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Original article

Fractionation-Guided Evaluation of *Justicia secunda* and *Kalanchoe pinnata* as Inhibitors of DNA Gyrase and Penicillin-Binding Proteins in Multidrug-Resistant *Acinetobacter baumannii and Klebsiella pneumoniae*

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ABSTRACT

Background: The increasing prevalence of multidrug-resistant (MDR) bacterial pathogens necessitates alternative antimicrobial agents; thus, this study combined fractionation-guided *in vitro* evaluation with *in silico* target-based analysis to assess the antibacterial potential and possible mechanisms of action of *Justicia secunda* and *Kalanchoe pinnata* against MDR *Acinetobacter baumannii* and *Klebsiella pneumoniae*.

Methods: Crude extracts of *J. secunda* and *K. pinnata* were prepared by maceration and subsequently fractionated. Phytochemical profiling was performed using high-performance liquid chromatography (HPLC). Antibacterial activity against MDR *A. baumannii* and *K. pneumoniae* was evaluated using agar well diffusion, while minimum inhibitory concentrations (MICs) were determined by broth dilution. Molecular docking with MM/GBSA refinement assessed interactions of identified compounds with DNA gyrase (*A. baumannii*) and penicillin-binding protein (*K. pneumoniae*). ADMET and drug-likeness properties were predicted in silico.

Results: HPLC analysis revealed diverse phenolic acids and flavonoids in both plants, with abundant compounds including naringin, myricetin, baicalin, apigenin, catechin, and mangiferin. Fractionation enhanced antibacterial activity relative to crude extracts. In *J. secunda*, the ethyl acetate fraction showed the strongest activity (zones up to 27.33 mm; MIC 3.125 mg/mL). In *K. pinnata*, n-hexane and ethyl acetate fractions produced inhibition zones up to 27.00 mm, while the aqueous fraction exhibited the lowest MIC (3.125 mg/mL). Docking studies identified several phytochemicals with higher predicted binding affinities than standard antibiotics, alongside favorable ADMET profiles and Lipinski compliance.

Conclusion: *Justicia secunda* and *Kalanchoe pinnata* contain bioactive phytochemicals with significant antibacterial activity against MDR pathogens, supporting their potential as sources of novel antimicrobial leads.

KEYWORDS: Fractionation-Guided Antibacterial Screening, Multidrug-Resistant Pathogens, *Justicia Secunda*, *Kalanchoe Pinnata*, Molecular Docking and Admet Analysis

INTRODUCTION

Bacterial infections remain a major global public health challenge, contributing substantially to morbidity and mortality worldwide. Although antibiotics revolutionized the management of infectious diseases, their effectiveness has been increasingly compromised by the rapid emergence and spread of antimicrobial resistance (AMR). Multidrug-resistant (MDR) Gram-negative pathogens now pose the greatest therapeutic challenge, with organisms such as carbapenem-resistant *Acinetobacter baumannii*, third-generation cephalosporin-resistant *Klebsiella pneumoniae*, and other ESKAPE pathogens accounting for a substantial proportion of hospital-acquired infections worldwide. In many low- and middle-income countries settings, resistance rates exceeding 50% have been reported, resulting in prolonged hospitalization, treatment failure, increased healthcare costs, and elevated mortality (Mulani *et al*., 2019; Murray *et al*., 2022; WHO, 2023; CDC, 2024). Without effective interventions, AMR-related deaths are projected to reach 10 million annually by 2050, with profound socioeconomic consequences (O'Neill, 2016; WHO, 2023).

Klebsiella pneumoniae is of particular concern due to its remarkable genomic plasticity, rapid acquisition of resistance determinants, and the emergence of hypervirulent lineages. The organism's polysaccharide capsule, biofilm-forming capacity, and efficient horizontal gene transfer mechanisms facilitate immune evasion, environmental persistence, and resistance to antibiotics and bacteriophages (Khaertynov *et al*., 2018; Patro and Rathinavelan, 2019; Moubareck and Hammoudi, 2020). Similarly, *Acinetobacter baumannii* has emerged as one of the most formidable nosocomial pathogens of the modern era. Notorious for its ability to survive desiccation, disinfectants, and oxidative stress, *A. baumannii* rapidly adapts to hospital environments and frequently exhibits resistance to nearly all available antibiotic classes, including last-resort carbapenems (Valcek *et al*., 2022; Whiteway *et al*., 2022; Yehya *et al*., 2025). Consequently, both organisms are classified by the World Health Organization as priority pathogens, underscoring the urgent need for novel therapeutic strategies.

Despite the escalating AMR crisis, the antibiotic development pipeline remains critically constrained, with few novel agents targeting WHO-priority Gram-negative pathogens. Existing antibiotics are further limited by toxicity, hypersensitivity reactions, immunosuppressive effects, high cost, and declining efficacy. These challenges necessitate the exploration of alternative antibacterial sources, particularly those offering structural diversity, novel mechanisms of action, and improved accessibility in resource-limited settings.

Medicinal plants represent a rich and largely untapped reservoir of antibacterial compounds. Approximately 80% of the world's population relies on plant-based remedies for primary healthcare, especially in biodiverse tropical regions (WHO, 2019). Natural products have historically underpinned modern drug discovery. Plant-derived secondary metabolites—including alkaloids, flavonoids, phenolics, terpenoids, glycosides, and lignans—exert antibacterial effects through diverse mechanisms such as membrane disruption, enzyme inhibition, efflux-pump suppression, and biofilm interference. The multiplicity of these mechanisms reduces selective pressure for resistance and enhances their relevance against MDR pathogens (Barbieri *et al*., 2017; Angelini, 2024).

To maximize the therapeutic potential of medicinal plants, fractionation-guided bioassays have emerged as a powerful strategy in natural product research. Unlike crude extracts, which may contain antagonistic or inactive constituents, solvent-based fractionation enables the enrichment and identification of bioactive components responsible for antibacterial activity. This approach has successfully yielded potent fractions and lead compounds with enhanced efficacy against resistant bacteria (Chassagne *et al*., 2021; Woo *et al*., 2023), yet remains underutilized for many ethnomedicinal plants.

Kalanchoe pinnata (Lam.) Pers. and *Justicia secunda* Vahl are two medicinal plants with strong ethnopharmacological relevance and emerging antimicrobial evidence. *K. pinnata*, widely distributed across Africa, Asia, South America, and the Caribbean, is traditionally used for the treatment of infections, wounds, inflammation, and respiratory disorders. Its phytochemical profile includes flavonoids, alkaloids, steroids, bufadienolides, and glycosides, compounds reported to possess antibacterial, antifungal, antiviral, and anti-inflammatory properties (Agarwal and Shanmugam, 2019; Ramon *et al*., 2023; Nascimento *et al*., 2023). However, systematic fractionation-guided evaluations of its antibacterial activity against MDR pathogens remain limited.

Similarly, *Justicia secunda* (Acanthaceae), commonly known as bloodroot, is widely used in African and Caribbean traditional medicine to manage anemia, hemorrhage, inflammation, wounds, fever, and infectious diseases. Phytochemical investigations have revealed the presence of phenolics, flavonoids, lignans (justisecundosides A–C), alkaloids, and terpenoids, some of which demonstrate promising antimicrobial activity (Koffi *et al*., 2022). Nonetheless, robust fractionation-guided studies specifically targeting WHO-critical Gram-negative pathogens are scarce.

Given the escalating global burden of AMR, the critical threat posed by *A. baumannii* and *K. pneumoniae*, and the stagnation of the antibiotic pipeline, there is an urgent need to identify alternative antibacterial agents with novel mechanisms of action. While previous studies have largely focused on crude extracts, this study uniquely integrates fractionation-guided evaluation to systematically identify bioactive antibacterial fractions from *K. pinnata* and *J. secunda* against MDR *A. baumannii* and *K. pneumoniae*. By aligning ethnomedicinal relevance with contemporary drug-discovery strategies, this work strengthens the translational potential of these plants and addresses a critical gap in natural product-based antibacterial research. Therefore, this study aims to combine fractionation-guided *in vitro* antibacterial evaluation with in silico molecular docking to investigate the activity and potential mechanisms of *Kalanchoe pinnata* and *Justicia secunda fractions* against *multidrug-resistant Acinetobacter baumannii* and *Klebsiella pneumoniae*, targeting DNA gyrase and penicillin-binding proteins.

MATERIALS AND METHODS

MATERIALS

Source of Test Organisms and Inocula Preparation

Multidrug-resistant (MDR) bacterial strains, namely *Acinetobacter baumannii* and *Klebsiella pneumoniae*, were obtained from the Microbiology Laboratory, Department of Microbiology, in our institution. The isolates were selected based on their established resistance profiles for antimicrobial susceptibility testing. Bacterial inocula were standardized to a **0.**5 McFarland turbidity standard, prepared using 0.05 mL of 1% barium chloride and 9.95 mL of 1% sulfuric acid**.** Fresh bacterial colonies were emulsified in sterile normal saline and visually adjusted to match the McFarland standard, as previously described (Oluyele *et al*., 2023).

Collection and Authentication of Plant Materials

Fresh leaves of *Justicia secunda* (Vahl) Griseb. (synonym: *Diathera secunda*; family Acanthaceae) and *Kalanchoe pinnata* (Lam.) Pers. (synonym: *Bryophyllum pinnatum*; family Crassulaceae) were collected from their natural habitats in Ondo State, Nigeria. *J. secunda* was collected from Akungba-Akoko, while *K. pinnata* was obtained from Oda Quarters, Akure.

The collected leaves were washed, air-dried under ambient laboratory conditions, and pulverized into fine powder prior to extraction. Identification and authentication of the whole plant specimens were subsequently carried out at the Plant Science and Biotechnology Departmental Herbarium and Taxonomic (PSBHT) Unit, Adekunle Ajasin University, Akungba-Akoko (AAUA), Ondo State, Nigeria. Voucher specimens were deposited in the departmental herbarium with accession numbers PSBHT-221 for *J. secunda* and PSBHT-219 for *K. pinnata*.

Crude Extraction and Fractionation of Plant Materials

Crude extraction was carried out using the maceration method as described by Oluyele *et al*. (2017). Briefly, the air-dried leaves of *Kalanchoe pinnata* and *Justicia secunda* were pulverized into fine powder. A total of 560 g of *K. pinnata* powder and 930 g of *J. secunda* powder were separately soaked in 4.0 L and 5.5 L of absolute ethanol, respectively, for five days with intermittent shaking to facilitate efficient extraction of bioactive constituents. The resulting mixtures were first sieved through muslin cloth and subsequently filtered using Whatman No. 1 filter paper. The filtrates were concentrated under reduced pressure using a rotary evaporator, and the crude extracts obtained were stored at 20 °C until further analyses.

Fractionation of the crude extracts was performed by liquid–liquid partitioning following the method of Oluyele (2025). Briefly, each crude extract was reconstituted in distilled water at a ratio of 1:10 (w/v) and transferred into a separatory funnel. The aqueous solution was allowed to equilibrate and then successively partitioned with n-hexane, ethyl acetate, and methanol to obtain the corresponding n-hexane, ethyl acetate, methanol, and residual aqueous fractions. These fractions were collected separately and concentrated for subsequent analyses.

Evaluation of Antibacterial Activity

Agar Well Diffusion Assay

The antibacterial activity of the plant extracts was evaluated using the agar well diffusion method (Oluyele *et al*., 2025a). Mueller–Hinton agar plates were aseptically and uniformly inoculated with bacterial suspensions standardized to 0.5 McFarland turbidity**.** Wells of 6 mm diameter were punched into the agar using sterile cork borers, and each well was loaded with 100 µL of the extract at concentrations ranging from 50 to 3.125 mg/mL**.** The inoculated plates were allowed to pre-diffuse at room temperature for 30 min and subsequently incubated at 37 °C for 24 h. After incubation, the diameters of the zones of inhibition were measured in millimeters (mm). All assays were performed in triplicate, and results were recorded as mean values. Appropriate solvent and antibiotic controls were included.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of the plant extracts was determined using the broth dilution method (Oluyele *et al*., 2025a). Serial two-fold dilutions of the extracts (50–3.125 mg/mL) were prepared in Mueller–Hinton broth in sterile test tubes. Each tube was inoculated with a standardized bacterial suspension adjusted to obtain a final inoculum density of approximately 5×10^5 CFU/mL**,** and the **final** assay volume in each tube was 2.0 mL**.** The tubes were incubated at 37 °C for 24 h**.** Following incubation, bacterial growth was assessed by visual inspection and further confirmed by measuring optical density at 600 nm using a spectrophotometer, with extract-only blanks included to correct for background absorbance. The MIC was defined as the lowest concentration of the extract that completely inhibited visible bacterial growth.

High-Performance Liquid Chromatography (HPLC) Analysis of Extracts

Approximately 2.0 g of each extract was weighed into an amber bottle, and 20 mL of acetonitrile:methanol was added. The mixture was agitated for 30 min**,** and the organic phase was collected into a 25 mL volumetric flask and brought to volume with the same solvent. The solution was filtered and used for HPLC analysis. Chromatographic separation was performed using a reversed-phase HPLC system (Agilent 1200) with a Hypersil BDS C18 column (250 mm × 4.0 mm) under gradient elution. The mobile phase consisted **of** 0.1% formic acid and acetonitrile, with **a** flow rate of **0.6 mL/min,** injection volume of 20 µL, and detection at 280 nm. Reference standards were first injected to establish retention times and peak profiles. Sample peaks were identified by comparing retention times and UV spectra with those of the standards (Oluyele *et al*., 2025b).

In silico studies

Generation and Preparation of Compound Library

The phytochemical compounds investigated in this study were obtained from *Kalanchoe pinnata* and *Justicia secunda* via HPLC analysis. Since the phytochemical profiles of both plants were highly similar, differing only in two compounds, the compound sets were combined into a single ligand library and treated as one screening pool for subsequent analyses against both protein targets. The chemical structures of these compounds, along with the standard drugs, were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov) in Structure Data File (SDF) format. The downloaded

molecules were imported into the Maestro workspace of Schrödinger Suite 2021-4 (Schrödinger, LLC, New York, USA) and prepared using the LigPrep module. Ligand preparation included generation of appropriate ionization states at physiological pH, stereoisomers where applicable, geometry optimization, and energy minimization using the OPLS4 force field.

Protein Preparation

The three-dimensional structure of DNA gyrase from *Acinetobacter baumannii* was retrieved from the Protein Data Bank (RCSB PDB, https://www.rcsb.org) with PDB ID: 7PQM. The penicillin-binding protein (PBP) of *Klebsiella pneumoniae* was obtained from the UniProt (https://www.uniprot.org/) database with UniProt ID: W8UA61. Both protein structures were prepared using the Protein Preparation Wizard of Schrödinger Maestro 2021-4. The preparation protocol involved assigning correct bond orders, adding missing hydrogen atoms, correcting missing side chains and loops, removing non-essential crystallographic water molecules, optimizing the hydrogen-bonding network, and performing restrained energy minimization using the OPLS4 force field to relieve steric clashes and optimize the structures for docking (Oluyele *et al*., 2025a).

Receptor Grid Generation

The receptor grids for both targets were generated using the Glide Receptor Grid Generation module in Schrödinger Maestro 2021-4. For *A. baumannii* DNA gyrase (7PQM), the grid was centered on the native binding pocket of the protein, while for *K. pneumoniae* PBP (W8UA61), the grid was generated around the predicted active site region. The grid boxes were defined to fully enclose the binding cavities and allow sufficient ligand flexibility during docking. These grids were subsequently used for all docking calculations (Oluyele *et al*., 2025a)

Structure-Based Molecular Docking

Structure-based virtual screening was carried out using the Extra Precision (XP) Glide docking protocol implemented in Schrödinger Maestro 2021-4. The combined ligand library (from *K. pinnata* and *J. secunda*) and the standard drugs were docked into the active sites of DNA gyrase (7PQM) and PBP (W8UA61). The XP mode was selected because of its improved accuracy and enhanced ability to discriminate true binders, albeit at a higher computational cost (Oluyele *et al*., 2025a). The docked poses were ranked based on their Glide docking scores, and the top-ranked conformations were selected for further analysis.

Prime/MM-GBSA Binding Free Energy Calculations

The docked protein–ligand complexes were subjected to binding free energy calculations using the Prime MM-GBSA module of Schrödinger Suite 2021-4. The complexes were refined using local optimization, and the binding free energy (ΔG_bind) was calculated using the OPLS4 force field (Lu *et al*., 2021). MM-GBSA rescoring was employed to improve the reliability of the docking results by providing a more accurate estimation of ligand binding stability. The binding free energy was computed using the equation:

$$\Delta G\_bind = G\_complex - (G\_protein + G\_ligand)$$

ADMET Screening

The pharmacokinetic and drug-likeness properties of the selected hit compounds were evaluated using the ADMETlab 2.0 online server (https://admetlab.cn). Parameters related to absorption, distribution, metabolism, excretion, and toxicity were predicted to assess the developability and safety profiles of the compounds (Xiong *et al*., 2021). These predictions were used to complement the docking and MM-GBSA analyses and to prioritize compounds with favorable drug-like properties.

Ethical approval for research methods

A statement must be included to indicate that the work was carried out according to all local, national, and international regulations and requirements, and that permission of the appropriate Ethical Committee has been obtained if the described research involved the use of animals or humans, genetically modified organisms or any other methods which may pose ethical issues

Statistical Analysis

All experiments were performed in triplicate (n = 3)**,** and results are presented as mean values with variability**.** Statistical analyses were conducted using SPSS version 22.0 (IBM Corp., Armonk, NY, USA)**.** Differences among groups were evaluated using one-way analysis of variance (ANOVA**)**, and significant differences between means were determined by Duncan's Multiple Range Test. Statistical significance was set at $p \leq 0.05$**.**

RESULTS AND DISCUSSION

HPLC analysis and Antibacterial Activity of Justicia secunda *Extract*

As presented in Table 1 and illustrated in Figure 1, HPLC analysis of *J. secunda* extract revealed the presence of multiple phenolic acids and flavonoid compounds. Identified constituents included caffeic acid, ferulic acid, maleic acid, naringin, curcumin, myricetin, baicalin, quercetin, apigenin, phloretin, kaempferol, and p-coumaric acid. The chromatogram showed well-resolved peaks, with flavonoids such as naringin, myricetin, baicalin, and apigenin exhibiting prominent peak areas, indicating their abundance and possible contribution to the observed antibacterial activity.

Table 1: Compound Identified in *Justicia secunda* Extract

RetTime	Type	Area	Amt/Area	Amount	Name
1.356	VV	2553.29321	1.00097e-1	255.57728	Caffeic Acid
1.487	VV	12.19482	4.69604e-3	5.72674e-2	Ferulic Acid
2.359	VVb	10.46182	3.18204e-3	5.63474e-2	Maleic Acid
4.533	BB	31.14853	1.39871e-1	4.35677	Naringin
5.321	vvb	1.8614e-3	8.46117e-1	1.23556e-3	Curcumin
5.821	VVb	31.621340	1.34390e-2	4.6159	Myricetin
6.470	MM	6.41116	4.17755e-2	2.67829e-1	Baicalin
8.080	MM	7.56710	2.00894e-2	1.52018e-1	Quercetin
8.236	MM	7.76476	8.18996e-2	6.35930e-1	Apigenin
9.875	VV	2.86410-3	7.23786	2.31390e-2	Phloretin
10.857	VV	3.489310-1	5.3217	2.46150e-2	Kaempferol
10.566	VV	9.92265e-3	6.03082e-2	5.98417e-4	p-Coumaric Acid

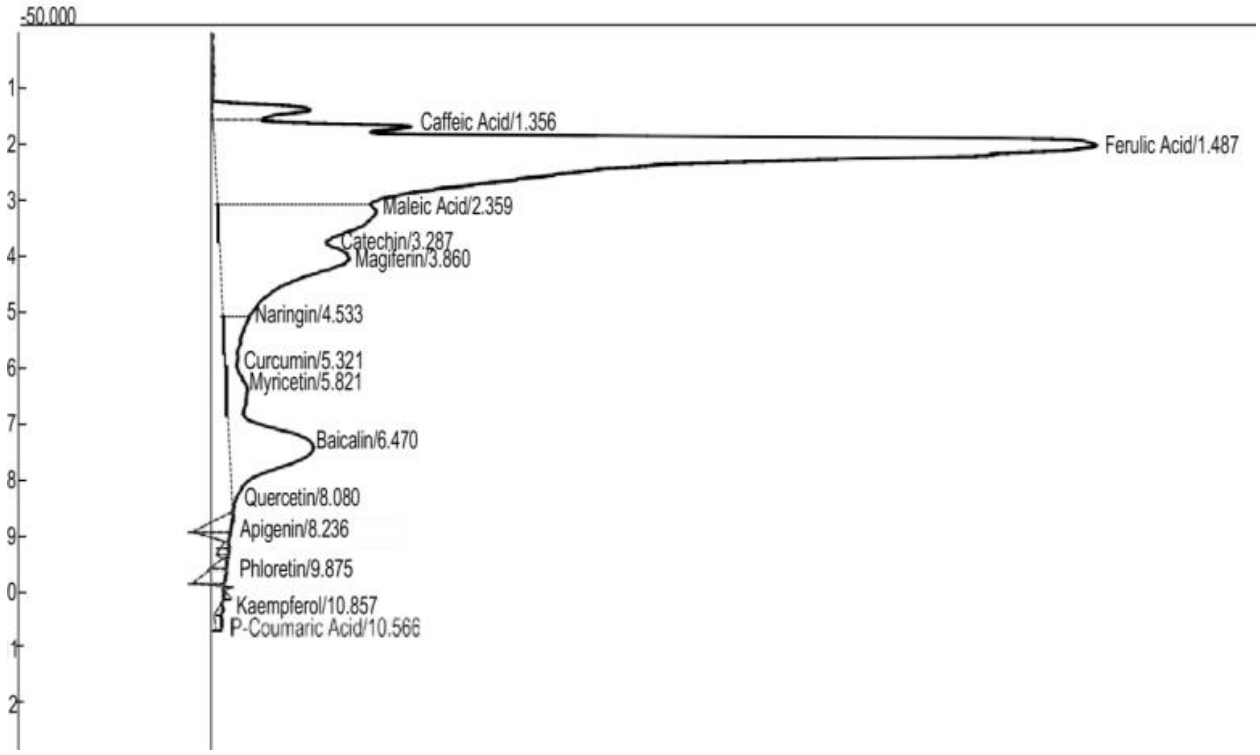

Figure 1: Chromatograph of *Justicia secunda* Extract

Antibacterial Activity of *Justicia secunda* Extracts against MDR Pathogens

As shown in Table 2, the crude extract of *Justicia secunda* exhibited appreciable antibacterial activity against the tested multidrug-resistant isolates. Zones of inhibition ranged from 17.67 ± 0.33 mm to 18.67 ± 0.33 mm, with *Acinetobacter baumannii* showing slightly higher susceptibility than *Klebsiella pneumoniae*. The n-hexane and ethyl-acetate fractions produced the largest inhibition zones, ranging from 25.67 ± 0.33 mm to 27.33 ± 0.33 mm, with the ethyl-acetate fraction demonstrating the strongest activity against both organisms. The methanol fraction showed moderate activity (24.33 ± 0.67–24.67 ± 0.33 mm), while the aqueous fraction exhibited comparatively lower but notable antibacterial effects. As presented in Table 3, the ethyl-acetate fraction recorded the lowest minimum inhibitory concentration (MIC) of 3.125 mg/mL against both *A. baumannii* and *K. pneumoniae*, indicating superior antibacterial potency. The methanol fraction exhibited MIC values of 6.25 mg/mL, whereas the n-hexane and aqueous fractions showed higher MIC values (12.5 mg/mL).

Table 2: Antibacterial Activity of *Justicia secunda* Extracts against MDR Pathogens

Test Organisms	Crude (mm)	n-hexane (mm)	Ethyl-acetate (mm)	Methanol (mm)	Aqueous (mm)
Acinetobacter baumannii	18.67 ± 0.33	26.33 ± 0.33	27.33 ± 0.33	24.67 ± 0.33	24.67 ± 0.67
Klebsiella pneumoniae	17.67 ± 0.33	26.67 ± 0.67	25.67 ± 0.33	24.33 ± 0.67	22.33 ± 0.67

Table 3: Minimum Inhibitory Concentration (MIC) of J. seconda Extract fractions against selected MDR pathogens

Test Organisms	n-hexane (mg/mL)	Ethyl-acetate (mg/mL)	Methanol (mg/mL)	Aqueous (mg/mL)
Acinetobacter baumannii	12.5	3.125	6.25	12.5
Klebsiella pneumoniae	12.5	3.125	6.25	12.5

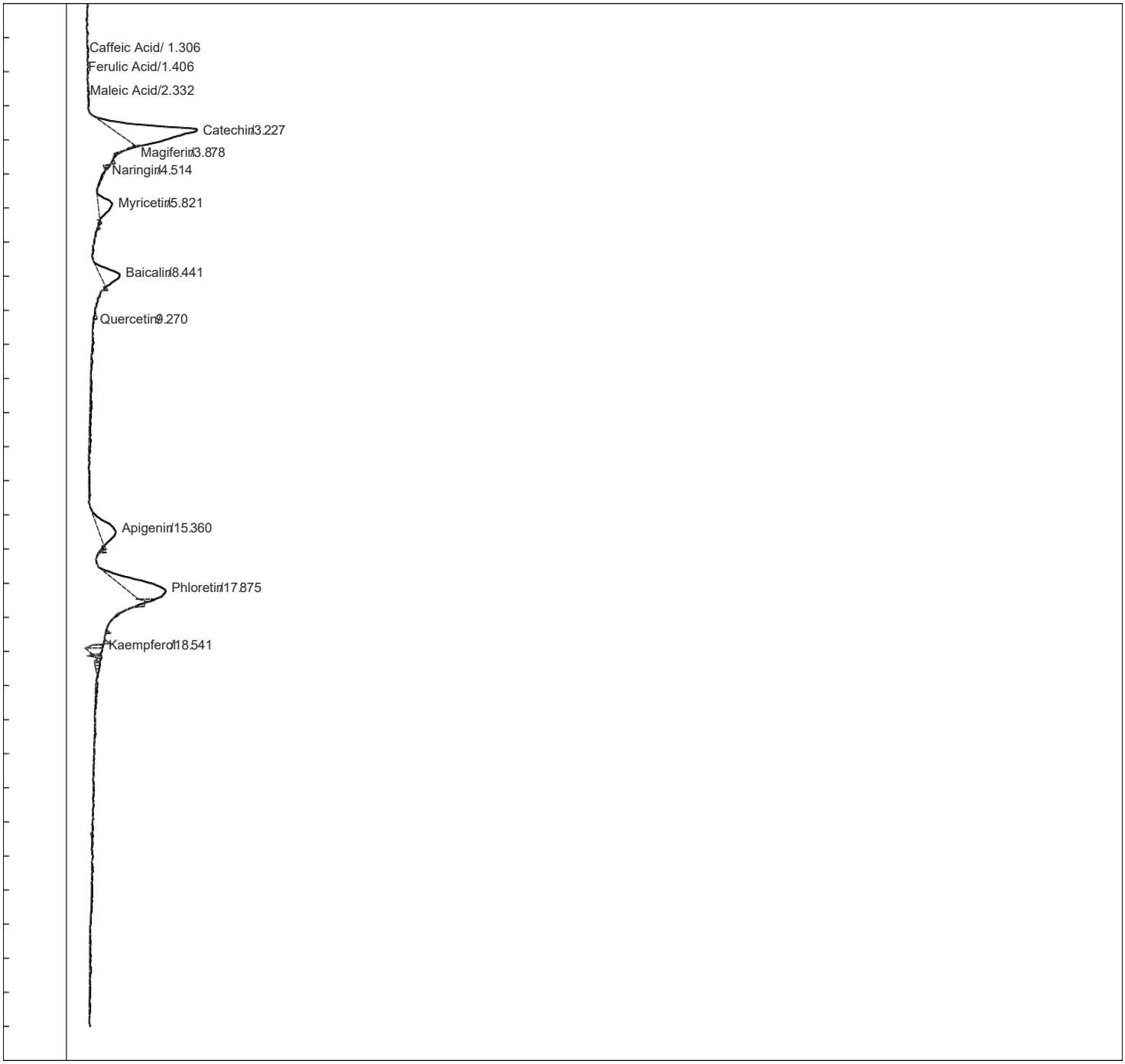

Figure 2: Chromatograph of *K. pinnata* Extract

HPLC Profile of Kalanchoe pinnata ***Extract***

As presented in Table 4 and illustrated in Figure 2, HPLC profiling of *K. pinnata* extract revealed a diverse array of phenolic acids and flavonoids, including caffeic acid, ferulic acid, maleic acid, catechin, mangiferin, naringin, myricetin, baicalin, quercetin, apigenin, phloretin, and kaempferol. The chromatogram displayed distinct and well-resolved peaks, with catechin, mangiferin, and myricetin showing relatively higher peak intensities, suggesting their abundance and potential contribution to the antibacterial potency observed in the fractionated extracts.

Table 4: Compound Identified in *K. pinnata* Extract

RetTime (min)	Type	Area (mAUs)	Amt/Area	Amount. (mg/L)	Name
1.306	VV x	27.63991	1.00097e-1	2.76667	Caffeic Acid
1.406	VV b	2.70424	4.69604e-3	1.26992e-2	Ferulic Acid
2.332	VV b	29.09355	3.20764e-2	9.33216e-1	Maleic Acid
3.227	MM	135.70905	2.51191e-1	34.08885	Catechin
3.878	VV	785.77502	6.70379e-3	5.26767	Magiferin
4.514	BV	26.68658	4.25662e-2	1.13595	Naringin
5.821	MM	136.28868	4.03751e-1	55.02670	Myricetin
8.441	BB	10.26320	4.17755e-2	4.28750e-1	Baicalin
9.270	MM	23.09195	2.00894e-2	4.63902e-1	Quercetin
15.360	MM	20.86465	8.18996e-2	1.70881	Apigenin
17.875	VV x	47.08201	6.54764e-2	1.5087	Phloretin
18.541	VV x	2.97546e-2	9.13517e-1	2.71814e-2	Kaempferol

Abbreviations: RetTime - retention time; min - minutes.

Antibacterial Activity of Kalanchoe pinnata ***Extracts***

As shown in Table 5, the crude extract of *Kalanchoe pinnata* demonstrated antibacterial activity against the tested MDR pathogens, with inhibition zones ranging from 12.67 ± 0.88 mm to 13.33 ± 0.88 mm. Fractionation significantly improved antibacterial efficacy. The n-hexane and ethyl-acetate fractions exhibited the highest activity, producing inhibition zones of up to 27.00 ± 0.58 mm against both *A. baumannii* and *K. pneumoniae*. The methanol fraction showed moderate activity (23.00 ± 0.58 to 25.00 ± 0.58 mm), while the aqueous fraction displayed comparatively lower inhibitory effects. As presented in Table 6, the aqueous fraction of *K. pinnata* demonstrated the lowest MIC value (3.125 mg/mL) against both test organisms, followed by the methanol and n-hexane fractions (6.25 mg/mL). The ethyl-acetate fraction recorded higher MIC values (12.5 mg/mL).

Table 5: Antibacterial Activity of *K. pinnata* Extracts

Test Organisms	Crude (mm)	n-hexane (mm)	Ethyl-acetate (mm)	Methanol (mm)	Aqueous (mm)
Acinetobacter baumannii	12.67 ± 0.88	27.00 ± 0.58	25.00 ± 0.58	23.00 ± 0.58	16.67 ± 0.67
Klebsiella pneumoniae	13.33 ± 0.88	27.00 ± 0.58	27.00 ± 0.58	25.00 ± 0.58	16.00 ± 0.58

Table 6: Minimum Inhibitory Concentration (MIC) of K. pinnata Extract fractions against MDR pathogens

Test Organisms	n-hexane (mg/mL)	Ethyl-acetate (mg/mL)	Methanol (mg/mL)	Aqueous (mg/mL)
Acinetobacter baumannii	6.25	12.5	6.25	3.125
Klebsiella pneumoniae	6.25	12.5	6.25	3.125

Molecular Docking, ADMET and Drug-Likeness Profile of Bioactive Compounds from *K. pinnata* and *J. secunda*

Molecular docking with MM/GBSA refinement was used to evaluate phytochemicals from Kalanchoe pinnata and *Justicia secunda* against DNA gyrase of *Acinetobacter baumannii* and penicillin-binding protein (PBP) of *Klebsiella pneumoniae*. Against *A. baumannii* (Figure 4, Figure 6, Table 7), several compounds showed stronger inhibitory potential than ceftriaxone and levofloxacin, while analysis against *K. pneumoniae* (Figure 5, Figure 7, Table 8) identified lead molecules with higher predicted affinities than ceftriaxone and meropenem.

ADME/TOX analysis showed that most bioactive compounds possessed favorable physicochemical and drug-likeness properties (Table 9). Several compounds, including apigenin, caffeic acid, catechin, ferulic acid, kaempferol, myricetin, and p-coumaric acid, complied fully with Lipinski's rule of five. High gastrointestinal absorption and good bioavailability were observed for most low-molecular-weight compounds, whereas larger glycosylated molecules exhibited reduced absorption. Minimal CYP2C9 and CYP2C19 inhibition was predicted for the majority of compounds, indicating low metabolic interaction potential

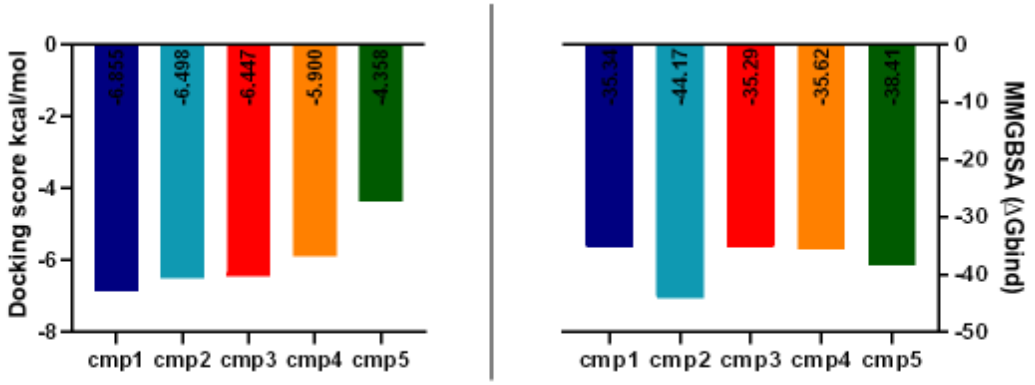

Figure 4: The graphical expression of the calculation of binding affinity and binding free energy of bioactive compounds of *K. pinnata* and *J. secunda* against the DNA gyrase protein of *Acinetobacter baumannii*

Legend: cmp1 (Myricetin), cmp2 (Mangiferin), cmp3 (Quercetin), cmp4 (Baicalin) and cmp5 (Levofloxacin)

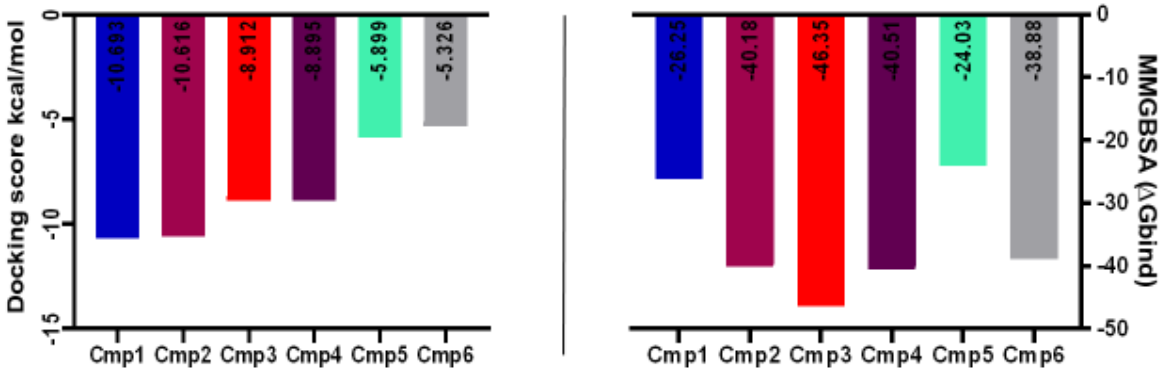

Figure 5: The graphical expression of the calculation of binding affinity and binding free energy of bioactive compounds of *K. pinnata* and *J. secunda* against the Penicillin binding protein of *Klebsiella pneumoniae*

Legend: cmp1 (Baicalin), cmp2 (Naringin), cmp3 (Catechin), cmp4 (Mangiferin), cmp5 (Ceftazidime), and cmp6 (Meropenem)

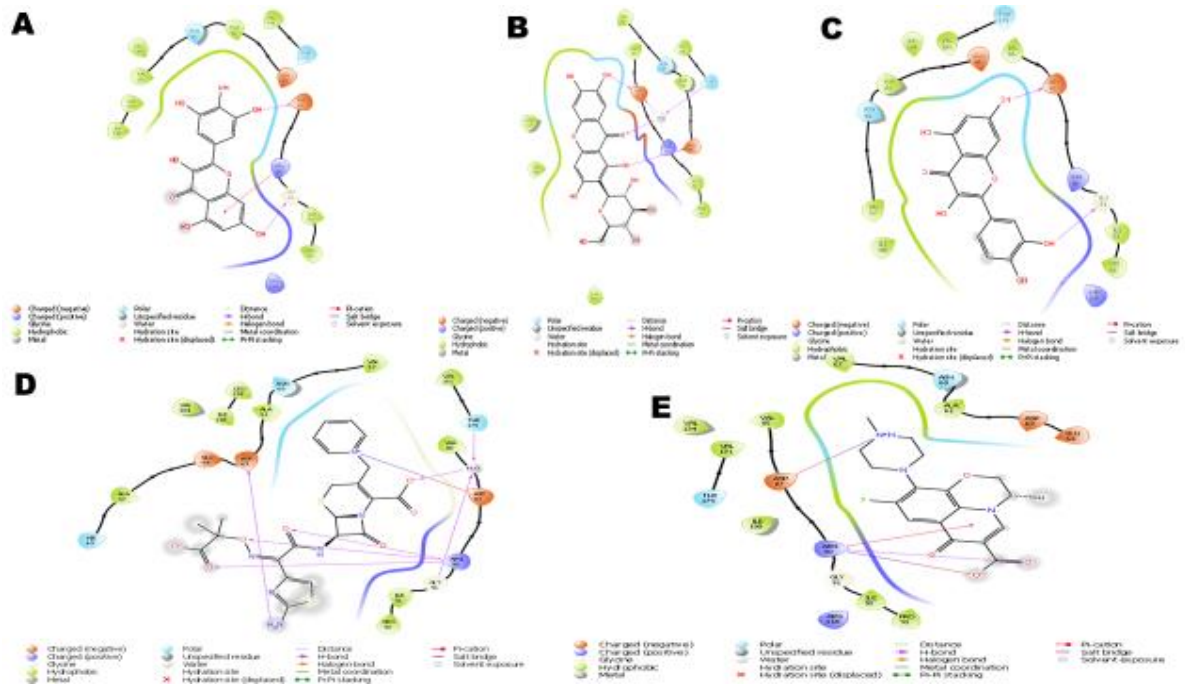

Figure 6. Two-dimensional (2D) molecular interaction maps of the binding modes of bioactive compounds of Kalanchoe pinnata and Justicia secunda in the active site of the DNA gyrase protein of Acinetobacter baumannii. The interactions reveal major hydrogen bonds, hydrophobic contact and other non-covalent interactions that aid in the stabilization of the ligand in the binding pocket. (A) Myricetin, **(B)** Mangiferin, **(C)** Quercetin, **(D)** Baicalin and **(E)** Levofloxacin (standard drug).

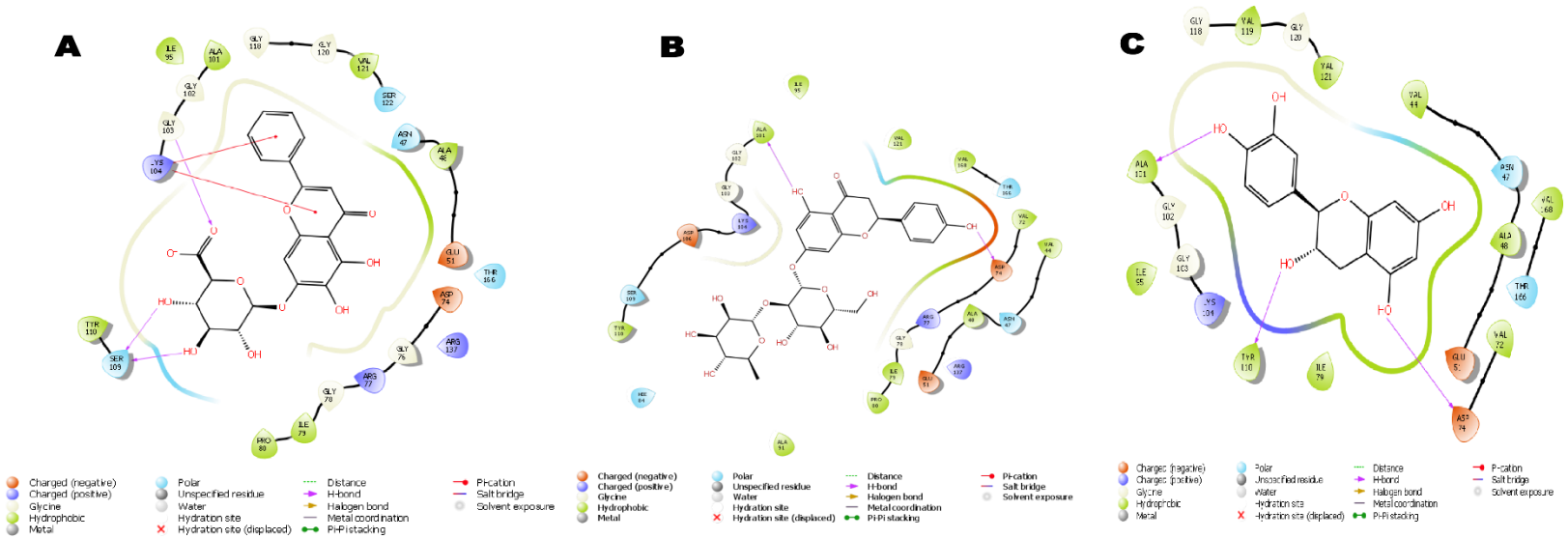

Figure 7a. Two-dimensional (2D) molecular interaction maps of the binding modes of bioactive compounds of Kalanchoe pinnata and Justicia secunda in the active site of the PBP of *Klebsiella pneumoniae.* The interactions reveal major hydrogen bonds, hydrophobic contact and other non-covalent interactions that aid in the stabilization of the ligand in the binding pocket. **(A)** Baicalin, **(B)** Naringin, **(C)** Catechin.

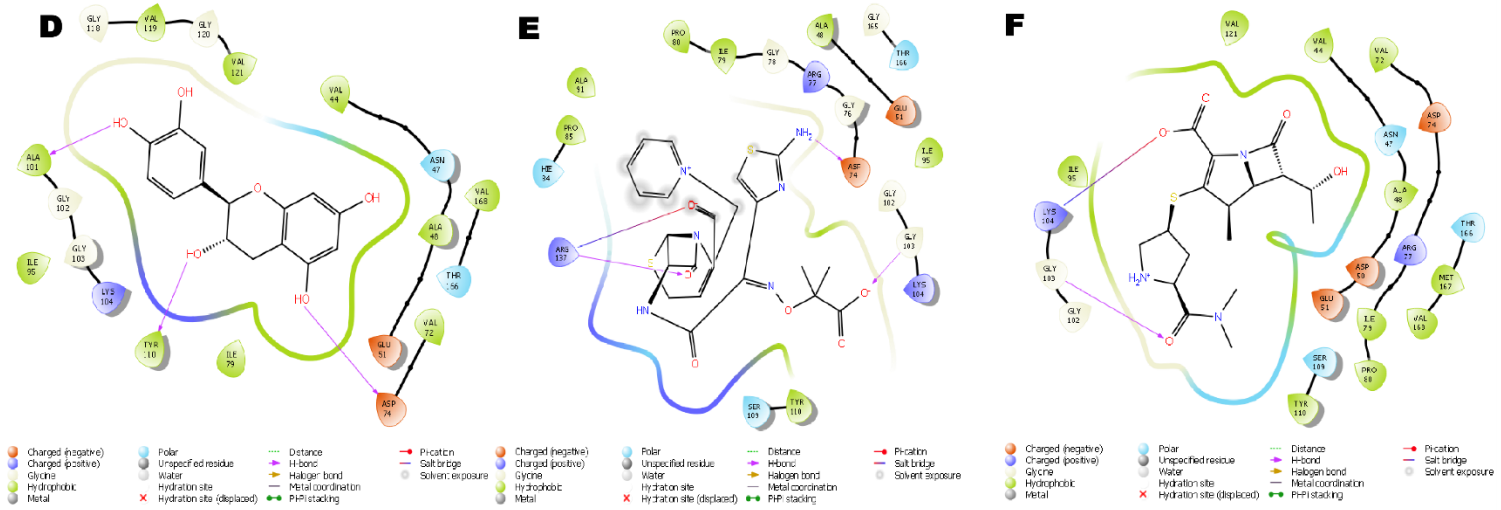

Figure 7b. Two-dimensional (2D) molecular interaction maps of the binding modes of bioactive compounds of Kalanchoe pinnata and Justicia secunda in the active site of the PBP of *Klebsiella pneumoniae.* The interactions reveal major hydrogen bonds, hydrophobic contact and other non-covalent interactions that aid in the stabilization of the ligand in the binding pocket. **(D)** Mangiferin, **(E)** Ceftazidime (standard drug), and **(F)** Meropenem (standard drug).

Table 7: Hydrogen Bonds and Hydrophobic interactions of the hit phytochemicals of bioactive compounds of *Kalanchoe pinnata* and *Justicia secunda* for *A. baumannii*

Compound Name	H-Bond	Hydrophobic interactions	Other Interactions
Myricetin	ASP 87, GLY91	ILE108, LEU109, VAL57, VAL134, ALA61, VAL181, ILE92, PRO93	Pi cation: ARG 90
Mangiferin	ASP87, THR179, GLU64,	VAL134, ILE108, ALA104, PRO93, ILE92, ALA61, VAL181, VAL85, VAL57	NONE
Quercetin	ASP87, GLY91	ILE 108, VAL57, ALA61, VAL134, VAL 181, ILE92, PRO93,VAL85	NONE
Baicalin	VAL134, ASN60, THR179, ASP87, GLY91	ILE108, VAL13, PRO93, ILE92	Pi cation : ARG90
Levofloxacin	ASP87, THR179, GLY91	VAL57, VAL134, ILE108, LEU109, PRO93, ILE92, VAL181, VAL85, ALA61	NONE

Table 8: Hydrogen Bonds and Hydrophobic interactions of the hit phytochemicals of bioactive compounds of *Kalanchoe pinnata* and *Justicia secunda* for *K. pneumoniae*

Compound Name	H-Bond	Hydrophobic interactions	Other Interactions
Baicalin	SER109, GLY103	ILE95, ALA101, VAL121, ALA48, ILE79, PRO80, TYR110, ALA101	Pi cation: LYS104
Naringin	ALA101, ASP74	ILE95, ALA101, VAL121, VAL168, VAL72, VAL44, ALA48, ILE79, PRO80, ALA91, TYR110	NONE
Catechin	ASP74, TYR110, ALA101	ILE 95, VAL72, VAL168, VAL 181, ILE95,VAL85, ALA101, ALA48, VAL44, ILE79	NONE
Mangiferin	ASP74	ILE95, VAL44, VAL121, ALA48, VAL168, ILE79, PRO80, TYR110	PI-PI Stacking: TYR110
Ceftazidime	ARG 137, GLY103, ASP74	PRO85, ALA91, TYR110, ILE95, ALA48, ILE79, PRO80	Salt Bridge: ARG137
Meropenem	GLY103	VAL121, ILE95, VAL44, VAL72, ALA48, ILE79, PRO80, TYR110, VAL168, MET167	Salt Bridge: Lys 104

Table 9: Druglikeness and ADMET profile of the bioactive compounds of *K. pinnata* and *J. secunda*

Compound Name	MW	HBA	HBD	TPSA	iLOGP	ROV	ESOL Log S	GIA	CYP2C19 inhibitor	CYP2C9 inhibitor	BA
Apigenin	270.05	5	3	90.9	2.980918	0	-4.21599	0.001684	0.112379424	0.00212802	0.83683
Baicalin	446.08	11	6	187.12	1.300846	1	-2.30448	0.008194	6.57E-06	7.80E-07	0.536646
Caffeic acid	180.04	4	3	77.76	1.189631	0	-1.83711	0.301189	1.89E-05	0.00422838	0.995638
Catechin	290.08	6	5	110.38	1.172588	0	-2.58137	0.016103	9.82E-06	2.47E-06	0.994156
Curcumin	368.13	6	2	93.06	2.1471	0	-3.61994	0.017107	0.490617067	0.26905319	0.478927
Ferulic acid	194.06	4	2	66.76	1.647679	0	-2.36392	0.249424	0.000782978	0.01841412	0.932978
Kaempferol	286.05	6	4	111.13	1.965317	0	-3.64796	0.015143	0.132401511	0.79935813	0.715565
Maleic acid	116.01	4	2	74.6	-0.02442	0	0.404371	0.949129	3.26E-10	5.12E-05	0.068982
Mangiferin	422.08	11	8	201.28	0.059778	1	-3.15526	0.751839	1.91E-08	0.00031125	0.992878
Myricetin	318.04	8	6	151.59	1.115212	0	-3.44294	0.31806	0.002221005	0.99521881	0.993408
Naringin	580.18	14	8	225.06	0.474727	1	-2.35286	0.750696	0.000258328	0.08677637	0.999825
P - coumaric acid	164.05	3	2	57.53	1.440249	0	-2.11364	0.082512	0.000589978	0.01600456	0.960873

DISCUSSION

This study confirms *Justicia secunda* and *Kalanchoe pinnata* as important sources of antibacterial phytochemicals with activity against multidrug-resistant *Klebsiella pneumoniae* and *A. baumannii*. Although crude extracts exhibited measurable antibacterial effects, fractionation consistently enhanced antibacterial potency. This observation aligns with reports demonstrating that solvent partitioning enriches active constituents and reduces antagonistic effects within complex phytochemical matrices (Bhattacharjee *et al*., 2011; Abuga, 2025).

For *J. secunda*, the ethyl-acetate fraction showed the highest antibacterial activity, yielding the largest inhibition zones and lowest MIC values (3.125 mg/mL) against *Acinetobacter baumannii* and *Klebsiella pneumoniae*. The reduced activity of the crude extract suggests dilution or masking of bioactive compounds, consistent with previous reports on *Justicia* species where moderately polar fractions rich in flavonoids and phenolic acids exhibited superior activity (Koffi *et al*., 2022; Sherif *et al*., 2024). The high susceptibility of *A. baumannii* agrees with studies indicating its vulnerability to polyphenol-induced membrane disruption and efflux pump inhibition (Dhiman *et al*., 2021).

In *K. pinnata*, enhanced antibacterial activity was observed in both n-hexane and ethyl-acetate fractions, while the aqueous fraction recorded the lowest MIC (3.125 mg/mL), suggesting contributions from both non-polar and polar constituents. Similar improvements following fractionation have been reported in other *Kalanchoe* species, attributed to enrichment of bioactive flavonoids, phenolic acids, and glycosides (Ramon *et al*., 2023; Nascimento *et al*., 2023). The lower activity of crude extracts likely reflects antagonistic interactions and reduced bioavailability of active compounds.

HPLC analysis confirmed the presence of caffeic acid, ferulic acid, quercetin, myricetin, apigenin, baicalin, naringin, phloretin, and kaempferol, all of which are known to exert antibacterial effects through membrane disruption, inhibition of nucleic acid synthesis, metabolic interference, and suppression of virulence factors (Cushnie and Lamb, 2021; Tyagi *et al*., 2015). Quercetin and myricetin have been shown to inhibit DNA gyrase and topoisomerase IV in Gram-negative bacteria (Nguyen and Bhattacharya, 2022), while caffeic and ferulic acids enhance bacterial susceptibility by inducing oxidative stress and destabilizing lipid bilayers (Bakrim *et al*., 2022). The enrichment of these compounds explains the superior activity of fractionated extracts, particularly in ethyl-acetate, methanol, and aqueous fractions.

MIC data further supported the enhanced potency of fractionated extracts, consistent with earlier studies demonstrating that fractionation concentrates active metabolites and eliminates inhibitory components (Chatterjee *et al*., 2011; Abuga *et al*., 2025). The comparatively higher MICs of n-hexane fractions in *J. secunda* suggest that non-polar constituents alone may be insufficient for optimal antibacterial activity.

The greater susceptibility of *A. baumannii* relative to *K. pneumoniae* may be attributed to differences in membrane structure and permeability, as *K. pneumoniae* possesses a thick polysaccharide capsule that limits phytochemical penetration (Borges *et al*., 2015; Dhiman *et al*., 2021).

Complementing the in vitro findings, molecular docking and MM/GBSA analyses demonstrated that several phytochemicals—particularly myricetin, mangiferin, quercetin, and baicalin—exhibited stronger predicted binding affinities to *A. baumannii* DNA gyrase than levofloxacin, with MM/GBSA refinement confirming complex stability. This concordance strengthens confidence in their inhibitory potential and is consistent with previous in silico studies of flavonoid-rich metabolites (Al Mashud *et al*., 2025). Similarly, docking against *K. pneumoniae* PBP showed that baicalin, naringin, catechin, and mangiferin bound more strongly than ceftazidime and meropenem, in agreement with reports that polyphenols form extensive stabilizing interactions within PBP active sites (Cai *et al*., 2016).

ADMET analysis revealed that smaller flavonoids displayed more balanced drug-like properties, whereas glycosylated flavonoids were highly polar with increased hydrogen-bonding capacity, potentially limiting

permeability despite strong target affinity (Ancuceanu *et al*., 2025). This trade-off is consistent with previous ADMET evaluations of flavonoid derivatives (Oluyele *et al*., 2025a).

Overall, the strong agreement between antibacterial assays, phytochemical profiling, and integrated computational analyses supports phytochemicals from *J. secunda* and *K. pinnata* as promising antibacterial scaffolds warranting further experimental validation against MDR pathogens.

CONCLUSIONS

This study demonstrates that *Justicia secunda* and *Kalanchoe pinnata* are rich sources of antibacterial phytochemicals with significant activity against multidrug-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Fractionation markedly enhanced antibacterial efficacy over crude extracts, highlighting its importance in enriching bioactive constituents. HPLC profiling confirmed the presence of key phenolic acids and flavonoids, including quercetin, myricetin, apigenin, caffeic acid, and ferulic acid, which likely act through multi-target antibacterial mechanisms.

In silico analyses further showed that several phytochemicals exhibited stronger predicted inhibitory interactions with DNA gyrase and penicillin-binding proteins than some conventional antibiotics, supporting their potential as antibacterial drug scaffolds. Future studies should focus on isolating and characterizing the most active compounds, followed by in vivo efficacy, toxicity, and synergy studies with existing antibiotics. Overall, these findings support the development of affordable, plant-derived antibacterial agents from *J. secunda* and *K. pinnata*, particularly for resource-limited settings.

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Conflict of interest

The authors declare that there are no competing interests associated with this study.

Authors contributions: O.O. conceived and designed the study. O.O., O.V.A. and S.F.O. performed the experimental procedures. O.O. and G.E. performed computational analyses. Data collection and analysis were carried out by O.O., O.V.A., and S.F.O. Overall supervision of the research was provided by O.O. Manuscript drafting and critical revision were undertaken by O.O. G.E. and E.E. All authors reviewed, approved, and agreed to the final version of the manuscript.

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Supplementary Materials

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