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ORIGINAL ARTICLE

Phytochemical Profile and Antibacterial Activity of *Vernonia amygdalina* Leaf Extracts against Bovine Mastitis Pathogens

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ABSTRACT

Vernonia amygdalina remains a veritable ethnoveterinary plant used for the treatment of bovine mastitis. In this study, 4 (4%) isolates of Escherichia coli and 3 (3%) of Staphylococcus aureus were recovered from a total of 100 bovine milk samples screened.In this study, 4 (4%) isolates of *E. coli* and 3 (3%) of *S. aureus* were detected from a total of 100 milk samples screened. Phytochemical analysis conductedrevealed dense profile of bioactive compounds among which are: flavonoid, saponin, alkaloid, glycoside, terpenoid, tannins, steroids, phenolic compound, anthraquinones and volatile oil. Crude methanolic leaf extract demonstrated better phytochemical richness and exhibited significantly higher zones of inhibition range (6.00 ± 0.00 - 24.4 ± 0.84 mm) compared to the aqueous extract (0.0 ± 0.0 - 18.22 ± 1.12 mm) at 12.5 - 100 mg/mL. One-way Analysis of Variance (ANOVA) revealed a statistically significant difference in the zones of inhibition across the four concentrations for both methanolic ($p < 0.05$) and aqueous ($p < 0.05$) extracts. The Minimum Inhibitory Concentration (MIC) ranged from 6.25 - 12.5 mg/mL for S. aureus and 12.5 – 25 mg/mL for E. coli in the methanolic extract, while the aqueous MIC range was 12.5 - 50 mg/mL for S. aureus and 25 – 50 mg/mL for E. coli isolates. The Minimum Bactericidal Concentration (MBC) values were 12.5-50 mg/mL in the methanolic extract and 25-100mg/mL in the aqueous extract, implying an MBC 1-2 fold higher than MIC, indicating bactericidal action at higher concentrations. The plant showed promise as a source of alternative therapy for bovine mastitis, but further purification, toxicity and in vivo studies are recommended.

Keyword: *Vernonia* amygdalina; Phytochemical screening; Bovine mastitis; Antibacterial activity; Bacterial.

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1.0 INTRODUCTION

Bovine mastitis is theinflammatory change of the mammary gland (udder) that occurs alongside physical, chemical, and microbiological changes. It is primarily characterized by increase in somatic cells (especially leukocytes) in the milk and pathological changes (reddening of udder tissues, reduced milk yield, altered milk quality, discomfort and pain) in the mammary tissue(Cheng & Han, 2020). The two major etiologic agents of bovine mastitis are *Staphylococcus aureus and Escherichia coli,* whose infectivity results in udder inflammation and culling of the lactating cow(Morales-Ubaldo et al., 2023; Robi et al., 2026). It is one of the most economically devastating and costly diseases for dairy farming globally due to lost milk production, discarded milk, and treatment expenses (Muturi, 2020).Due to its impact on animal welfare and economic viability of the dairy industry, mastitis is a major research priority, especially in Zamfara State, where dairy farming is a primary livelihood. Mastitis pathogensoriginates from rectal,ruminal and genital reservoir, spreading during milking,when infected milk makes contactwith uninfected teat (Cobirka et al., 2020; Oraguha, 2024). Mastitis is subdivided into environmental, if it is caused by pathogens from the environment, and contagious, if spread from infected to healthy animals (Adamu et al., 2020). Based on severity, the disease can be clinical, subclinical or chronic. Clinical mastitis (CM) is easily recognized by red, swollen udders, fever, and watery milk with flakes and clots (Ruegg, 2017); couple with high risk of recurrence in the same or next lactation (Abebe et al., 2023). Subclinical mastitis shows no visible abnormalities but decreased production and elevated somatic cell count of>200,000 cells/mL as defined by the International Dairy Federation (Sharma et al., 2011). Chronic mastitis is characterized by prolonged inflammatory process lasting months with irregular recurrent episodes (Cheng & Han, 2020). Once in the parenchymal tissue, mastitis bacteria elicit inflammation and immune response through the acquisition of various virulence factors including biofilm, outer membrane transport proteins and iron acquisition genes, which compromise treatment, fuel resistance and enable intracellular persistence (Gunther et al., 2017). As a result, humans can be directly infected with Extended-spectrum beta-lactam resistant *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and Enterobacter spp. (ESKAPE pathogens) which could be transmitted via contact with carrier animals or through the food chain, due to zoonotic spillover (Anueyiagu et al., 2025). If toxigenic *S. aureus* is involved, it produces enterotoxin Bin milk causing food poisoning characterized by nausea, vomiting, abdominal cramps and diarrhea (CDC, 2024).

Antibiotic administration remains a valuable mastitis management strategy (Robledo-Díaz et al., 2025). Drugs such as pirlimycin, cloxacillin, novobiocin, amoxicillin, dihydrostreptomycin, penicillin G, erythromycin and cefapirin must achieve concentrations above minimum inhibitory concentration (MIC) at the infection site when administered (Hossain et al., 2017). In practice however, this is difficult due to pathological changes in udder tissue, residual antibiotics in milk, injudicious prophylactic use and ineffective routes of administration (Hossain et al., 2017). This could

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result in the emergence of antibiotic-resistant bacteria including variants resistant to last-resort antibiotics (Dego, 2020).

Historically, the National Institute for Research in Dairying (NIRD) introduced the five-point mastitis control plan which focuses on recording and treating clinical mastitis cases, post-milking teat disinfection, dry cow therapy, culling chronically infected cows and milking machine maintenance (More et al., 2022). However, it has shown limited success against environmental pathogens due to high prevalence, multi-etiologic nature of the causal agents and their ability to cause infections between milkings (Hillerton & Booth, 2018). Vaccination, another touted preventive mastitis strategy has also failed to provide reliable protection against diverse strains within a herd (Cheng & Han, 2020). There are over 5,000 medicinal plant species (Alebachew et al., 2014) and *Vernonia amygdalina* is no exception. *V. amygdalina,* is also known as bitter leaf due to its bitter taste. It is a perennial tropical shrub widely recognized in African ethnomedicine and nutritional research (Jarmai et al., 2022).It is a potent ethnoveterinary plant with the ability to disrupt bacterial cell wall synthesis (Musyoki, 2024). It also exhibits anti-inflammatory and antioxidant properties that protect against tissue damage and promote healing (Osaghale et al., 2024). Its anti-inflammatory properties act on pathogens or their endotoxins by inactivating the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Cheng & Han, 2020). Inhibition of this pathway by *V. amygdalina* phytochemicals reduces pro-inflammatory cytokine production and alleviates mastitis symptoms (Osaghale et al., 2024). Its bioactive properties are due to various phytochemicals (terpenoids, tannins, alkaloids, saponins, flavonoids, steroids, glycosides, vernolide, vernodalol, vernomygdine and sesquiterpene lactone) stored in it secondary metabolites (Ugbogu et al., 2021).

Nigeria ranks third among cow milk producing countries in Africa, contributing about 1.7% to national GDP and 9% to agricultural value chain (Merem et al., 2022). Data from the National Agricultural Sample Census as reported by the National Bureau of Statistics (NBS) places Nigeria's total cattle population at 20.9 million and about 2.27 million are dairy cattle. Epidemiological reports indicated that this dairy sub-population remains highly susceptible to mastitis owing to prevailing milking and husbandry practice. extensive nomadic management practice by Fulani pastoralists, characterized by hand milking, absence of post-milking teat disinfection, zero-grazing constraints, and use of unhygienic milking vessels such as calabashes (Adamuet al., 2020;). Local data on the treatment costs of bovine mastitis in Zamfarastate is lacking. However, data from Zaria, both in Northwest Nigeria indicated that pastoral Fulani households manage an average herd size of 41.5 cattle, with estimated cost per case of clinical mastitis at 35.2% base risk incidence put at ₦5,005.85(US $15.87) (Moru*et al*., 2018; Ibrahim *et al*., 2018). If this figure is adjusted to the current inflationary rate, its equivalence is ₦20,821.44. Information on the burden of bovine mastitis caused by *Staphylococcus aureus and Escherichia coli* is crucial but lackingin the study area. Despite the use of various classes of antibiotics for the treatment of bovine mastitis,modern veterinary inputs remain prohibitively expensive or inaccessible for pastoralists, which often leads to lack of udder disinfection, inadequate handwashing and poor shelter/housing management, risk of exposure to mastitis pathogens, self-treatment, delayed care and the immergence of antibiotics resistance (Bolajoko et al., 2020).Hence the exploitation *V. amygdalina*asa critical therapeutic alternative. Coincidentally, *V. amygdalina* is used traditionally for the treatment of mastitis. However, limited scientific data exist on its phytochemical profile and antibacterial activity against mastitis pathogens isolated from bovine milk samples in the study area. The objectives of this study therefore is to determine the phytochemical profile, percentage occurrence, antibacterial activity, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *V. amygdalina* crude leaf extracts against mastitis pathogens, isolated from bovine milk samples in the study area. This is with a view to providing insight into its ethnomedicinal value and empirical data for novel drug discovery.

2.0 MATERIALS AND METHODS

2.1 Study area

This study was conducted in the Microbiology Department, Federal University Gusau, Zamfara State. Gusau is the capital city of Zamfara state mainly populated by Hausa/Fulani people and other ethnic minorities. The state has an average precipitate of 61.41mm and an average annual temperature of 30.22°C. The state has an area of 39,762 km^2 with an estimated population of 9,278,873 (National Population Commission-NPC, 2017).

2.2 Study design

Both the cross-sectional and experimental study design was applied in data collection and analysis. The study was used to establish the relationship between the concentrations of the crude extracts and the diameter of zone of inhibition (DZI). The planning of this study in volved plant leaf processing, extraction, varying the concentrations of the leaf extracts, measurement of anti-bacterial activity of the extracts and deducing the relationships between cause and effects. While this was the first reported study of the phytochemical profile and antibacterial activity of crude leaf extracts of *Vernonia amygdalina* against *Staphylococcus aureus and Escherichia coli* isolated from bovine milk samples in the study area. The study was conducted from January 1st 2025- 10th July, 2026.

2.3 Inclusion criteria

Only milk samples from cow suspected of having mastitis by the veterinarian was included in this study.

2.4 Ethical approval

Ethical clearance was obtained from the Animal Health Research Ethics Committee, Department of Veterinary Services, Federal Ministry of Agriculture, GusauZamfara State, with reference number MAN/SUB/475/Vol. III

2.5 Procedure of milk collection

A purposive sampling technique was used for milk sample collection. Samples were collected from dairy cows presented for treatment at five major veterinary and dairy centers in the state, namely: Samaru dairy cluster, Tudun Wada livestock station, UnguwarGwaza veterinary unit, SabonFegi cluster and Kwata livestock hub. These centers were high-traffic locations for ruminant livestock and served as collection points for cattle with clinical, subclinical and chronic mastitis cases. Cow teat and udder surface was first washed with water and thereafter disinfected with 70% ethanol before sample collection to prevent contamination from external contaminants. The first few squirts of milk was discarded. Then, 10mL of milk was collected mid-stream into sterile screw capped container. A total of 100 milk samples was collected and were stored in an ice-packed cooler before being transported to the Microbiology Laboratory of the Federal University Gusau for further analysis. The sampling procedure used complied with the guideline of the National Research Council-NRC (2011), Guide for the Care and Use of Laboratory Animals.

2.6 Determination of sample size

The sample size of bovine mastitic milk collected was determined based on the formula for estimating prevalence in a cross-sectional study as described by Heston (2023).

$n = Z^2 \times Pexp (1 - Pexp) / d^2$

Where; n = required sample size, Z = standard normal deviate at 95% confidence interval (1.96), Pexp = expected prevalence, d = desired absolute precision. Since the true prevalence of bovine mastitis associated with *Staphylococcus aureus* and *Escherichia coli* in the study area was unknown, an expected prevalence of 50% was used. Hence:

$n = (1.96)^2 \times 0.5(1-0.5) / (0.1)^2$

$n = 3.8416 \times 0.25 / 0.01$

$n = 96.04 \approx 96$ samples

Therefore 100 bovine milk samples was collected to increase precision, robustness of the study and account for 4% non-response, including statistical power, and the potential for sample loss due to contamination

2.7 Media preparation

All microbiological media used were prepared according to manufacturers' instructions accompanying the label. The media used were selective and general purpose media and include: Mannitol Salt Agar (MSA), Eosin Methylene Blue Agar (EMB), Mueller Hinton Agar and Nutrient Agar.

2.7.1 Isolation and identification of test bacteria from bovine milk samples

Milk samples collected were cultured on mannitol salt agar medium and eosin methylene blue agar medium for the isolation of *Staphylococcus aureus* and *Escherichiacoli* respectively. The isolates were identified using selective media because it selectively allow their proliferation on the growth medium when trying to isolate the test bacteria (Garga, 2017). They

culture plates were incubated at 37°C for 24 h. Pure colonies of the test bacteria were subcultured on nutrient agar slants and used for further analysis.

2.7.2 Biochemical characterization of isolated bacteria

Biochemical assay was further used to confirm the *Staphylococcus aureus* and *Escherichia coli* isolates from the milk samples analyzed as described by Thaker et al. (2013). The biochemical panel used include both primary and secondary biochemical assay which were used to identify the test bacterial isolates, these include: Gram staining, catalase test, coagulase test, citrate test, indole test, methyl red and VogesProskauer test.

2.7.2.1 Gram staining

This was carried out according to Garga (2017). A drop of distilled water was placed on a clean glass slide and a colony of bacterial growth was taken from a 24 h old culture, to make a thin smear. The smear was air dried and then flooded with crystal violet for 60 s (primary stain), after which it was rinsed with distilled water. Logol's iodine (mordant) was added for 60 s, it was rinsed with distilled water and was decolorized with 95% alcohol for 30 s. The smear was finally flooded with Safranin (secondary stain) for 60 s; it was then rinsed with distilled water and allowed to air dry. Oil immersion was added to air dried stained smear and viewed under ×100 objective lens. Bacterial cells which retains the colour of the primary dye and stain purple were reported as Gram positive cells while Gram negative cells retained the color of the secondary dye and therefore stains pink.

2.7.2.2 Catalase test

This was carried out according to the method described in Thaker et al. (2013). Few colonies were emulsified on a clean glass slide using a sterile wire loop. About 2 drops of 3% hydrogen peroxide was added on the slide containing the colonies and was thoroughly mixed. Positive test was indicated by bubble formation.

2.7.2.3 Coagulase test

Coagulase test was conducted as described in Thaker et al. (2013). A drop of distilled water was placed on two slides; a colony of bacteria was emulsified in the drop of water to make a thick suspension. A loop full of plasma was added to the thick suspension on one slide and mixed gently and observed within ten min. Clumping indicated a positive test while absence of clumping indicated a negative test. The other slide was used as control.

2.7.2.4 Citrate utilization test

A prepared slant of sterile Simon's citrate agar in test tube was streaked and butt-stab with three colonies of the test bacterium. The test tube was covered with cotton wool and aluminum foil and incubated at 37°C for 24h. A positive reaction was indicated by development of blue color on the reagent slant layer (Thaker et al., 2013).

2.7.2.5 Indole test

This was carried out according to the method described in Thaker et al. (2013). Colonies of the test organism were inoculated into 5mL of nutrient broth and incubated at 37°C. After 24h a Kovac's indole reagent was added. A positive reaction was indicated by development of red color in the reagent layer above the broth within 1min.

2.7.2.6 Methyl Red and Voges Proskauer Test

This was carried out according to the method described in Thaker et al. (2013). Colonies of the test bacteria were inoculated into 5mL of sterile glucose phosphate broth at 37^0C. After 48h of incubation, 1mL of the phosphate broth was transferred in to a serological tube and a drop of methyl red was added. A positive result was indicated by appearance of bright red coloration. To the rest of the broth in the original tube, a drop of potassium hydroxide was added. The mixture was shaken and placed in a slopping position. Development of red colour starting from liquid air interface within 1h indicated positiveVogesProskauertest. Absence of colour change indicates a negative test.

2.7.2.7 β-Haemolysis Test

Milk samples were inoculated and streaked onto 5% sterile sheep blood agar medium. The plates were incubated aerobically at 37°C for 24h. A clear transparent zone surrounding directly the *S. aureus* colonies where red blood cells were completely lysed was reported as β-Haemolysis (Aryl, 2024)

2.8 Plant collection and identification

Fresh leaves of *Vernonia amygdalina* were collected aseptically in Gusau, Zamfara state an6d transported in a sterile polyethene bag to the herbarium unit of the Biological Science Department, Federal University Gusau for authentication and was assigned a voucher number FUG/2025/0123.Leaves were washed, shade dried for 14days and pulverized to fine powder as described by Obialo et al. (2024).

2.8.1 Preparation of crude extracts

The method described by Fitri et al. (2025) was used for the extraction of the pulverized leave of the plant.

2.8.2 Aqueous extract

A total of 50 g of powdered leaf was soaked in 500 mL of distilled water for 72 h with intermittent shaking. The mixture was filtered with Whatman No.1 filter paper and the filtrate was concentrated using a water bath at 45°C.The extract was stored in airtight bottles at 4°C until needed.The extract was thereafter standardized to 500 mL.

2.8.3 Methanolic extract

A 50 g of powdered leaf was soaked in 500 mL of 95% methanol for 72 h. The mixture was filtered and concentrated using a rotary evaporator at 40°C. The extract was stored in airtight bottles at 4°C until needed. The extract was standardized to 500 mL. The powdered leaf material of *V. amygdalina* was standardized to 50 g in 500 mL of solvent (1:10 w/v) for both methanolic and aqueous extraction in accordance with WHO guidelines for medicinal plant standardization and as described by Habtamu and Melaku (2018). This ratio was adopted to ensure comparability between solvents, control for extraction variables, and eliminate bias in antibacterial activity assessment. Standardization ensures that any observed difference in zone of inhibition is attributable to solvent polarity and phytochemical solubility rather than variation in starting material.

2.9 Qualitative Phytochemical Screening of *Vernonia amygdalina* Leave Extracts

Qualitative phytochemical screening was carried out to test for the presence of alkaloids, flavonoids, saponins, tannins, phenols, steroids, terpenoids, anthraquinone, volatile oil and glycosides using standard procedures (Garga, 2017; Obialo et al. 2024)

2.9.1 Test for Flavonoid

Extracts were treated with a few drops of sodium hydroxide solution. The formation of an intense yellow color, which became colorless with the addition of dilute acids, indicated the presence of flavonoids (Obialo et al., 2024).

2.9.2 Tests for Tannins

A 1-2 drops 5% Ferric chloride solution was added to 3mL of the extract in a test book. The development of a deep blue-black color was reported as confirmation for tannins (Garga, 2017)

2.9.3 Tests for Glycosides

Two mL of extract was mixed with 1 mL of glacial acetic acid containing a drop of 1% ferric chloride solution. Afterward, 1 mL of concentrated sulfuric acid was added carefully along the side of the test tube to form a layer. The formation of a brown ring at the interface indicated the presence of deoxy sugars, was reported as confirmation for cardiac glycosides (Obialo et al., 2024).

2.9.4 Tests for Alkaloids

2mL of each extract was stirred with 2mL of 10% aqueous hydrochloric acid solution in a test tube. One (1) mL of the suspension was treated with a few drops of Wagners reagent and the second portion was treated similarly with Mayers reagent. Turbidity or precipitation with either of these reagents was taken as evidence for the presence of alkaloids (Garga, 2017).

2.9.5 Tests for Steroids
A 0.5g of the extract was dissolved in 2mL of chloroform. Two milliliter (2mL) of sulphuric acid was carefully added to form lower layer. A reddish – brown color at the interface indicated the presence of a steroidal ring Garga, (2017).

2.9.6 Test for Phenolic Compounds
The powdered plant sample was taken and dissolved in 20mL of distilled water in a test tube, after which it was boiled and then filtered. A 3-4 drops of 0.1 iron chloride was added to the filtered sample. A brownish green or blue colour was reported as a positive test for phenols (Obialo et al., 2024).

2.9.7 Test for Saponin glycosides
To 2.5mL of Fehling's solution A and B, a bluish green precipitate was reported as saponin glycosides (Garga, 2017).

2.9.8 Test for Anthraquinones
A 0.5g plant extract was shaken with 10mL benzene, and 5mL of 10% ammonia solution. The mixture was shaken and the presence of a pink, red, or violet colour in the ammoniacal (lower) phase was reported as a confirmation of the presence of anthraquinones (Garga, 2017).

2.9.9 Test for Terpenoids
Five mL of the extract was mixed with 2 mL of chloroform. Then, 3 mL of concentrated sulfuric acid was added gently along the side of the test tube. A reddish-brown or grayish color at the interface was taken as evidence for the presence of terpenoids (Obialoet al., 2024).

2.9.10 Tests for Volatile Oils
One milliliter (1mL) of the extract was mixed with diluted HCl. A white precipitate was formed which indicated the presence of volatile oils (Garga, 2017).

2.10 Preparation of extracts concentrations
A stock solution of 100 mg/mL was prepared by dissolving 1 g of each crude extract in 10 mL of 10% Dimethyl Sulphoxide (DMSO). Theworking concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL were prepared from the stock by two-fold dilution.For Minimum Inhibitory Concentration (MIC), two-fold serial dilution was done to obtain concentrations ranging from 100 mg/mL to 0.78 mg/mL.

2.12 Determination of Antibacterial Activity of Crude Leave Extracts of *Vernonia amygdalina*.
Antibacterial screening was done using the agar well diffusion method as described by CLSI (2020) with slight modifications. Sterile Mueller Hinton agar plates were seeded with standardized bacterial inoculum (0.5 McFarland) using surface streaking for confluent growth with a sterile bent glass rod. The plates were kept for 5 min to set. Using a 6mm cork borer,three wells were made on each plate. The bottom of each hole was sealed using a molten agar medium. Various concentrations of the extract, 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL were prepared from the stock by two-fold dilution and dispensed into each well in triplicates. Ciprofloxacin (5 µg) was used as positive control and 10% DMSO as negative control. Plates were incubated at 37°C for 24 h after whichthe mean zones of inhibition were measured and recorded in millimeters.The data obtained were expressed as mean ± standard deviation. A One-way ANOVA was used to compare zones of inhibition at $p < 0.05$ and 95% confidence limit.

2.12 Determination of MIC of the Extracts
Minimum inhibitory concentration of the crude leave extracts was determined by the broth dilution method according to CLSI (2020).Two-fold serial dilution was done to obtain concentrations ranging from 100 mg/mL to 0.78 mg/mL. An overnight culture of each of the isolates matching 0.5 McFarland scale was mixed with the extract following standard procedureand incubated at 37°C for 24 h. The lowest concentration of the extracts with no visible turbidity was taken as the MIC.

2.13 Determination of MBC of the Extracts
Minimum bactericidal concentration of the extracts was determined by selecting tubes that showed no growth during MIC determination. A loop-full was taken from the test tubes and was inoculated on sterilized Muller Hinton Agar plates by streaking. The plates were incubated at 37ºC for 24 h. The lowest concentration of the extracts that showed no colony growth on the solid medium was regarded as the MBC (CLSI, 2020).

3.0 RESULTS

3.1 Colonial Characteristics, Cell Micromorphology and Biochemical Characteristics of Bacteria Isolated from Mastitic Milk Samples
Table 1 presents the colonial characteristics, cellular morphology, Gram reaction and biochemical characteristics of bacteria isolated from mastitic milk samples. Two bacterial species, Staphylococcus aureus and Escherichia coli, were identified based on their cultural, morphological and biochemical characteristics. Three isolates were identified as S. aureus (SA1, SA2 and SA3), while four isolates were identified as E. coli (EC1, EC2, EC3 and EC4). The recovery of these organisms indicates that both Gram-positive and Gram-negative bacteria were represented among the mastitic milk samples examined.

Table 1: Colonial Characteristics, Cell Micromorphology and Biochemical Assay of Isolated Bacteria from Mastitic Milk Samples

Colonial characteristics / Cell micromorphology	Gram reaction	Biochemical characteristics	Isolated bacteria	Isolated code
Round, smooth, golden yellow colonies, cocci in shape	+ve	Cat +ve Coa +ve Haem +ve Mann +ve Urea +ve	*Staphylococus aureus*	**SA1, SA2, SA3**
Round, smooth colonies with greenish metallic sheen rod-like in shape	-ve	Lact Fer. Indole Test Methyl Red VP Test Citrate Test	*Escherichia coli*	**EC1, EC2, EC3, EC4**

(+ve)-Positive; (—ve)-Negative; Cat- Catalase Test; Coa- Coagulase Test; Hae-(β) HaemolysisTest; Man-Mannitol Fermentation; Lact.-Lactose Fermentation;VP- VogesPrauskauer(Garga, 2017; Obialo et al.2024).

3.2 Phytochemical Screening of Crude Methanolic and Aqueous Leaf extracts of *Vernonia amygdalina*
Table 2 presents the phytochemical constituents detected in crude methanolic and aqueous leaf extracts of V. amygdalina. The results revealed the presence of several bioactive compounds, including alkaloids, flavonoids, cardiac glycosides, tannins, anthraquinones, volatile oils, terpenoids, steroids and phenolic compounds, although their reported abundance varied between the extraction solvents. The methanolic extract showed a broader range of detected constituents, with highly abundant flavonoids, terpenoids and steroids. Alkaloids, cardiac glycosides, saponins and volatile oils were reported at moderate abundance, while tannins and phenolic compounds were slightly abundant.

Table **2.** Phytochemical screening of crude methanolic and aqueous leaf extracts of *Vernonia amygdalina*

Bioactive compounds	Methanolic Extract	Aqueous extract
Alkaloid	++	+
Flavonoid	+++	+
Cardiac glycoside	++	++
Tannins	+	+
Anthraquinone	+	+
Saponin	++	-
Volatile oil	++	-
Terpenoids	+++	-
Steroids	+++	-
Phenolic compounds	+	+

(+)-Slightly abundant; (++)-Moderately abundant; (+++)-Highly abundant; (-) = absent

3.3 Antibacterial Activity/ Diameter of Zone of Inhibition (mm) of Crude Methanolic and Aqueous Leaf Extracts of *V.amygdalina* against *S. aureus* and *E. coli* isolated from Mastitic Milk Samples

Table 3 presents the antibacterial activity of methanolic and aqueous leaf extracts of V. amygdalina against seven bacterial isolates obtained from mastitic milk samples. The results were expressed as mean diameters of zones of inhibition in millimetres, with the corresponding variation indicated by the reported values after the ± symbol. Both extracts demonstrated inhibitory activity against several isolates, although the magnitude of inhibition varied according to extract type, concentration and bacterial isolate. The methanolic extract generally produced larger inhibition zones than the aqueous extract at corresponding concentrations, particularly at 100 mg/mL

Table 3 Antibacterial Activity/ Diameter of Zone of Inhibition (mm) of Crude Methanolic and Aqueous Leaf Extracts of *V.amygdalina* against *S. aureus* and *E. coli* isolated from Mastitic Milk Samples

	Methanolic extract (mg/mL)				Aqueous Extract (mg/mL)				Positive	Negative
Isolated Bacteria	100	**50**	**25**	**12.5**	**100**	**50**	**25**	**12.5**	**Cipro (5µ)**	**10% DMSO**
SA1	20.0 ± 0.67	15.60 ± 2.11	9.60 ± 1.60	6.40 ± 2.11	16.02 ± 2.32	10.12 ± 0.00	6.72 ± 0.23	6.22 ± 0.84	30.2 ± 2.24	0.00 ± 0.0
SA2	24.4 ± 0.84	16.00 ± 0.00	7.50 ± 0.67	6.0 ± 2.62	14.81 ± 3.31	12.62 ± 1.22	8.12 ± 2.44	6.60 ± 0.00	28.4 ± 1.22	0.00 ± 0.0
SA3	18.2 ± 1.12	12.60 ± 0.67	8.20 ± 1.22	5.00 ± 0.0	18.22 ± 1.12	16.24 ± 0.00	7.22 ± 1.44	8.00. ± 1.24	26.4 ± 2.00	0.00 ± 0.0
EC1	20.0 ± 2.12	10.12 ± 0.23	9.22 ± 1.33	7.0 ± 0.00	14.00 ± 0.00	10.68 ± 3.40	7.24 ± 1.22	6.00 ± 0.00	28.6 ± 3.12	0.00 ± 0.0
EC2	20.0 ± 1.12	10.40 ± 1.22	8.2 ± 0.00	6.00 ± 0.00	14.23 ± 2.66	10.00 ± 1.20	6.22 ± 2.31	0.00 ± 0.00	24.0 ± 0.67	0.00 ± 0.0
EC3	20.4 ± 2.22	12.04 ± 0.82	7.22 ± 0.12	7.00 ± 0.00	16.82 ± 2.14	8.82 ± 1.00	7.68 ± 1.24	0.00 ± 0.64	22.4 ± 2.26	0.00 ± 0.0
EC4	16.0 ± 2.22	8.22 ± 1.12	6.80 ± 2.22	6.00 ± 0.00	12.00 ± 0.00	9.00 ± 0.00	6.00 ± 0.00	0.00 ± 0.00	24.2 ± 3.44	0.0 ± 0.0

SA1-SA3- *Staphylococcus aureus;* EC1-EC4- *Escherichia coli; Cipro-Ciprofloxacin*
DMSO- Dimethyl Sulphoxide

Figure 1 presents the minimum inhibitory concentration (MIC) of methanolic and aqueous leaf extracts of V. amygdalina against the seven bacterial isolates. Extracts demonstrated MIC at 50 mg/mL when sensitized with the crude aqueous and methanolic leaf extracts of the plant, highlighting their bacteriostatic effect at that concentration. Potency assessment as described in Noor et al. (2026) was further reported according to various thresholdas: Strong Crude Extract- MIC ≤ 100 mg/mL; Moderate = 100-500 mg/mL; Weak: > 500 mg/mL

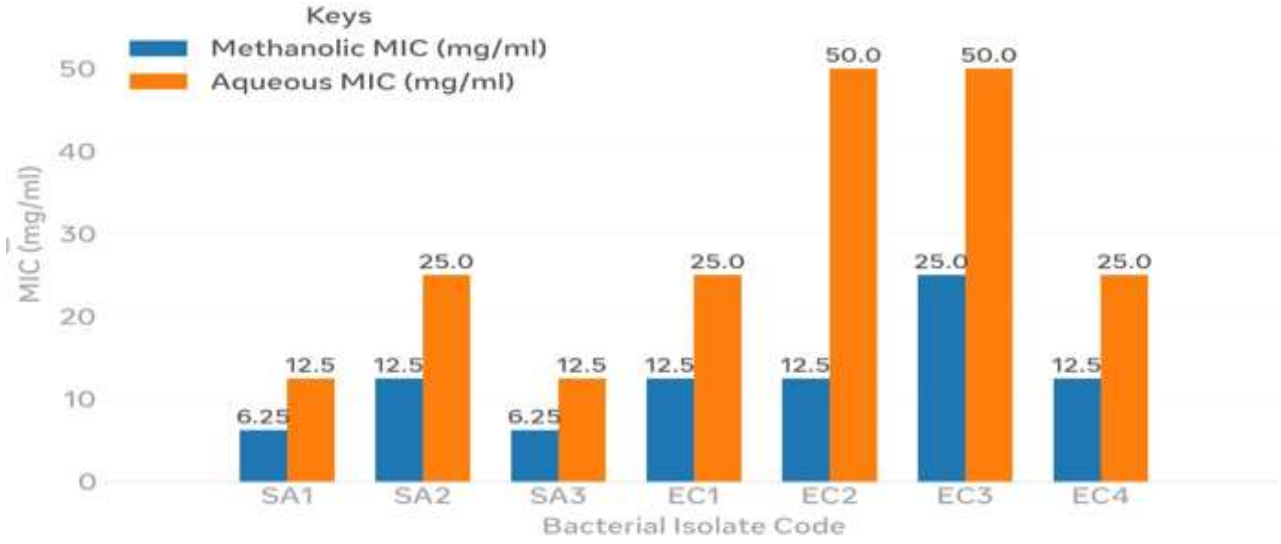


*Figure **1**. Minimum Inhibitory Concentration (mg/mL) of Vernonia amygdalinaCrude Leave Extracts. Blue: Methanolic MIC (mg/mL); Orange: Aqueous MIC (mg/mL); SA1-SA3- Staphylococcus aureus; EC1-EC4- Escherichia coli; Potency Assessment Criteria: Strong Crude Extract: MIC ≤ 100 mg/mL; Moderate = 100-500 mg/mL; Weak: > 500 mg/mL (Noor et al., 2026).*

Figure 2: shows the result of the Minimum Bactericidal Concentration (MBC of Vernonia amygdalina crude Leaf Extracts against the isolated bacteria. The MBC was observed among the isolates at 12.5-50 mg/mL in the methanolic leave extract and 25-100 mg/mL in the aqueous leave extract, highlighting their bactericidal effect at these concentration.

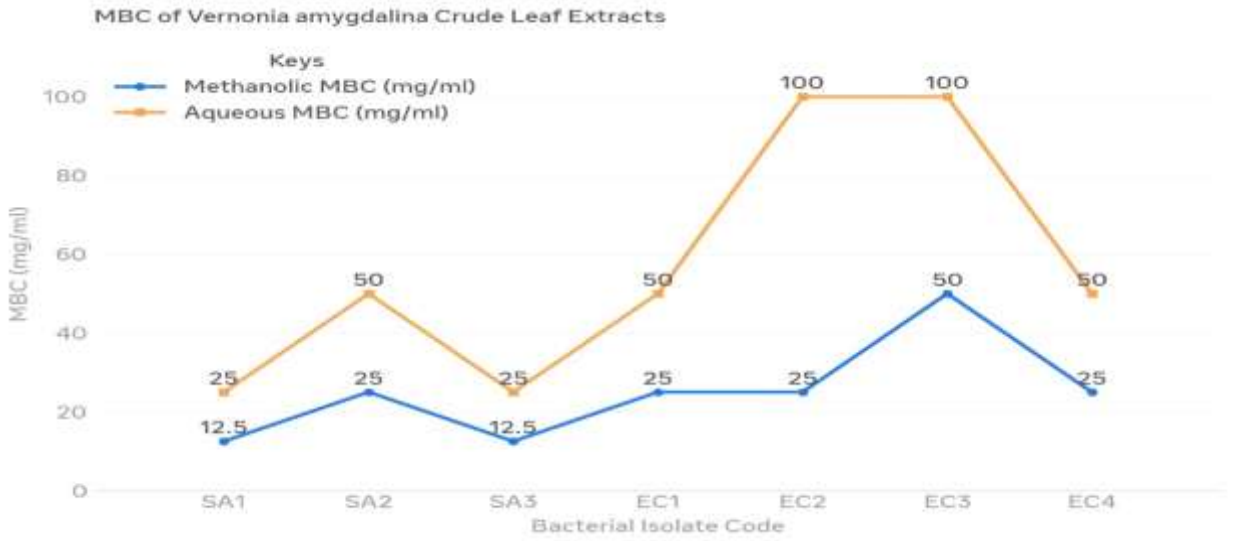


Figure 2. Minimum bactericidal concentration (mg/mL) of Vernonia amygdalina crude leave extract. Blue line: Methanolic MBC (mg/mL); Orange line: Aqueous MBC (mg/mL); X-axis: Isolate Code - SA1-SA3 = Staphylococcus aureus, EC1-EC4 = Escherichia coli. Interpretation: MBC = 2 × MIC, indicating bactericidal activity. Methanolic extract demonstrated more potency across all isolates. Highest resistance seen in EC2 and EC3 with aqueous extract at 100 mg/mL.

4.0 DISCUSSION

In this study, the low bacterial recovery rate of 7% from 100 clinical mastitis milk samples analyzed is lower than the 30-80% reported in most bovine mastitis surveys adduced by Ahmad et al. (2026) in Nigeria, Abebe et al. (2023) in Southern Ethiopia and Ndivhuwo (2020) in South African. This could be attributed to prior antibiotic treatment of cases presented at veterinary centres and the use of only aerobic culture without enrichment. However, the predominance of *Staphylococcus aureus* (3%) and *Escherichia coli* (4%) is consistent with the role of these organisms as major contagious and environmental mastitis pathogens as reported by Santos et al. (2020) and Umar et al. (2023) in Northern Nigeria as well as Agredo-Campos et al. (2023) in Columbia. Findings from the phytochemical screening conductedin themethanolic and aqueous leave extracts revealed dense profile of bioactive compounds among which are: alkaloids, flavonoids, saponins, tannins, phenols and glycosides, with steroids and terpenoids detected only in the methanolic extract. The higher intensity (+++) of flavonoids,terpenoids and steroids in the methanolic extract compared to the aqueous extract is expected, as methanol is a better solvent for polar secondary metabolites. This finding agrees with Adewuyi (2025) who reported similar phytochemical profile for *V. amygdalina* in Niger State Nigeria. In addition, findings from this studymirrors the result reported by Oyema-ilo et al. (2018) at Nnewi, Jarmai et al. (2022) in Borno State and Maryam et al. (2024) in Kano State, Nigeria who adduced to detectingsimilar phytochemicals reported in this study. The antibacterial assessment showed a dose-dependent activity for both extracts. The methanolic extract exhibited significantly higher zones of inhibition (6.00 ± 0.00- 24.4 ± 0.84 mm) than the aqueous extract (0.0 ± 0.0 - 18.22 ± 1.12 mm) at 12.5 - 100 mg/mL. At higher concentrations (100 mg/mL), there is a greater quantity of these antibacterial compounds particularly flavonoids, terpenoids and steroids which were highly abundant in the crude plant extracts. Flavonoid exerts its antibacterial activity by cell membrane disruption, enzyme and ATPsynthase inhibition, efflux pump suppression and biofilm retardation. Terpenoids is involved in membrane destruction and permeabilization**,** protein coagulation and denaturation, oxidative phosphorylation perturbation, nucleic acid and protein synthesis inhibition and anti-quorum sensing. Steroids on the other hand play critical role in virulence and toxin downregulation particularly critical bacterial toxin genes such as the *hla* and *hlb* in *Staphylococcus aureus* and equally suppress adhesion factors as well asefflux pump blockade, synergy and ultimately cell death (Shamsudin et al., 2022)The Gram-positive *S. aureus* was more susceptible (24.4 ± 0.84 mm at 100 mg/mL) than Gram-negative *E. coli* (20.4 ± 2.22 mm at 100 mg/mL), which is attributable to the outer membrane barrier in Gram-negative bacteria that limits penetration of bioactive compounds. Methanol can dissolve both polar and moderately nonpolar compounds while water can only dissolve highly polar compounds (Forster & walsh, 2017). This accounted for the higher antibacterial activity of methanol, demonstrated by wider mean zone of inhibition when compared to the aqueous extract with Gram-positive *S. aureus* –demonstrating 18.22 ± 1.12 mm at 100 mg/mL and Gram-negative *E. coli* 16.82 ± 2.14 mm at 100 mg/mL. This is consistent with the study conducted by Musyoki (2024) at Kampala in Uganda. The researcher was able to demonstrate that the plant leave extract caused zone of inhibition against isolates of *Staphylococcus aureus* but no inhibition zone against *Escherichia coli; w*hich also agrees with the findings of Raji et al. (2026) in Jigawa State, Nigeria. In addition, the difference in the mean inhibition zone observed in this study is most likely due to specie level polymorphism associated with the isolated bacteria, caused by single nucleotide polymorphism (SNP), complex copy number variation, presence/absence of efflux pumps genes or target site mutation, which enabled the isolated bacteria to differentially respond to the antibacterial agents used against them Bala et al. (2018). One-way Analysis of Variance (ANOVA) revealed a statistically significant difference in the mean zones of inhibition across the four concentrations for both methanolic ($p < 0.05$) and aqueous ($p < 0.05$) extracts. Furthermore, there was a significant difference between methanolic and aqueous extracts at 100 mg/mL ($p = 0.0025$, $p < 0.05$), with methanolic extract exhibiting significantly higher activity. Ciprofloxacin (positive control) gave 22.4 ± 2.26 -30.2 ± 2.24 mm inhibition zone while 10% DMSO (negative control) demonstrated no inhibition (0.00 mm). Since the one-way ANOVA revealed a statistically significant difference among the four concentration groups ($p < 0.05$), a post-hoc pairwise comparison using Bonferroni correction was performed to determine which specific concentrations differed significantly. The result showed that all pairwise comparisons for both methanolic and aqueous extracts were statistically significant ($p < 0.05$), confirming a dose-dependent antibacterial activity. The antibacterial activity decreased significantly with decreasing concentration, with 100 mg/mL showing the highest zone of inhibition compared to 12.5 mg/mL.

The Minimum Inhibitory Concentration (MIC) ranged from 6.25 - 12.5 mg/mL for *S. aureus* and 12.5 – 25 mg/mL for *E. coli* in the methanolic extract, while the aqueous MIC range was 12.5 - 50 mg/mL for *S. aureus isolates and* 25 – 50 mg/mL for *E. coli*isolates. The Minimum Bactericidal Concentration (MBC) values were 1-2 folds higher than MIC, indicating bactericidal action at higher concentrations. The lower MIC/MBC of the methanolic extract further confirms its superior extraction efficiency and supports its traditional use in ethnoveterinary medicine. The fact that the methanolic extract showed larger mean differences compared to the aqueous extract at equivalent concentrations further supports the superiority of methanol as an extracting solvent. This is attributable to methanol's amphiphilic nature, which enables it to extract both polar and non-polar bioactive compounds, unlike water which predominantly extracts polar constituents (Doughari, 2012). This finding is consistent with the reports of Magaji (2026) and Obioha et al. (2023) who reported higher antibacterial activity for methanolic extracts of *V. amygdalina* compared to aqueous extracts. The variation in MIC and MBC values observed in this study (6.25-50 mg/mL) compares to lower values (1.56-12.5 mg/mL) reported by Ojiako et al. (2022) in Imo State and higher values (50-100 mg/mL) reported by Abubakar et al. (2021) in Sokoto. This

is attributed to differences in climatic conditions, soil composition, seasonof collection and chemotype of *Vernonia amygdalin*. Zamfara State falls within the Sudan Savanna with high temperature, low rainfall and nutrient-poor soils, which can affect the accumulation of flavonoids and saponins, thereby influencing antibacterial potency. Other factors such as extraction time, plant age, inoculum size and agar medium can also contribute to this variation.Ciprofloxacin (5 µg) gave zones of 22.40 - 30.20 mm, confirming the viability of the test isolates, while 10% DMSO showed no inhibition, confirming it has no antibacterial activity at the concentration used.

Limitations

The present study has some limitations. Isolation of *S. aureus*and*E. coli*was restricted to aerobic bacteria using conventional culture and biochemical characterization assays, which are time-intensive and insufficient for molecular discrimination of strains. The sample size drawn exclusively from the veterinary centers may not be representative of mastitis pathogens circulating in smallholder farms within the study area. Additionally, the absence of fractionation of crude extracts would have resulted in antagonistic interactions from inactive matrix components or excipients, potentially masking the full antibacterial potential of the bioactive compounds.

Conclusion

Vernonia amygdalina remains a veritable ethnoveterinary plant used for the treatment of bovine mastitis. In this study,4(4%) isolates of *E. coli* and 3(3%) of *S. aureus* were detected from a total of 100 milk samples screened. Phytochemical analysis conductedrevealed dense profile of bioactive compounds among which are:flavonoid, saponin, alkaloid, glycoside, terpenoid, tannins, steroids, phenolic compound, anthraquinones and volatile oil. Crude methanolic leaf extract demonstrated better phytochemical richness and exhibited significantly higher zones of inhibition range (6.00 ± 0.00- 24.4 ± 0.84mm) compared to the aqueous extract (0.0 ± 0.0 - 18.22 ± 1.12mm) at 12.5 - 100 mg/mL. One-way Analysis of Variance (ANOVA) revealed a statistically significant difference in the zones of inhibition across the four concentrations for both methanolic ($p < 0.05$) and aqueous ($p < 0.05$) extracts. The Minimum Inhibitory Concentration (MIC) ranged from 6.25 - 12.5 mg/mL for *S. aureus* and 12.5 – 25 mg/mL for *E. coli* in the methanolic extract, while the aqueous MIC range was 12.5 - 50 mg/mL for *S. aureus*and25 – 50 mg/mL for *E. coli*isolates. The Minimum Bactericidal Concentration (MBC) values were 12.5-50 mg/mL in the methanolic extract and 25-100mg/mL in the aqueous extract, implying that the BBC`is 1-2 folds higher than MIC, indicating bactericidal action at higher concentrations. The plant showed promise as a source of alternative therapy for bovine mastitis, but further purification, toxicity and in vivo studies are recommended.

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Competing Interests

Author has declared that no competing interests exist.

Authors' Contributions

All the authors conceived and designed the study, carried out the sample collection and laboratory investigations, analyzed and interpreted the data, and prepared and approved the final manuscript.

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