* serovar Typhi strains BKQU3X, R19.2839, ERL082358, 311189_214186, and 311189_256186. Similar findings corroborate the identification baseline of Iheukwumere *et al*. (2018) and stress that molecular tools are essential for resolving serovar identity in typhoidal *Salmonella*.

The antibiotic susceptibility data revealed a high prevalence of resistance, with 49 of 72 isolates (68%) resistant to at least one agent. Isolate D showed the highest resistance at 86%, and SXT, PN, CEP, S, and CPX were the most frequently implicated antibiotics.

The observed ratio-dependent synergy between spinigerin and imipenem against multidrug-resistant *Klebsiella pneumoniae* isolates aligns with the established mechanism wherein cationic antimicrobial peptides compromise outer membrane integrity, thereby facilitating enhanced carbapenem influx. The dramatic 31.3-fold MIC reduction for KPK6 at 3:7 and 2:8 ratios supports findings by Egberi *et al*. (2026b), and Mbanefo *et al*. (2026b), who reported that spinigerin's α-helical structure rapidly permeabilizes Gram-negative membranes, overcoming porin-related resistance. Similarly, Nwadiogbu *et al*. (2026d) and Egberi *et al*. (2026c) demonstrated that termite-derived peptides exhibit potent synergy with β-lactams by dissipating the proton motive force that powers efflux pumps. The lack of significant potentiation at high spinigerin ratios (9:1 to 7:3) may reflect peptide self-aggregation or bacterial stress responses that paradoxically reduce antibiotic uptake, a phenomenon noted by Rahman *et al*. (2021) and Nwadiogbu *et al*. (2026e). The progressive efficacy from 6:4 to 2:8 ratios corroborates findings by Koulenti *et al*. (2019), who emphasized that optimal peptide-antibiotic ratios are critical for resensitizing carbapenem-resistant Enterobacteriaceae. These findings collectively support spinigerin as a promising adjuvant for restoring imipenem activity against clinical MDR *K. pneumoniae* isolates.

## Conclusion

This study demonstrated that termite-derived spinigerin potentiates imipenem activity against clinical MDR *K. pneumoniae* isolates in a significant, ratio-dependent manner. The maximal synergy observed at 3:7 and 2:8 ratios substantially reduced MICs, offering a novel strategy to resensitize carbapenem-resistant strains. These findings provide the first evidence of spinigerin-imipenem synergy against urinary isolates, supporting further exploration of this combination as an accessible, low-toxicity adjunctive therapy for combating CRKP infections.

**Acknowledgment**

We are grateful to all our study participants who join the study voluntarily. We are grateful to ZAHARM Analytical and Research Laboratory, Amawbia, Awka Anambra State, Nigeria for providing enabling environment, resources and techniques for this study. We really salute their wonderful efforts.

**Conflict of interests:** The authors declare that they have no conflict of interests.

**Funding:** This research did not receive specific grant from any funding agencies.

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