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FTIR Fingerprinting Unravels Solvent-Dependent Antibacterial Potentials of *Vernonia amygdalina* Leaf Extracts Against Selected Multidrug-Resistant ESKAPE Isolates

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Abstract

Plant-derived antimicrobials can potentially counteract the increasing ineffectiveness of conventional antibiotics against multidrug-resistant (MDR) ESKAPE pathogens. This study compared the extraction yield, phytochemical profile, functional-group signatures, and antibacterial activity of aqueous and n-hexane leaf extracts of *Vernonia amygdalina* against selected MDR ESKAPE pathogens from Katsina State, Nigeria. The fresh leaves were subjected to maceration, the obtained yield was calculated, and phytochemical constituents were screened using both standard qualitative assays and FTIR spectroscopy. Antibacterial activity was evaluated by agar well diffusion technique. Statistical significance was determined using the Welch test and two-way ANOVA. The n-hexane extract produced a significantly higher yield than the aqueous extract (28.81% vs. 9.87%; $p < 0.0001$). Conventional screening detected cardiac glycosides, saponins, steroids, and flavonoids, while FTIR analysis indicated bioactive functional groups consistent with terpenoids, steroids, polyacetylenes, cyanogenic glycosides, triglycerides, and lipophilic alkaloids. Both extracts inhibited the test pathogens, with no significant difference relative to the positive control ($p = 0.066$). These findings indicate solvent-dependent variation in bioactive metabolites present in *V. amygdalina* leaves. These compounds exhibited antibacterial activity against MDR ESKAPE pathogens and can hence be exploited as potential leads for discovering novel antibacterials to tackle these pathogens in the study area and beyond.

Keywords: African bitterleaf (*Vernonia amygdalina*) leaf; antibiotic resistance; ESKAPE pathogens; n-hexane; phytochemical screening

Academic Editor: Athanasios Tsakris

Received: 3 June 2026

Revised: 30 June 2026

Accepted: 5 July 2026

Published: 8 July 2026

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1. Introduction

The ESKAPE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*

spp.) are named as such because of their ability to escape or evade the bactericidal effects of commonly prescribed antibiotics [1]. This is mediated by the array of diverse drug resistance mechanisms acquired by these pathogens, including biofilm formation, efflux pumps-mediated extrusion, and beta-lactamase production [2]. Consequently, ESKAPE pathogens had emerged as a significant public health challenge due to their high prevalence, virulence, nosocomial nature, and threats of therapeutic failure [3]. The rise in antimicrobial resistance (AMR) among these pathogens has compounded this problem into a global public health concern [4]. This is thought to be fueled by plasmid-mediated gene exchange, mobile genetic elements, carbapenemases production and modifications in cell permeability [5].

Recent meta-analyses of data from Africa indicate that ESKAPE pathogens are widely distributed in numerous reservoirs in clinical, food, animal, and environmental settings, with the highest pooled prevalence in West Africa [6]. This underscores the need for One Health-oriented antimicrobial discovery and surveillance [7].

AMR among ESKAPE pathogens is particularly alarming when contextualized against the overall burden of bacterial infections, especially in Africa. Bacterial infections are responsible for an estimated 7.7 million deaths annually, making them the second-leading cause of death worldwide. Out of these, 4.95 million deaths are directly associated with antimicrobial resistance (AMR), and 1.27 million deaths are directly attributable to highly resistant bacterial strains. In this group, the ESKAPE pathogens dominate, with *K. pneumoniae* and *S. aureus* ranking as top killers [8,9]. Similarly, the burden is worse in Africa, as it bears the highest regional burden of AMR mortality globally. An estimated 27.3 deaths per 100,000 population in Africa is directly attributable to drug-resistant infections. This is heavily driven by a high prevalence of third-generation cephalosporin-resistant *Enterobacterales* (*K. pneumoniae*) and MRSA in clinical environments [6,10].

The Nigerian situation is equally concerning. It has been established that carbapenem-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* are already prevalent in Nigerian hospitals, with the burden increasing over time. However, the published evidence only concentrated in a few geopolitical zones, particularly the South-West [11]. This pattern simultaneously points to a real AMR problem as well as an evidence gap, that is, carbapenem resistance is present, but surveillance is uneven. Hence, locally accessible and scientifically defensible practical alternatives remain urgently needed [12].

Plant-derived antimicrobials remain one of the most promising complementary routes for addressing MDR bacteria, in the sense that they enable identifying molecules with multi-target activity rather than single-site antibacterial action [13,14]. In this regard, *Vernonia amygdalina* is particularly relevant considering that it is rich in flavonoids, alkaloids, saponins, tannins, triterpenoids, sesquiterpene lactones, steroids, cardiac glycosides, cyanogenic glycosides, and phenols [15]. Consequently, the antibacterial as well as broad pharmacological activities of this plant are well-documented [15,16]. Similarly, recent studies have shown that solvent extraction alters the phytochemical profile and FTIR spectrum of *V. amygdalina* extracts. In other words, the polarity of the extraction solvent used influences which bioactive compounds are extracted as well as their biological activity [13,17,18]. In the same vein, recent studies also demonstrated the antibacterial activity of *V. amygdalina* extracts against multidrug-resistant enteric pathogens [16,19]. Consequently, the foregoing supports the candidacy of *V. amygdalina* extracts as novel anti-MDR bacteria leads [20].

Mechanistically, the antibacterial activity of the phytoconstituents of *V. amygdalina* occurs through membrane disruption, enzyme inhibition, efflux modulation, antibiofilm effects, and interference with bacterial signaling [21]. As explained earlier, solvent fractionation can enrich either polar or lipophilic compounds with different target spectra, and this matters for *V. amygdalina* because lipophilic constituents such as steroids, terpe-

noids, and polyacetylenes, are more likely to partition into non-polar solvents, whereas polar phenolics and glycosides are better represented in aqueous fractions [14]. Therefore, FTIR offers a rapid fingerprinting approach that can link the chemistry of extracts to function [22,23].

Against this backdrop, the present study was designed to test two related hypotheses. Firstly, FTIR fingerprinting can quantitatively and qualitatively resolve distinct constituents of *V. amygdalina* leaf extracts influenced by the extraction solvent. Secondly, these differences would translate into measurable variation in antibacterial activity against MDR ESKAPE isolates from Katsina State, Nigeria. The study combines conventional phytochemical screening with FTIR fingerprinting and bioassay-guided antibacterial testing to clarify whether aqueous and n-hexane fractions differ in extract yield, functional-group composition, and antibacterial activity against MDR ESKAPE pathogens. Accordingly, this study provides a broadly relevant contribution in the search for plant-based antibacterial leads against MDR ESKAPE pathogens.

2. Materials and Methods

2.1. Study Area, Sample Collection and Herbarium Studies

The *Vernonia amygdalina* leaf sample (Supplementary Figure S1) was collected from a garden within Katsina Metropolis (location: longitude 12.975561° N, 7.633454° E) during the harmattan period of the dry season. All experiments were carried out at the Microbiology and Central Laboratories, Umaru Musa Yar'adua University, Katsina, Nigeria.

Herbarium studies were carried out by a botanist in the Department of Biology, Umaru Musa Yar'adua University, Katsina. The specimen was identified and assigned a voucher number (umyuh1819).

2.2. Sample Processing and Extraction of Phytochemical Compounds

The collected samples were dried under shade at room temperature and finely pulverized into a powder using a mortar and pestle [24].

Phytochemical compounds were extracted using a maceration technique modified from Lubis et al. [13]. Both distilled water (H₂O) and n-hexane were used for the extraction, using 100 mL to dissolve 10 and 5.50 g of the leaf powder, respectively. The variation in starting masses is due to extraction ability of each solvent, solubility differences, and the expected yield for downstream analyses based on the starting mass (15.5 g). The phytochemicals were extracted for 3 days, and then the mixture was filtered, heated in water bath, and then left to evaporate. Afterwards, the extract was weighed and the percentage yield was calculated as follows:

$$\text{Percentage Yield} = \frac{\text{Weight of extract obtained}}{\text{Weight of starting material}} \times 100$$

2.3. Phytochemical Analysis

2.3.1. Conventional Phytochemical Tests

The extracts were subjected to tests for saponins, cardiac glycosides, steroids, and flavonoids, as follows. Cardiac glycosides were tested by dissolving 0.50 g of the balance and dissolving into 2 mL of glacial acetic acid in a test tube. Thereafter, three drops of ferric chloride solution were added followed by 1 mL of concentrated H₂SO₄. A brown ring at the interface indicates the presence of deoxysugars, a characteristic of cardenoloids [25]. For flavonoids, the same amount of *Vernonia amygdalina* leave extracts (0.50 g) was added followed by 3 drops of 10% of ferric chloride solution. A green or blue color indicates the presence of phenolic nucleus [26]. Meanwhile, the test for saponins involved mixing 10 mL distilled water with the same amount of extract (0.5 g). The preparation was

allowed to stand for 15 min with regular shaking. The emergence of a layer of foam indicates the presence of saponins [27]. Steroids were tested by adding 2 mL of acetic anhydride to 0.50 g of the extracts, followed by 2 mL of H₂SO₄. A color change from violet to blue indicates the presence of steroids [28]. Finally, terpenoids were tested by adding 0.50 g of the extracts in 5 mL of water. This was mixed with 2 mL of chloroform, and then 3 mL of concentrated H₂SO₄. A reddish-brown colorization of the interface indicates the presence of terpenoids [29].

2.3.2. FTIR Analysis

The FTIR analysis of the extracts was conducted as modified by Alara et al. [30] and Kale and Dhabe [31]. The analysis was conducted using an Agilent Cary 630 FTIR spectrometer. Sample preparation involved carefully preparing the samples and incorporating them as KBr pellets. For each sample, 64 scans were recorded to ensure reproducibility and reliability of the spectral data. Background scans were also performed to account for any atmospheric interference or noise, allowing for a more accurate comparison with the sample spectra. The resolution was 4 cm⁻¹, and the analytical spectral range was 4000 to 650 cm⁻¹. The apodization function was set to Happ–Genzel to reduce the side lobes of the spectral peaks and improve the overall quality of the spectral data. This minimizes distortions that could compromise the interpretation of the spectra.

After acquiring the data, the resulting spectra were analyzed to identify the characteristic peaks associated with different functional groups present in the samples [32].

2.4. Antibigram/Multidrug Resistance Assay

2.4.1. Preparation of Media and McFarland Standards

Four clinical ESKAPE isolates (*Klebsiella pneumoniae*, KP; *Enterobacter* species, ES; *Staphylococcus aureus*, SA and *Pseudomonas aeruginosa*, PA) were collected from the culture collection at the Department of Microbiology Laboratory, UMYUK. These were subjected to antibiogram testing using prepared 0.5 mL McFarland standard based on CLSI (2024) protocols [33]. Similarly, Mueller–Hinton Agar (Oxoid, Basingstoke, UK) was prepared according to the manufacturer's instructions.

2.4.2. Sensitivity Testing

Sensitivity testing was done using disk diffusion assay, as adopted by Mahuta et al. [34]. Initially, 0.1 mL of the McFarland standard for each ESKAPE isolate was spread on respective Mueller–Hinton agar plates, and a 30 min absorption period was allowed. Thereafter, the following antibiotics disks: SXT = Septrin (30 µg), CH = chloramphenicol (30 µg), SP = Sparfloxacin (30 µg), CPX = Ciprofloxacin (30 µg), AM = Amoxacin (30 µg), AU = Augmentin (10 µg), CN = Gentamicin (30 µg), PEF = Pefloxacin (30 µg), OFX = Tarivid (10 µg) and S = Streptomycin (30 µg) were placed on the surface of the plates, which were then incubated for 18 h. The zone of inhibition was measured using digital Vernier Caliper, and the results were interpreted as sensitive, intermediate or resistant, based on an interpretative chart, to enable the classification of the isolates and the identification of those that are multidrug-resistant, as adapted by Riko et al. [35].

2.4.3. Extract Bioassay (Agar Well Diffusion Technique)

Four specific concentrations of both aqueous and n-hexane extracts (50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL) were prepared by dissolving 0.5 g of the extract in 5 mL of distilled water to obtain the 100 mg/mL stock, after which the respective dilutions were prepared serially [36].

Thereafter, six plates of Mueller–Hinton Agar were prepared (two plates for each MDR ESKAPE isolate). Each plate was inoculated with 0.1 mL of the McFarland standard and five holes were punched in each plate using a sterile cork borer, into which 0.1 mL of the extract from each test tube was added. Additionally, 0.1 mL of distilled water was included as a negative control, while a ciprofloxacin disk was placed in the center of each plate as a positive control. This procedure was replicated for all bacterial isolates tested (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.), using both aqueous and n-hexane extracts. Following inoculation, the plates were incubated for 18 h, after which the zones of inhibition were measured using a digital Vernier Caliper, providing a quantitative assessment of the antimicrobial efficacy of the extracts [35,37].

2.5. Statistical Analysis

All values were stated as average $\pm$ standard deviation of triplicate measurements. Statistical analyses were conducted using AnalyStat software (version 1.6.50). Welch test was used to compare the yield obtained from the two solvents. ANOVA and Mann–Whitney tests were used to evaluate significant differences in the zones obtained from the aqueous and n-hexane fractions. Differences were considered statistically significant at $p \leq 0.05$ level of significance [38].

3. Results

3.1. Properties of the Extracts

The properties of the leaves/extract are presented in Table 1. The physical properties of the extract (color, texture and odor) as well as the yield varied between the aqueous and n-hexane extracts. The percentage yield differs significantly based on the solvent used for the extraction (Welch test: $p < 0.0001$), with the aqueous extract yielding 9.87%, while that of n-hexane was 28.81%, even though the starting mass in the aqueous treatment was approximately twice that of the n-hexane.

Table 1. Properties of the *V. amygdalina* extracts.

Parameter	Detail
Collection point	Local garden within Katsina, Nigeria Co-ordinates: 12.9756° N, 7.6334° E
Herbarium reference number	UMYUH1819
Physical properties of the extract	Color: dark green (aqueous)/bluish black (n-hexane) Texture: sticky Odor: odorless
Percentage yield	9.87% (aqueous); 28.81% (n-hexane)

3.2. Phytochemical Composition of the Extract

Table 2 shows the physicochemical composition of the extracts. More phytochemical classes were present in the aqueous extract (4: cardiac glycosides, saponins, steroids, flavonoids) than in the n-hexane fraction (3: saponins, steroids and flavonoids).

Table 2. Physiochemical composition of *V. amygdalina* aqueous and n-hexane leaf extracts.

S/No	Phytochemicals	Aqueous Extract	n-Hexane Extract
1	Cardiac Glycosides	+	–
2	Saponins	+	+
3	Steroids	+	+
4	Flavonoids	+	+
5	Terpenoids	–	–

Key: + = Presence of phytochemical, – = Absence of phytochemical.

3.3. FTIR Analyses of the Extracts

Table 3 presents the FTIR spectra analyses for the aqueous extracts. The results showed the presence of functional groups that are potentially indicative of terpenoids; polyacetylenes, cyanogenic glycosides, nitrile alkaloids and fatty acid nitriles; phenols and flavonoids, alkaloids and saponins. These are the likely bioactive compounds present in the extract based on the peak number, wave number and intensity, based on the formulae shown in the table. Meanwhile, for the n-hexane extracts, the likely bioactive compounds were terpenoids, steroids and triglycerides; polyacetylenes, lipophilic alkaloids and fatty acid nitriles; phenols and amine compounds, as deduced in the same manner (Table 4).

Table 3. FTIR spectra analysis of aqueous *V. amygdalina* leaf extract.

Peak Number	Wavenumber (cm) ⁻¹	Intensity	Interpretation and Formula	Likely Bioactive Compound(s)
1	1640.03	65.18	C=C/C=O stretching	Terpenoids
2	2124.58	95.83	C≡C, C≡N (nitrile)	Polyacetylenes, terpenoids, cyanogenic glycosides, nitrile alkaloids and fatty acid nitriles
3	2303.49	97.16	CO ₂ asymmetric overtone	NA (atmospheric carbon)
4	3265.15	42.64	O-H/N-H stretching	Phenols and flavonoids, alkaloids and saponins

Table 4. FTIR spectra analysis of the n-hexane extracts of *V. amygdalina*.

Peak Number	Wavenumber (cm) ⁻¹	Intensity	Interpretation and Formula	Likely Bioactive Compound(s)
1	1640.03	66.72	C=C/C=O stretching	Terpenoids, steroids and triglycerides
2	2117.13	95.68	C≡C, C≡N (nitrile)	Polyacetylenes, lipophilic alkaloids and fatty acid nitriles
3	3265.15	43.56	O-H/N-H stretching	Phenols and amine compounds

3.4. Antibigram of the Test Bacteria

The antibiogram of the ESKAPE clinical isolates is presented in Table 5. All three isolates are classified as MDR. The most effective antibiotics varied by organism, with chloramphenicol showing some potential against *Enterobacter* sp., amikacin against *Pseudomonas aeruginosa*, and penicillin against *Staphylococcus aureus*, although all were still classified as resistant or intermediate. The MARI values were very high (0.80 for *Enterobacter* sp. and 0.90 for both *Pseudomonas aeruginosa* and *Staphylococcus aureus*), indicating a concerning level of resistance that poses significant challenges in clinical settings (Table 5).

Specifically, *Enterobacter* sp. isolates are resistant to all tested antibiotics except for chloramphenicol (CH) and ofloxacin (OFX), which showed intermediate susceptibility. The highest zone of inhibition recorded was 15.40 mm for CH. In contrast, the other antibiotics, including SXT, SP, CPX, AM, AU, CN, and PEF, showed no effective inhibition, with all the zones of inhibition classified as resistant. Similarly, the *Pseudomonas aeruginosa* isolates also demonstrated multidrug resistance, as they resisted most antibiotics tested. The zone of inhibition of 14.45 mm for amikacin (AM) is intermediate. Other antibiotics, such as SXT, CH, SP, CPX, AU, and CN, showed no inhibition, indicating complete resistance. The highest inhibition observed was 12.60 mm for AU, which is still under the resistant category. On the other hand, the *Staphylococcus aureus* isolates also exhibited multidrug resistance, resisting most antibiotics tested. The most effective antibiotic for this isolate was penicillin (PEF), which showed a zone of inhibition of 16.45 mm, which none-

theless falls under the resistant group. Other antibiotics, including SXT, SP, AM, AU, and CN, showed no inhibition, indicating complete resistance. Intermediate responses were noted for chloramphenicol (CH) and ofloxacin (OFX), suggesting limited activity (Table 5).

Statistically, two-way ANOVA showed that there is no statistically significant difference between the resistance profiles of the bacteria ($p = 0.57$), indicating uniform multi-drug resistance status. Likewise, the bacteria exhibited similar resistance to all tested antibiotics ($p = 0.24$), which indicates that no specific antibiotic demonstrates specific efficacy against the test strains.

Table 5. Antibiogram, Multiple Antibiotic Resistance Index and multidrug resistance status of *Enterobacter* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus* from Katsina, Nigeria.

Antibiotics and Concentrations	Organisms, Zones of Inhibition (mm $\pm$ Standard Deviation) and Interpretations		
	<i>Enterobacter</i> sp.	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
SXT (30 μ g)	5.50 $\pm$ 1.50 (R)	0.00 $\pm$ 0.00 (R)	0.00 $\pm$ 0.00 (R)
CH (30 μ g)	15.40 $\pm$ 0.30 (I)	0.00 $\pm$ 0.00 (R)	5.90 $\pm$ 1.20 (R)
SP (30 μ g)	0.00 $\pm$ 0.00 (R)	6.50 $\pm$ 1.50 (R)	3.75 $\pm$ 0.95 (R)
CPX (30 μ g)	7.60 $\pm$ 0.50 (R)	0.00 $\pm$ 0.00 (R)	9.70 $\pm$ 2.40 (R)
AM (30 μ g)	0.00 $\pm$ 0.00 (R)	14.45 $\pm$ 2.15 (I)	0.00 $\pm$ 0.00 (R)
AU (10 μ g)	0.00 $\pm$ 0.00 (R)	12.60 $\pm$ 1.30 (R)	0.00 $\pm$ 0.00 (R)
CN (30 μ g)	0.00 $\pm$ 0.00 (R)	0.00 $\pm$ 0.00 (R)	0.00 $\pm$ 0.00 (R)
PEF (30 μ g)	15.55 $\pm$ 1.15 (R)	6.90 $\pm$ 1.20 (R)	16.45 $\pm$ 2.45 (R)
OFX (10 μ g)	15.85 $\pm$ 1.65 (I)	0.00 $\pm$ 0.00 (R)	14.90 $\pm$ 0.10 (I)
S (30 μ g)	0.00 $\pm$ 0.00 (R)	0.00 $\pm$ 0.00 (R)	0.00 $\pm$ 0.00 (R)
MARI (max = 1.00)	0.80	0.90	0.90
MDR Status	MDR	MDR	MDR

Key: R = Resistant, I = Intermediate, SXT = Trimethoprim–Sulfamethoxazole, CH = chloramphenicol, SP = Sparfloxacin, CPX = Ciprofloxacin, AM = Amoxicillin, AU = Amoxicillin–Clavulanic acid, CN = Gentamicin, PEF = Pefloxacin, OFX = Ofloxacin, S = Streptomycin. MARI = Multiple Antibiotic Resistance Index. MDR = Multidrug Resistance. A MARI Index of ≥ 0.30 is considered indicative of multidrug resistance. Two-way ANOVA: (bacteria: $p = 0.57$, antibiotics: $p = 0.24$).

3.5. Bioassay (Agar Well Diffusion Assay)

The results of the antibacterial activity assay of the extracts are presented in Figure 1, and they showed that the aqueous extract was effective particularly against *S. aureus* at higher concentrations. Similarly, the n-hexane extract was more effective than the aqueous extract at 50 mg/mL against *Enterobacter* sp. and *P. aeruginosa*, but it was less effective against *S. aureus* at the same concentration. Thus, while both extracts exhibited varying degrees of antibacterial activity, the n-hexane extract generally showed wider zones of inhibition compared to the aqueous extract, particularly at higher concentrations.

Specifically, for *Enterobacter* sp., the n-hexane extract at 50 mg/mL produced the widest inhibition zone of 22.40 ± 1.00 mm, while the aqueous extract showed a slightly narrower inhibition zone of 17.40 ± 0.10 mm. On the other hand, the aqueous extract showed no inhibition at 6.25 mg/mL. Similarly, for *P. aeruginosa*, the n-hexane extract was more effective at 50 mg/mL (18.90 ± 1.10 mm) compared to the aqueous extract (18.20 ± 0.50 mm). However, both extracts failed to inhibit bacterial growth at the lowest concentration tested (6.25 mg/mL). In the case of *S. aureus*, the aqueous extract was the most effective at 50 mg/mL, yielding a zone of inhibition of 25.15 ± 3.95 mm, while the n-hexane extract showed a significantly lower inhibition of 15.25 ± 1.95 mm. At lower concentrations, the aqueous extract lost its antibacterial activity entirely at 12.5 mg/mL, while the n-hexane extract maintained some activity, with a zone of inhibition of 13.65 ± 0.05 mm.

Statistically, the Welch test showed that the aqueous and n-hexane extracts exhibited similar inhibition zones ($p = 0.24$); likewise, the zones of inhibition generated were statistically similar to those of the positive control ($p = 0.066$).

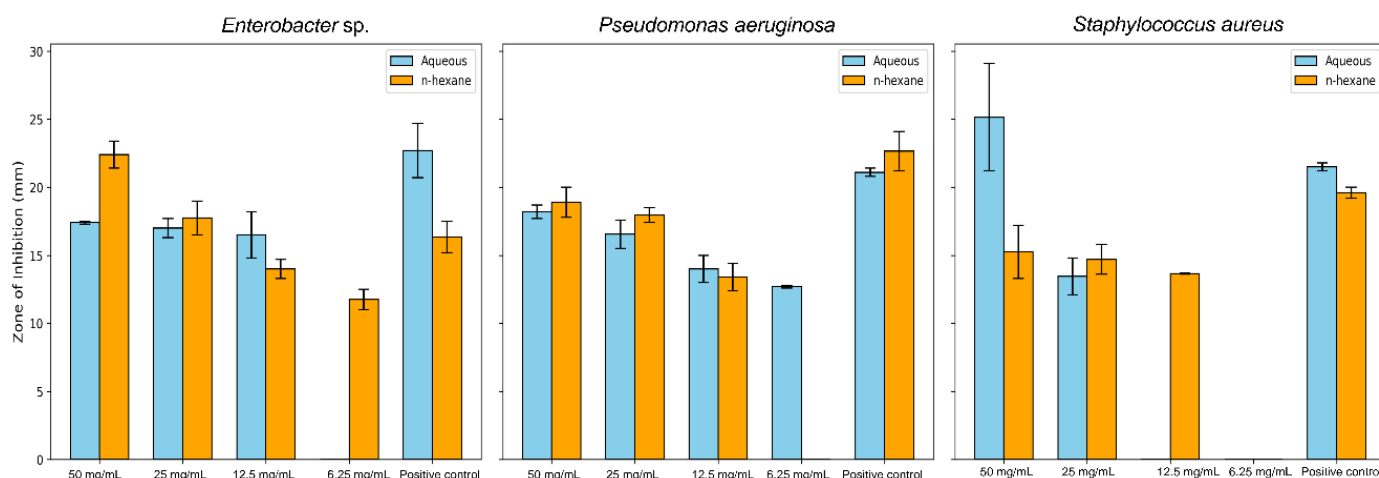


Figure 1. Antibacterial activity of the aqueous and n-hexane extracts of *Vernonia amygdalina* against MDR ESKAPE isolates from Katsina, Nigeria. Legend: Error bars represent standard deviation. Positive control = ciprofloxacin. Variation in positive control values is due to separate measurements for each isolate and extract. Mann–Whitney test for aqueous vs. n-hexane ($p = 0.24$). One-way ANOVA for aqueous vs. n-hexane vs. positive control ($p = 0.066$).

4. Discussion

This study utilized FTIR to demonstrate the solvent-dependent chemical fingerprinting of *Vernonia amygdalina* leaf extracts, as well as their meaningful antibacterial activity against MDR ESKAPE isolates from Katsina State, Nigeria. ESKAPE pathogens are known to be widely distributed across clinical and non-clinical reservoirs in Africa, with West Africa carrying a substantial burden [6]. Similarly, recent reviews portray specific members of the ESKAPE group, including *Klebsiella pneumoniae*, as established and growing problems in the study area [11]. In the same vein, MDR *Pseudomonas aeruginosa* had been reported from residential sewage in Katsina [39]. Numerous previous studies had also established the presence of multidrug-resistant Gram-negative bacteria with clinical implications from Katsina, Nigeria, further highlighting the resistance ecology in both hospital and environmental settings [34,35,40]. This justifies the search for locally available, plant-derived antibacterial leads that can complement conventional therapy [41].

In this study, the n-hexane fraction was found to produce the greater mass yield, whereas the aqueous fraction showed broader qualitative phytochemical diversity. This indicates that the quantity of soluble material recovered is independent of the classes of metabolites, which are typically partitioned by solvent polarity [42]. Previous studies on *V. amygdalina* also support this interpretation [13,15], as the species is known to contain flavonoids, alkaloids, saponins, steroids, terpenoids, cardiac glycosides, cyanogenic glycosides and phenolic compounds [15,20]. Moreover, solvent-dependent extraction has been shown to alter FTIR fingerprints and biological activity [43]. Thus, the two fractions in this study complementarily gave an insight into the total phytochemical properties of the test plant.

The FTIR data strengthens that conclusion. The aqueous extract was associated with signals of functional groups associated with terpenoids, polyacetylenes, cyanogenic glycosides, nitrile alkaloids, fatty acid nitriles, phenols, flavonoids, alkaloids and saponins, whereas the n-hexane fraction yielded spectra associated with terpenoids, steroids, triglycerides, polyacetylenes, lipophilic alkaloids, fatty acid nitriles, phenols and amines

[13,44]. The chemical fingerprinting of these extracts based on the spectra is coherent with the known phytochemical richness of *V. amygdalina* [15]; however, further analyses, such as HPLC, are necessary to definitively confirm the exact metabolic profile of the extracts [45]. The findings of the study are also in agreement with recent evidence that specific phytochemicals from *V. amygdalina* belonging to the aforementioned classes fingerprinted by FTIR, including vernolide, vernodalol and isorhamnetin, have documented antibacterial activity. The sesquiterpene lactones (vernolide and vernodalol) exert their antibacterial activity through alkylation of nucleophilic sites in microbial proteins and enzymes, hence, disrupting essential signaling pathways such as ERK, STAT3, and NF- κ B. This interference reduces bacterial proliferation and survival, and ensures selective toxicity because of differences in redox balance and stress response systems between microbes and mammalian host cells [46]. On the other hand, isorhamnetin is a flavonoid that exhibits antibacterial activity by inducing oxidative stress in bacterial cells. It promotes reactive oxygen species (ROS) accumulation, leading to lipid peroxidation and membrane damage. It can also inhibit bacterial efflux pumps and enzymes involved in DNA replication, thereby sensitizing bacteria to oxidative damage and impairing their growth [47]. In this sense, FTIR functions as a rapid screening layer that links extract chemistry to bioactivity, while compound-level confirmation still requires GC-MS or LC-MS analyses [48].

The antibiogram data from this study indicated that all three isolates were MDR, with MARI values of 0.80 for *Enterobacter* sp. and 0.90 for both *P. aeruginosa* and *S. aureus*, far above the conventional threshold used to flag high-risk resistance [35,49]. The absence of significant differences across bacteria and antibiotics suggests a uniformly elevated resistance burden which is explainable within the context of one health, in the sense that drivers of antimicrobial resistance are systemic and interconnected across clinical, environmental, agricultural, etc., settings, rather than being isolated to specific pathogens or drug classes [50]. These results further underscore the need for locally adaptable alternatives to conventional antibiotics in the study area, considering the intense resistance pressure exhibited by the ESKAPE isolates.

From a mechanistic viewpoint, the antibacterial activity exerted by the extracts in this study against the MDR ESKAPE pathogens is plausibly multi-target. Based on the identified classes of phytochemicals, other studies had shown that these compounds can trigger membrane disruption, altered membrane permeability, inhibited specific enzymes, modulated oxidative stress and interfered with bacterial signaling. Specifically, saponins and some phenolic classes contribute to membrane destabilization, while terpenoids, steroidal and alkaloid fractions may contribute to broad-spectrum antimicrobial effects [13,15]. The stronger performance of the n-hexane fraction against some organisms is consistent with enrichment of lipophilic metabolites that traverse or perturb bacterial envelopes more effectively, whereas the aqueous fraction may better capture polar molecules that remain highly active against specific targets such as *S. aureus* [51]. Hence, organism–solvent interactions reinforce the value of solvent partitioning towards resolving the metabolic composition of the extracts and, hence, predicting antibacterial activity against specific members of the ESKAPE group [52].

Going deeper, based on the FTIR fingerprinting, the actual synergy between the metabolites which translates into antibacterial activity can be hypothesized as follows: The aqueous extract likely utilized the combination of the hydrophilic and amphiphilic secondary metabolites to collapse cellular structural integrity and metabolic pathways of the bacteria. Specifically, phenols and flavonoids initiate the attack by forming strong hydrogen bonds with cell wall proteins and outer membrane porins, effectively cross-linking and deactivating critical transport proteins while destabilizing the lipopolysaccharide (LPS) layer of the Gram-negative bacteria. Concurrently, saponins, which are natural surface-active agents, are inserted into the phospholipid bilayer to cause severe membrane

permeability. This facilitates the rapid influx of cyanogenic glycosides, nitrile alkaloids, and fatty acid nitriles. Once internalized, these nitrile and glycosidic compounds undergo enzymatic cleavage, liberating toxic traces (such as prussic acid/cyanide derivatives) that bind tightly to cytochrome c oxidase, freezing the bacterial electron transport chain and terminating ATP synthesis. Furthermore, alkaloids intercalate directly into the bacterial DNA architecture, halting replication enzymes, while the polar terpenoids and polyacetylenes generate intracellular reactive oxygen species (ROS) that induce severe lipid peroxidation and DNA strand breakage, collectively bypassing existing antibiotic resistance efflux mechanisms [15,53,54].

Meanwhile, the n-hexane extract might have exhibited superior antibacterial efficacy against two of the MDR ESKAPE strains compared to the aqueous fraction primarily due to its lipophilic composition, which easily overcomes the highly evolved hydrophobic barriers characteristic of drug-resistant strains. While the hydrophilic elements of the aqueous extract are often resisted by the outer membrane and tight porin channels of Gram-negative *K. pneumoniae* or *A. baumannii*, the n-hexane fraction is concentrated with highly lipophilic terpenoids, steroids, and polyacetylenes. These non-polar molecules have partition coefficients that favor immediate dissolution into the bacterial lipid domains. Similarly, hydrophobic interactions allow steroids and triglycerides to fluidize and alter the structural packing of the inner and outer cell membranes, leading to uncontrollable membrane expansion, structural fragmentation, and the immediate leakage of critical intracellular ions like K^+ and Mg^{2+} . This massive structural collapse is reinforced by lipophilic alkaloids and amine compounds. Because they are not restrained by charge constraints, these compounds penetrate deeply through the protective lipid envelope to disrupt inner membrane potential ($\Delta\Psi$) and deactivate vital membrane-bound respiratory enzymes. Thus, physical barrier of the bacterial cell envelope becomes permanently compromised. This enables the n-hexane extract to achieve superior target site accumulation, which effectively prevents the efflux pumps of the pathogen from ejecting the active compounds [15,55,56]. These mechanisms might explain why the extracts exhibited no statistically significant difference in antibacterial activity compared to the ciprofloxacin positive control ($p = 0.066$) under the experimental conditions.

Nevertheless, the study is limited in the sense that FTIR provides functional-group information, not exact compound identity. In addition, only three out of the six members of the ESKAPE group were assessed. Furthermore, MIC/MBC, time-kill kinetics, antibiofilm activity and cytotoxicity were not assessed. These limitations do not weaken the central outcome of this study; instead, they should form the basis for investigation in further research. We therefore recommend that future studies should identify and quantify the exact bioactive constituents present in each extract by GC-MS, HPLC or LC-MS, test alternative solvents, evaluate toxicity, and conduct bioassay-guided fractionation to identify which metabolite classes drive the strongest antibacterial effect to guide future novel drugs development.

5. Conclusions

This study revealed that the n-hexane fraction has a higher yield ($p < 0.0001$) and exhibited stronger antibacterial activity against three MDR ESKAPE pathogens compared to the aqueous fraction. However, both the aqueous and n-hexane fractions of the *V. amygdalina* had no statistically significant difference in antibacterial activity compared to the ciprofloxacin positive control ($p = 0.066$) against the three MDR ESKAPE pathogens, under the experimental conditions, indicating their potential for exploration in novel drugs development.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/amh71030019/s1>, Table S1: Antibacterial activity of the aqueous and n-hexane extracts of *Vernonia amygdalina* against *Enterobacter* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Figure S1: *Vernonia amygdalina* plant used for sample collection. Figure S2. FTIR spectra of aqueous and n-hexane extracts of *Vernonia amygdalina*.

Author Contributions: Conceptualization, Y.Y.R. and Z.N.S.; methodology, Y.Y.R. and M.K.; software, Y.Y.R.; validation, Y.Y.R.; formal analysis, Z.N.S. and M.K.; investigation, Z.N.S. and M.K.; resources, Y.Y.R., Z.N.S. and M.K., data curation, Z.N.S. and Y.Y.R.; writing—original draft preparation, Z.N.S. and Y.Y.R.; writing—review and editing, A.S. and E.O.-A.; visualization, Y.Y.R.; supervision, Y.Y.R.; project administration, Y.Y.R., A.S. and E.O.-A.; funding acquisition, Z.N.S., M.K. and Y.Y.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets reported in this study are included herein and in the Supplementary Materials. The original FTIR spectra are available from the corresponding author upon reasonable request.

Acknowledgments: We acknowledge the support and enabling environment provided by the Microbiology and Central Laboratories, UMYU, Katsina, Nigeria. SciSpace Diagrams Agent (SciSpace, Version 2026; Typeset Inc., Austin, TX, USA) was used to generate the clustered bar charts (Figure 1) based on input mean $\pm$ standard deviations. The graphic output was exported as a PNG file and then imported into Microsoft PowerPoint (Microsoft Office LTSC 2021, Version 2108; Microsoft Corp., Redmond, WA, USA) for final structural modifications, labeling adjustments and typographic alignment to meet journal specifications.

Conflicts of Interest: The authors declare no conflicts of interest.

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