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Original Article

PHYTOCHEMICAL PROFILE, ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF SOLVENT EXTRACTS OF *Vernonia amygdalina*

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ABSTRACT

This study was conducted to determine the antibiofilm and antibacterial potential of *Vernonia amygdalina* leaf and bark against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. The bioactive compounds in *V. amygdalina* were extracted with ethyl acetate, *n*-hexane, and ethanol and evaluated by phytochemical analysis. Antibacterial activity of the extracts was determined by agar well diffusion, while broth microdilution was used to determine the minimum inhibitory concentration (MIC). The pour plate technique was used to determine the minimum bactericidal concentration. Biofilm formation was established, and antibiofilm activity was evaluated using the p-iodonitrotetrazolium colourimetric assay. The phytochemical screening indicated saponin as the only compound in the leaf, while saponin, flavonoids, alkaloids, phenolics, and triterpenes were present in the bark. Ethyl acetate extract exhibited the highest antibacterial activity at the lowest concentration. It had MIC values between 8 and 16 mg/mL and inhibited biofilm formation at 87.96%, especially against *K. pneumoniae*. The ethanol extract had a moderate antimicrobial effect, and that of *n*-hexane was negligible. The results show that polar solvents such as ethyl acetate are effective for extracting antimicrobial agents from *V. amygdalina*. They also highlight *V. amygdalina* as an effective natural agent against biofilm-forming and antibiotic-resistant pathogens.

Keywords: Keywords: AMR, Antibacterial activity, Antibiofilm activity, phytochemical constituents, *Vernonia amygdalina*.

1. INTRODUCTION

Antimicrobial resistance (AMR) results from years of antibiotics misuse and overuse. Resistance in microorganisms occurs when they develop adaptations that allow them to survive despite exposure to antibiotics. These adaptations are mainly caused by genetic mutations that make infections difficult to treat (Ferraz, 2024). Globally, an estimated 4.95 million deaths were associated with bacterial AMR annually, including approximately 1.27 million deaths directly attributable to resistant infections, according to the World Health Organisation (2023). This has increased the number of calls and efforts to develop novel strategies to treat infectious diseases. Hence, plant-derived antimicrobials that have been shown to be beneficial have received renewed consideration in research (Inusa *et al.*, 2018).

The West African region has used medicinal plants to treat resistant infections for decades. *Vernonia amygdalina* (bitter leaf) has been highly successful in this regard (Inusa *et al.*, 2018). *V. amygdalina* is a perennial shrub and member of the family Asteraceae that grows to about 2–6 m. The plant is native to West Africa (Nigeria and Cameroon) and is widespread in tropical regions (Edo *et al.*, 2023). A recent study identified diverse bioactive compounds of medicinal importance in all parts of the plant (Sileshi *et al.*, 2024). These bioactive compounds have medicinal and protective effects against various diseases (Talwar *et al.*, 2024). Despite these properties, it is often neglected when selecting medicinal plants for commercial use. This study targeted *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, which are important multidrug-resistant pathogens (Mancuso *et al.*, 2021). These pathogens are a major threat to public health and are implicated in several hospital-acquired infections. Therefore, the aim of this study was to investigate the antibacterial and antibiofilm potential of *V. amygdalina* extracts against clinically relevant *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* isolates.

2. MATERIALS AND METHODS

Plant Collection and Identification

Collection and identification of *V. amygdalina* was carried out in May, 2025. The plant's leaves and bark were collected from a home garden in Ilorin, Kwara State, Nigeria. The collected parts were submitted to the University of Ilorin Herbarium, Department of Plant Biology, for confirmation, and a voucher number, UIL/001/972/2025, was assigned.

Preparation of Plant Material and Extraction

The samples were first washed and air-dried. They were then blended into powder using an electric blender. Bioactive constituents

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were extracted from the powdered substance using *n*-hexane, ethyl acetate, and ethanol as the solvents. 100 g of the powdered sample was macerated in 1000 mL of each extraction solvent in a tight-lid jar. The mixture was shaken for 48 hours and then filtered through a muslin cloth. Re-extraction was also conducted using each solvent to ensure complete extraction. The extracts were concentrated to dryness using a rotary evaporator. Dried extracts were transferred into sterile McCartney bottles. The samples were preserved at 4°C until further use (Inusa *et al.*, 2018; Zakariyah *et al.*, 2017).

Source and Standardization of test organisms

Test bacterial isolates were obtained from the Department of Microbiology and Parasitology at the University of Ilorin Teaching Hospital in Kwara State. They included *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*. The test microorganisms were inoculated into sterile broth in suspension and standardized to a McFarland standard of 0.5 or 1×10^8 CFU mL^{-1} according to the CLSI M07 guidelines for dilution antimicrobial susceptibility testing.

Establishment of biofilm formation in test organisms

The Tube Adherence Test, as described by Sathish *et al.* (2017), was performed. The organisms were inoculated into Tryptone Soy Broth containing 1% glucose. The culture was then incubated at 37°C for 48 hours. The supernatant was poured out, and the test tubes were stained with 0.5% w/v safranin solution. Subsequently, the used tubes were cleaned with distilled water. A layer of a stained substance on the tube walls indicated a positive test. A negative result was indicated by a clear, stained ring confined to the surface of the liquid/air mixture. The experiment was conducted three times, and the results were grouped as absent, weak, moderate, or strong.

Reagents

These include synthetic compounds and standard antibiotics, such as Gentamicin sulfate (catalogue number GA7939-5G), sourced from Glentham Life Sciences. Tetrazolium salt ($C_{19}H_{15}ClN_4$; Product code: 26520) was purchased from MOLYCHEM. Erythromycin disc sourced from Oxoid Limited. Other reagents include ethanol, DMSO, phosphate-buffered saline, normal saline, and hydrogen peroxide.

Sterility test of the plant extracts

The extracts were checked for sterility by inoculating 1 mL of each extract into sterile nutrient agar. The plates were incubated at 37°C for 24 hours. Following this, the plates were checked for microbial growth. The plates showed no growth, indicating that the extracts were sterile (Inusa *et al.*, 2018).

Phytochemical screening

Qualitative analysis:

The preserved extracts as prepared above were obtained for phytochemical screening. Standard protocols were followed to determine the bioactive constituents in the plant samples, including alkaloids, phenolics, triterpenoids, steroids, glycosides, reducing sugars, and proteins (Babarossa *et al.*, 2026; Harborne *et al.*, 1998; Rao *et al.*, 2023). Blank was used as a negative control, and a known extract or a standard compound was used as a positive control.

Phenolics: Add 1–2 drops of 5% (w/v) $FeCl_3$ to 2 mL of the plant sample in a tube. The presence of green or blue colour indicated the presence of phenolics.

Flavonoids: Add Shinoda reagent, consisting of magnesium turnings and 1–2 mL concentrated HCl (36% w/w), to 0.5 g plant sample in a test tube and shake. A pink-to-magenta coloration indicated flavonoids.

Tannins: Some drops of lead acetate were combined with 1 g of the powdered plant sample inside a tube. White precipitate indicated tannins.

Saponins: 2 mL of the extract was placed in a tube and shaken vigorously for 15–30 seconds. Persistent froth ($\geq$1 cm) lasting for more than 15 minutes indicated saponins.

Glycosides: The powdered sample was hydrolyzed with concentrated HCl. A few drops of Molisch's reagent were added, followed by concentrated H_2SO_4 along the internal walls of the tube. A purple ring at the acid interface indicated the presence of glycosides.

Triterpenes: Salkowski test was carried out. Chloroform and H_2SO_4 were added to 1 g of the plant sample in a 2 mL volume. The presence of reddish-brown coloration indicated the presence of triterpenes.

Steroids: Liebermann–Burchard reagent was used. Acetic anhydride and H_2SO_4 were combined with 2 mL of the aqueous extracts. The presence of blue/green rings indicated the presence of steroids.

Alkaloids: Mayer's reagent was prepared by dissolving 1.36 g of mercuric chloride and 5.0 g of potassium iodide in water and diluting the solution to 100 mL. A few drops of the prepared reagent were added to 2 mL of the aqueous plant extract. The formation of a creamy white precipitate indicated the presence of alkaloids in the extract.

Reducing sugars: 2 mL of Fehling's solution was mixed with 2 mL of the aqueous extract and heated for 5 minutes in a boiling water bath. A red precipitate indicated the presence of reducing sugars.

Protein: 1% NaOH and 0.1% $CuSO_4$ were added to 1 g of the plant sample. The purple coloration indicated the presence of proteins. This occurs due to peptide bonds.

Quantitative analysis

Determination of Total Phenolic Content: The method that was used to estimate the total phenolic content (TPC) of the plant sample was the Folin–Ciocalteu method, which was slightly modified (Trease & Evans, 2009). Gallic acid was used to make a calibration curve at a concentration between 0.1 and 0.5 mg/mL. The Folin–Ciocalteu reagent was then mixed with sodium carbonate and the plant sample under alkaline conditions. The blue coloration that showed up was then measured three times using a UV-visible spectrophotometer (765 nm). The unit used to express the TPC was Gallic Acid Equivalent in milligrams per gram (mg GAE/g) of the plant material.

Determination of Triterpenoids: The Vanillin–perchloric acid colorimetric method was used to estimate the total triterpenoid content (TTC) of the plant sample (Kunina *et al.*, 2018). A calibration curve was prepared using Ursolic acid at concentrations ranging from 1 to 5 mg/mL. Vanillin and perchloric acid were then mixed with the plant sample under alkaline conditions. A coloured complex formed, which was measured three times at 548 nm using a UV-visible spectrophotometer. Milligrams of Ursolic acid per milliliter of extract (mg UAE/mL) were used as the unit to express the triterpenoid content.

Determination of Saponin: The colorimetric method described by Le Bot *et al.* (2022) was carried out. 1 g of the powdered material was measured into a conical flask. 80% methanol was measured as 25 mL and added to the conical flask. The flask was shaken, refluxed for 2 hours, and then filtered. The extraction was repeated once, and the filtrates were combined. The solvent was then evaporated, and the resulting dry residue was reconstituted with methanol to a final volume of 10.00 mL. A Diosgenin stock solution at 1 mg/mL in methanol was prepared. Serial standards (0.10, 0.20, 0.40, 0.80 mg/mL) were made. Each standard was measured to 0.5 mL in separate containers, and 0.5 mL of a vanillin reagent (8% in MeOH) was added and mixed gently. A slow addition of 5 mL concentrated H_2SO_4 was made, ensuring it was added to the sides of each tube and mixed.

The tubes were then incubated for 10 minutes in a water bath at 60°C, cooled, and the absorbance was read against the blank reagent (vanillin + H_2SO_4 + methanol) at 544 nm.

Reconstitution of extracts

A total of 1.28g of crude extract was dissolved in 2.5 mL of absolute dimethyl sulfoxide (DMSO). This was done to obtain a stock solution at 512 mg/mL. Serial dilutions were performed from the first concentration to the fourth sub-concentration. The concentrations prepared were in increments of 64 mg/mL, 128 mg/mL, 256 mg/mL, and 512 mg/mL (Cowan, 1999).

Antimicrobial susceptibility testing

Determination of antimicrobial activity of extracts: *V. amygdalina* leaf and bark were tested for their antibacterial activity using the extracts obtained with the different solvents. These include ethanol, ethyl acetate, and *n*-hexane. The test was conducted using agar-well diffusion techniques against *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* (Zakariyah *et al.*, 2017). The process involved aseptically pouring sterile Mueller-Hinton agar into sterile Petri dishes and allowing it to solidify. A sterile swab stick was used to streak standardized organisms on the surface of the solidified media. A sterile cork borer was used to create 8 mm holes in the agar medium, and sterile forceps were used to carefully remove the agar discs. 100 μL of each extract concentration was dispensed into the holes created on the inoculated agar medium. 512 mg/mL of Gentamicin sulphate was used as a positive control. Culture plates were incubated for 24 hours at 37°C. The antibacterial potential of the extracts was assessed by measuring inhibition zones formed on the agar surface. The zones of inhibition were measured to the nearest 0.1 millimeters to express the antimicrobial activity.

Determination of Minimum Inhibitory Concentration (MIC): As reported by Zakariyah *et al.* (2025), analysis of the MIC of the plant leaf and bark extracts using ethyl acetate and ethanol was done through microbroth dilution. This involved using 96-well microtiter plates with round bottoms. 50 μL of Mueller–Hinton broth (MHB) was dispensed into wells 2–9. 256 mg/mL Gentamicin sulphate (Glentham Life Sciences) was added to the 9th well as the positive control, and MHB containing DMSO, diluted to 100 μL, was added to well 10 as the negative control. The 256 mg/mL extract was diluted to a final volume of 50 μL and dispensed into wells 1 and 2. A twofold serial dilution was performed by transferring 50 μL of suspension from well 2 to subsequent wells through well 8, discarding 50 μL. The concentrations prepared were in increments of 4 mg/mL, 8 mg/mL, 16 mg/mL, 32 mg/mL, 64 mg/mL, and 128 mg/mL. Standardized inoculum was dispensed into wells 1-10. MIC values were determined using a solution of p-iodonitrotetrazolium chloride (INT) as the indicator.

Determination of Minimum Bactericidal Concentration (MBC): The MBC of the optimized extracts was determined by pipetting 10 μL of MIC and higher concentrations into separate sterile Petri dishes; sterile nutrient agar was poured, swirled, and solidified. Culture plates were incubated for 24 hours at 37°C. The MBC was the lowest concentration of the extracts that eliminated the initial bacterial population, with no visible growth of the organism (Zakariyah *et al.*, 2025).

Biofilm Inhibition Assay

The antibiofilm extract potential was evaluated *in vitro* using the colorimetric tetrazolium salt (INT) assay, as described by Babarossa *et al.* (2025). A 96-well microtiter plate was used, and each well was inoculated with 200 μL of the 0.5 McFarland standard bacterial culture. To achieve a uniform distribution of bacterial cells, the plate was first incubated on a rocker table at 37°C for 4 h. Following this, the plates were incubated for 18 hours at 37°C to facilitate cell attachment and biofilm formation. Each well received 200 μL of each extract at concentrations of 32, 64, or 128 mg/mL. The positive control was Gentamicin sulphate; the negative control was a sterile bacteria-free nutrient agar containing DMSO. After incubation, the material in the wells was discarded, and the wells were washed with 200 μL of sterile, pH 7.4-adjusted phosphate-buffered saline. The washed wells were first inoculated with 200 μL of the MIC and MBC extracts, then with 200 μL of INT solution. Incubation of the plates was set to 2 hours at 37°C, and reading of the plates was done at a wavelength of 490 nm using a microplate reader. The percentage reduction in the biofilm formed was calculated in percentages as follows:

$$\text{Percentage of biofilm reduction} = \frac{\text{OD control} - \text{OD experimental}}{\text{OD control}} \times 100 \quad (1)$$

Statistical Analysis

The antibacterial activity of various *V. amygdalina* extracts against test organisms was evaluated by determining the inhibition zones. Each treatment was replicated three times ($n = 3$). The mean and standard deviation (SD) were calculated for the zones of inhibition to summarize the average and spread for each treatment. A one-way Analysis of Variance (ANOVA) was conducted in Microsoft Excel Analysis ToolPak to test the null hypothesis (H_0) that *V. amygdalina* extracts do not differ in antibacterial activity against the test organisms and the alternative hypothesis (H_1) that at least one extract differs significantly from the others.

3. RESULTS

Biofilm formation in Test Organisms

Table 1 shows the strength of how the test organisms formed biofilms.

Table 1: Biofilm Establishment in Test Organisms

Test Organism	Biofilm Formation
Staphylococcus aureus	strong
Klebsiella pneumoniae	moderate
Pseudomonas aeruginosa	strong

Phytochemical Constituents of *V. amygdalina* Leaf and Bark

Table 2 shows the phytochemicals of *V. amygdalina* leaf and bark used in this research. Saponin was the only constituent present in the leaf. However, the bark contained saponin, phenolics, flavonoids, alkaloids, and triterpenes.

Table 2: Qualitative phytochemical Constituents of *V. amygdalina* Leaf and Bark

Phytochemical constituents	Leaf	Bark
Saponin	+	+
Tannins	–	–
Phenolics	–	+
Flavonoids	–	+
Triterpenes	–	+
Glycosides	–	–
Steroids	–	–
Alkaloids	–	+
Reducing sugars	–	–
Proteins	–	–

KEY: + = Present, – = Absent

Table 3 below shows the concentrations of saponins, phenolics, and triterpenoids in the samples. A distinct concentration difference was observed between the leaf and bark, with the bark showing significantly higher total phytochemical content. Triterpenes were the most abundant constituent in the bark, reaching a maximum concentration of 78.23 mg/mL in the *n*-hexane extract. In contrast, Saponins in the leaf remained relatively consistent across all solvents, with a mean concentration of 9.84 mg/mL.

Table 3: Concentration of Saponin, Phenolics, and Triterpenes present in *V. amygdalina* Leaf and Bark

V. amygdalina Parts	Extract Solvent	Saponins (mg GAE/g)	Phenolics (mg GAE/g)	Triterpenes (mg GAE/g)
Leaf	Ethyl acetate	9.91 ± 0.06	ND	ND
	n-hexane	9.79 ± 0.06	ND	ND
	Ethanol	9.83 ± 0.06	ND	ND
Bark	Ethyl acetate	ND	7.97 ± 0.06	77.00 ± 1.22
	n-hexane	ND	7.85 ± 0.06	78.23 ± 1.22
	Ethanol	ND	7.95 ± 0.06	75.80 ± 1.22

KEY: ND = Not Detected

3.1 Antibacterial Activities of *V. amygdalina* Leaf and Bark Extracts Against Test Organisms

Figures 1, 2, and 3 illustrate the antibacterial activity of the leaf and bark extracts of *V. amygdalina* leaf and bark extracts against the selected test organisms. The activity of the extracts increased with increasing concentration. However, *n*-hexane extracts of both the leaf and bark exhibited limited antibacterial activity against the test organisms and were therefore excluded from the MIC/MBC and biofilm inhibition tests.

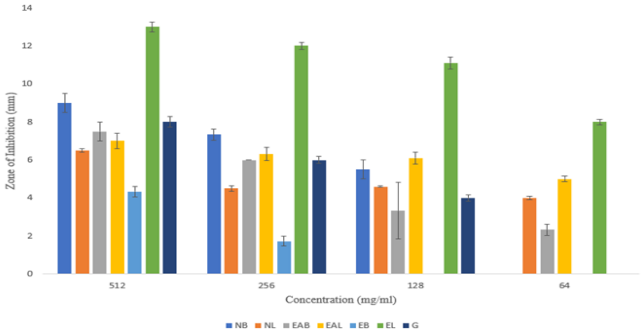

Figure 1: Antibacterial Activity of *V. amygdalina* extracts on Staphylococcus aureus

Values are means of triplicates ($n = 3$).

Legend: NB = *V. amygdalina* bark + *n*-hexane, NL = *V. amygdalina* leaf + *n*-hexane, EAB = *V. amygdalina* bark + Ethyl acetate, EAL = *V. amygdalina* leaf + Ethyl acetate, EB = *V. amygdalina* bark + Ethanol, EL = *V. amygdalina* leaf + Ethanol, G = Gentamicin.

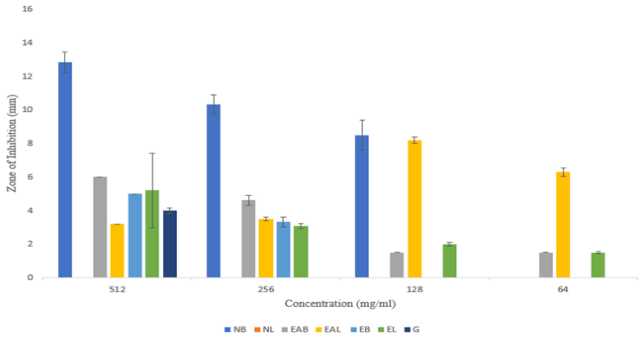

Figure 2: Antibacterial Activity of *V. amygdalina* extracts on *Klebsiella pneumoniae*

Values are means of triplicates ($n = 3$) ± SD.

Legend: NB = *V. amygdalina* bark + *n*-hexane, NL = *V. amygdalina* leaf + *n*-hexane, EAB = *V. amygdalina* bark + Ethyl acetate, EAL = *V. amygdalina* leaf + Ethyl acetate, EB = *V. amygdalina* bark + Ethanol, EL = *V. amygdalina* leaf + Ethanol, G = Gentamicin

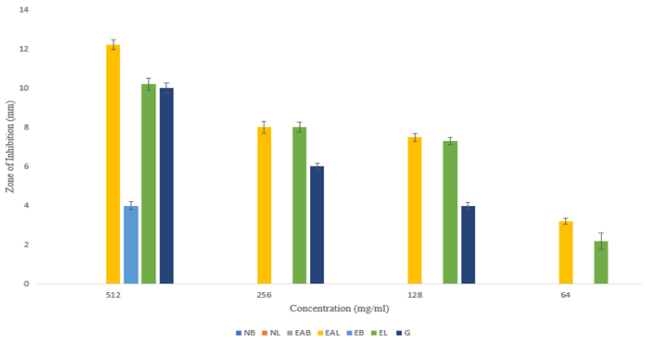

Figure 3: Antibacterial Activities of *V. amygdalina* Leaf and Bark Extracts Against Test Organisms

Values are means of triplicates ($n = 3$)

Legend: NB = *V. amygdalina* bark + *n*-hexane, NL = *V. amygdalina* leaf + *n*-hexane, EAB = *V. amygdalina* bark + Ethyl acetate, EAL = *V. amygdalina* leaf + Ethyl acetate, EB = *V. amygdalina* bark + Ethanol, EL = *V. amygdalina* leaf + Ethanol, G = Gentamicin.

Minimum Inhibitory and Bactericidal Concentration Results

The least concentration at which the extracts were effective against the test organisms is given in Table 4. No minimum bactericidal concentration was recorded for any extract against any test organism.

Table 4: Minimum Inhibitory and Bactericidal Concentrations (mg/mL)

	MIC					
Test Organisms	**EAB**	**EB**	**EAL**	**EL**	**Gentamicin**	**MBC**
Staphylococcus aureus	6	32	16	128	256	ND
Klebsiella pneumoniae	8	16	16	128	256	ND
Pseudomonas aeruginosa	32	8	4	32	256	ND

KEY

ND: Not Detected

Note: Values represent the mean of three independent replicates ($n = 3$).

Legend: EAB = *V. amygdalina* bark + Ethyl acetate, EB = *V. amygdalina* bark + Ethanol, EAL = *V. amygdalina* leaf + Ethyl acetate, EL = *V. amygdalina* leaf + Ethanol, MIC = Minimum Inhibitory Concentration, Control = Gentamicin sulphate, ND = Not Detected.

Biofilm Inhibition

Table 5 shows the percentage reduction in biofilm formation by the extracts in the test organisms.

Table 5: Percentage of Biofilm Reduction

Extracts	Concentration	% Biofilm Reduction (%)		
		Sa	Kp	Pa
EAB	MIC	24.11	36.61	32.63
	Sub-MIC	22.83	32.14	30.53
EB	MIC	29.39	26.34	27.75
	Sub-MIC	25.81	21.15	23.42
EAL	MIC	28.28	87.96	38.81
	Sub-MIC	22.70	60.57	36.81
EL	MIC	48.47	69.61	24.79
	Sub-MIC	0.13	64.34	23.76

Legend: Sa = *Staphylococcus aureus*, Kp = *Klebsiella pneumoniae*, Pa = *Pseudomonas aeruginosa*, EAB = *V. amygdalina* bark + Ethyl acetate, EB = *V. amygdalina* bark + Ethanol, EAL = *V. amygdalina* leaf + Ethyl acetate, EL = *V. amygdalina* leaf + Ethanol, MIC = Minimum Inhibitory Concentration, Sub-MIC = Sub Minimum Inhibitory Concentration (MIC/2).

Statistical Comparison of Antibacterial Efficacy of Extracts

A one-way ANOVA showed statistically significant differences in antibacterial activity among the extracts for all three organisms (Table 6). The results indicate highly significant differences between the treatment groups for *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*.

Table 6: ANOVA Summary of the Antibacterial Activity of the Extracts against the Test Organism

Organism	Source of Variation	Sum of Squares (*SS*)	*df*	Mean Squares (*MS*)	*F*-value	*P*-value
S. aureus	Between Groups	128.68	5	25.74	189.10	< 0.001
	Within Groups	1.63	12	0.14		
K. pneumoniae	Between Groups	269.68	5	53.86	62.14	< 0.001
	Within Groups	10.40	12	0.87		
P. aeruginosa	Between Groups	485.16	5	91.63	2856.06	< 0.001
	Within Groups	0.39	12	0.03		

A post-hoc (Tukey HSD) test was conducted to identify specific differences between the treatment means for each isolate. The comparative zones of inhibition (mm), including the statistical groupings for *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*, are presented in Table 7. The result indicated that the ethyl acetate bark (EAB) and leaf (EAL) extracts were significantly more effective than *n*-hexane and ethanol extracts. Specifically, EAB showed the highest antibacterial activity against *S. aureus* and *K. pneumoniae*, while EAL had a slightly higher activity against *P. aeruginosa*.

Table 7: Comparative Zones of Inhibition of the Extracts against the Test Organisms

Treatment Group	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*
NB	9.00^{c}	12.83^{d}	0.00^{a}
NL	6.50^{b}	0.00^{a}	0.00^{a}
EAB	7.50^{b}	6.00^{c}	0.00^{a}
EB	4.33^{a}	5.00^{bc}	4.00^{b}
EL	13.00^{d}	5.20^{bc}	10.20^{c}
EAL	7.00^{b}	3.20^{b}	12.20^{d}

Note: Values represent the mean of three independent replicates ($n = 3$). Means within the same column followed by different superscript letters (a, b, c, d) are significantly different ($p < 0.05$).

Legend: NB=*V. amygdalina* bark + *n*-hexane, NL=*V. amygdalina* leaf + *n*-hexane, EAB=*V. amygdalina* bark + Ethyl acetate, EAL=*V. amygdalina* leaf + Ethyl acetate, EB=*V. amygdalina* bark + Ethanol, EL=*V. amygdalina* leaf + Ethanol,

4. DISCUSSION

This study aimed to determine the phytochemical profile of *V. amygdalina* and the antibiofilm and antibacterial potential of its leaf and bark extracts obtained using *n*-hexane, ethyl acetate, and ethanol. The extracts were evaluated against *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*, known for their multidrug resistance and biofilm-forming capabilities.

Phytochemical analysis was conducted and revealed that phenolics, triterpenes, flavonoids, saponins, and alkaloids are present in *V. amygdalina* bark. However, only saponin was observed in the leaf, similar to findings reported by Adeoye *et al.* (2018). This observation may be due to environmental factors that prevented the synthesis of other compounds, as *Iwuanyanwu et al.* (2025) suggested. *V. amygdalina* typically grows well in areas with access to light and warmth. Soils rich in nutrients and moisture are essential for plant growth (Dega *et al.*, 2024). These conditions differ from those in local farmlands in Kwara State, where the plant was obtained for this study. The weather conditions in Kwara State are highly variable, with long periods of both wet and dry seasons that may have contributed to the lack of diversity of compounds in the leaf (Adeniyi & Abubakar, 2020). In addition, plant bark has generally been shown to contain more compounds that support plant defence and protection. Leaves often contain mainly compounds that support photosynthesis (Xu *et al.*, 2022), which may explain the higher triterpenoid content and the observed presence of other compounds in the bark. Despite our finding, the ethyl acetate leaf extract still produced significant inhibition zones. This suggests the likely presence of other compounds at undetectable levels, as well as the superior role of saponins alone in inhibiting bacterial cell membranes (Zhou *et al.*, 2017). Our study demonstrates that *V. amygdalina* exhibits moderate antibacterial activity against resistant pathogens, an effect attributable to its bioactive constituents. The *V. amygdalina* parts also showed good antioxidant potential, with triterpene content (mean = 77.01 mg/mL) and phenolic content (7.92 mg/mL) (Udochukwu *et al.*, 2015). Onifade *et al.* (2024) reported similar findings showing that *V. amygdalina* extracts with high phytochemical concentrations greatly inhibited resistant pathogens.

The extracts of *V. amygdalina* exhibited varying antibacterial activity against *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*, with inhibition decreasing as the concentration decreased. This indicates a concentration-dependent response against the test organisms. The results for *S. aureus* in Figure 1 show that the Ethanol leaf extract exhibited the highest antibacterial activity, achieving a maximum zone of inhibition of about 13mm. This was closely followed by *n*-hexane bark extract and the extracts obtained using ethyl acetate. The finding suggests that the polar constituents in the leaves are highly effective against *S. aureus*. In contrast, the ethanol bark extract showed the lowest bioactivity, with the organism showing resistance to the compound at 128 mg/mL. As shown in Figure 2, the bark *n*-hexane extract showed the highest antibacterial activity against *K. pneumoniae*, producing the largest inhibition zone. This suggests that non- or semi-polar phytochemicals, including phenolics, triterpenes, flavonoids, and alkaloids, in the bark may contribute strongly to the observed antibacterial effect. Similar findings have been reported by Romano *et al.* (2025), who presented that the antibacterial and antioxidant properties of phenolic compounds and triterpenes in *Arctium lappa* L. Ethyl acetate extracts displayed moderate activity, with the leaf extract maintaining noticeable bioactivity at low concentrations. The ethanol extracts had the lowest antibacterial activity against the organism. For *P. aeruginosa* shown in Figure 3, the ethyl acetate leaf extract showed the highest activity, producing the largest zone of inhibition, reaching approximately 13mm. Following this is the ethanol leaf extract, while the *n*-hexane bark extract showed no activity against the organism at all concentrations. These results suggest that semi-polar and polar extraction solvents may contribute to the inhibitory activity of bioactive compounds from *V. amygdalina* against *P. aeruginosa*. The limited activity of the *n*-hexane bark extracts is likely attributed to the ability of the solvent to solubilize only compounds with low polarity, like steroids and triterpenes. This may have contributed to the lower direct antimicrobial activity compared to the other solvent fractions. The findings of Nwokocha *et al.* (2024) align with ours, reporting that *n*-hexane is effective for extracting non-polar compounds from *Azanza garckeana* leaves. Generally, our study found that the solvents were less effective against *P. aeruginosa* than against *S. aureus* and *K. pneumoniae*. *P. aeruginosa* is known to be highly resistant, often using intrinsic and acquired mechanisms to bypass the effects of antibacterial agents. Specifically, an array of over 12 outer membrane proteins, efflux pumps, resistance genes, and antibiotics-inactivating enzymes characterizes the organism's resistance profile (Pang *et al.*, 2019). These mechanisms likely contributed to *P. aeruginosa*'s

observed resistance to *V. amygdalina*. However, this contrasts with the results obtained by Bibi *et al.* (2025), who studied the antibacterial activity of five medicinal plants against *P. aeruginosa*. The authors highlighted the organism's susceptibility to all five plants, with *C. arvensis* being highly effective.

Overall, the results suggest that the extraction solvents and plant parts influence the antibacterial activity of *V. amygdalina*. We suggest that ethyl acetate is efficient in extracting antibacterial constituents from *V. amygdalina*, likely due to its polarity, which allows it to solubilize both polar (e.g., phenolics) and moderately non-polar compounds (e.g., triterpenes). The *n*-hexane extract showed the lowest antimicrobial activity across the three organisms and failed to meet CLSI-defined thresholds for effective antimicrobial agents. The statistical analysis further correlated the antibacterial susceptibility test results for *n*-hexane. Hence, the other experiments did not include the *n*-hexane extracts due to their limited utility for the study. For *S. aureus*, the analysis showed a significant effect of extract type on inhibition zones, indicating that at least one extract differed significantly from the others. The EAB extract exhibited the largest zones of inhibition, significantly higher than those of *n*-hexane and ethanol extracts. Similarly, for *K. pneumoniae*, the analysis revealed that the EAB and EAL extracts were significantly more effective than *n*-hexane and ethanol extracts, with EAB showing the highest antibacterial activity. For *P. aeruginosa*, the result indicated that the EAL and EB were significantly more inhibitory than the *n*-hexane extracts. Due to the lower activity of the *n*-hexane extracts against the test organisms, further analysis was discontinued.

According to CLSI (2022), a zone of inhibition $\geq$ 15 mm is considered indicative of susceptibility for test organisms when tested with erythromycin. In this study, the extracts produced smaller zones of inhibition, with a maximum of 12.83 mm, indicating that the plant extracts exhibit intermediate antibacterial activity by CLSI standards and may also reflect a lack of purification compared to the positive control. Furthermore, CLSI (2022) reported that a MIC breakpoint $\geq$ 16mg/mL means the organism is resistant to gentamicin. In this study, the ethyl acetate extracts had significant MIC values across all three test organisms, ranging from 4-32 mg/mL (*P. aeruginosa*), 16 mg/mL (*S. aureus*), and 8-16 mg/mL (*K. pneumoniae*). This is compared with the MIC values of the ethanolic extracts, which ranged from 8 to 32 mg/mL (*P. aeruginosa*), 32 to 128 mg/mL (*S. aureus*), and 16 to 128 mg/mL (*K. pneumoniae*). These values highlight the potent antibacterial efficacy of ethyl acetate extract of *V. amygdalina* compared to gentamicin (128-512 mg/mL). Our study is also consistent with previous reports that plant-derived antimicrobials often inhibit but do not outrightly kill pathogens (Onifade *et al.*, 2024).

Biofilm inhibition, expressed by the percentage of biofilm reduction, demonstrated that both extracts reduced biofilm formation. Ethyl acetate extract of the leaf at sub-MIC concentration (MIC/2) achieved up to 22.70% reduction in *S. aureus*, 60.57% in *K. pneumoniae*, and 36.81% in *P. aeruginosa*, while ethyl acetate extract of the bark at sub-MIC concentration (MIC/2) achieved up to 22.83% reduction in *S. aureus*, 32.14% in *K. pneumoniae*, and 20.52% in *P. aeruginosa*, respectively. This supports the findings of Barbarossa *et al.* (2025), who reported that plant extracts showed >20% biofilm inhibition against *S. aureus*. The Sub-MIC concentration of the leaf ethanolic extract was ineffective against *S. aureus*. The results are consistent with those of Onifade *et al.* (2024), who reported that *V. amygdalina* bark extract inhibited resistant bacteria, with MIC values similar to those reported here. These findings are attributed to the phytochemicals present in the ethyl acetate extract, for which GC-MS analyses have identified several bioactive compounds. Narayanan *et al.* (2023) noticed 14 major constituents in the ethyl acetate extract of *Cymodocea serrulata* including alkaloids, flavonoids, saponins, phenols, quinones, steroids, and terpenoids. The authors further demonstrated *S. aureus* susceptibility to the extract at 75 mg/mL. Specifically, the mechanisms of biofilm-associated inhibition by these phytochemicals may include preventing quorum sensing and motility, which are especially crucial for biofilm formation, structure, and integrity (Gonçalves *et al.*, 2022). Other strategies include disrupting the extracellular matrix, microbial membranes, and DNA replication (Joseph, Baby & Murugadasan, 2024). These findings demonstrate the broad-spectrum activity of *V. amygdalina*, making the plant a promising source of antimicrobial agents.

5. DISCUSSION

This study showed that *V. amygdalina* has effective antibacterial and antibiofilm activities against *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*. The results also indicate the importance of solvent polarity in extracting antimicrobial compounds. The semi-polar ethyl acetate solvent was optimal for extracting various constituents, including triterpenoids and phenols. This contributed to the ethyl acetate extracts having the highest antimicrobial and antibiofilm activities. Overall, the superior performance of the ethyl acetate fractions makes them suitable plant-based antimicrobial agents. Our finding supports the continued use of *V. amygdalina* for combating persistent and resistant infections. This study has a few limitations that warrant discussion. First, the lack of MBC results could have provided more insights into the potential of the extracts. In addition, the lack of biochemical and molecular characterizations of the test organisms and plant extracts limit this study to preliminary results. For future research, we recommend enhanced compound isolation using high-performance liquid chromatography, Fourier Transform Infrared Spectroscopy to identify the functional groups of the extracts, and synergy testing with other antimicrobial agents against multidrug-resistant pathogens.

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Ethical statement

This study did not involve human participants, animals, or sensitive data. Therefore, ethical approval was not required.

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Author Contributions

R. F. Zakariyah; Conceptualization, Design, Methodology, Data curation, Writing — original draft, Project administration, Resources, Supervision. O. P. Ojelakin; Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing — review & editing. O. E. Olorunfemi; Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing — review & editing. W. A. Husain; Methodology, Investigation, Data curation, Formal analysis, Software, Validation, Visualization, Writing — review & editing. E. P. Samuel; Data curation, Formal analysis, Validation, Writing — review & editing.

Data Availability

The generated dataset will be provided upon request.

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