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Antibacterial activity of *Moringa oleifera* leaves extract against vancomycin resistant *Enterococcus faecalis* Clinical Isolates

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Abstract

Vancomycin-resistant *Enterococcus faecalis* (VRE) is a high-priority pathogen associated with limited therapeutic options, necessitating the exploration of alternative antimicrobial agents. Medicinal plants represent promising candidates because of their diverse bioactive constituents and potential multi-target mechanisms. This study evaluated the antibacterial activity of ethanolic *Moringa oleifera* leaf extract against clinical isolates of vancomycin-resistant *E. faecalis*. Clinical isolates were phenotypically identified using Gram staining and standard biochemical tests, while vancomycin resistance was determined by the disk diffusion method. Ethanolic leaf extract of *M. oleifera* was prepared and evaluated for antibacterial activity using agar well diffusion, broth microdilution for minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. Qualitative phytochemical screening of the extract was also performed. All three phenotypically confirmed *E. faecalis* isolates (100%) exhibited resistance to vancomycin, with inhibition zones ≤ 14 mm. Phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, saponins, and steroids. The extract demonstrated concentration-dependent antibacterial activity, with inhibition zones increasing from 9-11 mm at 25 mg/mL to 20-24 mm at 200 mg/mL. The MIC and MBC were 6.25 mg/mL and 12.5 mg/mL, respectively, yielding an MBC/MIC ratio of 2.0 and indicating bactericidal activity. However, molecular confirmation of vancomycin-resistance genes was not achieved. These findings demonstrate that *M. oleifera* leaf extract possesses bactericidal activity against phenotypically confirmed VRE isolates and may represent a potential source of alternative antimicrobial agents. Further studies involving bioactive-compound isolation, antimicrobial synergy, molecular characterization, and in vivo efficacy are warranted.

Keywords

Moringa Oleifera,
Vancomycin-Resistant
Enterococcus Faecalis,
Antibacterial Activity,
Phytochemicals,
Antimicrobial Resistance.



INTRODUCTION

Enterococcus faecalis is a Gram-positive, facultatively anaerobic, catalase-negative coccus that normally forms part of the gastrointestinal microbiota of humans and other animals. Under normal conditions, it exists as a commensal organism and does not usually cause disease. However, its ability to survive under a wide range of environmental conditions allows it to become an opportunistic pathogen when host defences are weakened or the normal microbial flora is disturbed. This shift from commensalism to pathogenicity is particularly important among hospitalized and immunocompromised patients, where *E. faecalis* has been associated with infections such as urinary tract infections, wound infections, bloodstream infections, endocarditis, and intra-abdominal infections (García-Solache & Rice, 2019; Murray et al., 2020; Sood et al., 2020).

The clinical importance of *E. faecalis* is further increased by its ability to acquire resistance to several antimicrobial agents. Enterococci have intrinsic characteristics that make them less susceptible to many antibiotics, while exposure to antimicrobial drugs can promote the acquisition of additional resistance mechanisms. Their ability to acquire and transfer resistance genes through mobile genetic elements and horizontal gene transfer has contributed to the emergence of multidrug-resistant strains (Miller et al., 2020). As a result, enterococcal infections can be increasingly difficult to treat, particularly in healthcare settings where antimicrobial use and selection pressure are high. The emergence of strains resistant to important therapeutic agents therefore presents a significant challenge to effective infection management and control.

One of the most clinically important forms of enterococcal resistance is resistance to vancomycin. Vancomycin has long been an important treatment option for serious infections caused by Gram-positive bacteria, including enterococcal infections. However, the emergence and spread of vancomycin-resistant enterococci (VRE) have reduced the effectiveness of this treatment option. Vancomycin resistance commonly results from changes in the bacterial cell-

wall precursor, which reduce the ability of the antibiotic to bind to its target and interfere with cell-wall synthesis. Resistance genes such as *vanA* and *vanB* are particularly important because they can confer high-level glycopeptide resistance and contribute to the persistence and spread of resistant strains (Ahmed & Baptiste, 2018; Miller et al., 2020).

Vancomycin-resistant *E. faecalis* is of particular concern because infections caused by these organisms may leave clinicians with limited treatment options. Drugs such as linezolid, daptomycin, and tigecycline may be used in the management of VRE infections, but their use can be limited by factors such as adverse effects, cost, pharmacological considerations, and the possible development of additional resistance (Yim et al., 2021). Dependence on a relatively small number of effective antimicrobial agents creates further concern as resistance continues to increase. This situation highlights the need to investigate alternative and complementary sources of antimicrobial agents that may help address infections caused by resistant organisms.

The increasing global burden of antimicrobial resistance has renewed interest in medicinal plants as potential sources of antimicrobial compounds. Plants have been used in traditional medicine for centuries, and many contain secondary metabolites with biological activities that may be useful against pathogenic microorganisms. Plant-derived compounds may act through several mechanisms, including disruption of bacterial cell membranes, interference with enzymes, changes in cellular permeability, and inhibition of essential cellular processes. Phytochemicals such as flavonoids, alkaloids, tannins, saponins, phenolic compounds, and steroids have been associated with various antimicrobial activities (Górniak et al., 2019; Khameneh et al., 2019; Othman et al., 2019).

One medicinal plant that has attracted considerable interest is *Moringa oleifera* Lam., commonly known as the "miracle tree." *M. oleifera* is widely distributed and is valued for its nutritional, medicinal, and economic importance. Different parts of the plant,

including the leaves, seeds, bark, roots, and flowers, have been investigated for their biological properties. The leaves are of particular interest because they contain a variety of bioactive compounds and are commonly consumed or used in traditional preparations. Studies have reported antioxidant, anti-inflammatory, nutritional, and antimicrobial properties associated with *M. oleifera*, making it a potential source of compounds with therapeutic value (Leone et al., 2015; Vergara-Jimenez et al., 2017).

The antimicrobial activity of *M. oleifera* has been linked, in part, to its diverse phytochemical composition. Flavonoids and other phenolic compounds may interfere with bacterial structures and metabolic processes, while alkaloids may exert antimicrobial effects through interactions with cellular components. Tannins can bind to proteins and interfere with microbial enzymes and other cellular functions, while saponins have been associated with disruption of bacterial membranes. The presence of several classes of phytochemicals may therefore contribute to the antimicrobial activity of *M. oleifera* extracts (Amabye & Tadesse, 2016; Górniak et al., 2019; Mukhtar et al., 2017). However, the activity of a plant extract can vary depending on factors such as the plant part used, geographical location, extraction solvent, concentration, and susceptibility of the target organism.

Previous studies have shown that *M. oleifera* extracts can inhibit several clinically important bacterial pathogens. Antibacterial activity has been reported against organisms such as *Staphylococcus aureus*, *Bacillus subtilis*, and other pathogenic bacteria, suggesting that the plant contains compounds with activity against both Gram-positive and Gram-negative organisms (Amabye & Tadesse, 2016; Muhammad et al., 2016; Mukhtar et al., 2017). This antimicrobial potential is particularly relevant in the context of antimicrobial resistance because plant-derived compounds may provide additional sources of antimicrobial agents for further research and development. However, information on the activity of

M. oleifera against highly resistant enterococci remains relatively limited.

Investigating medicinal plants against VRE is important because the treatment of resistant enterococcal infections requires alternatives to conventional antibiotics. Previous studies have reported antimicrobial activity of plant-derived compounds against vancomycin-resistant enterococci and other multidrug-resistant bacteria, suggesting that phytochemicals may have potential as alternative or complementary antimicrobial agents (Lee et al., 2011; Tanaka et al., 2004). Studies involving *M. oleifera* have also suggested that its antimicrobial activity may be enhanced when combined with conventional antibiotics, raising the possibility of future applications involving synergistic antimicrobial therapy (Mabrouk et al., 2022). However, the activity of *M. oleifera* leaf extract against phenotypically confirmed vancomycin-resistant *E. faecalis* clinical isolates remains an area that requires further investigation.

An important aspect of evaluating antimicrobial activity is distinguishing between bacterial growth inhibition and bactericidal activity. The agar well diffusion method provides an indication of the ability of an extract to inhibit bacterial growth, while the minimum inhibitory concentration (MIC) determines the lowest concentration capable of preventing visible bacterial growth. The minimum bactericidal concentration (MBC), on the other hand, provides an indication of the concentration required to kill viable bacteria under the conditions of the test. Using these methods together provides a more complete assessment of the antibacterial activity of a plant extract. In the present study, these approaches were used to evaluate the activity of ethanolic *M. oleifera* leaf extract against VRE clinical isolates.

Phytochemical screening also provides useful preliminary information about the classes of compounds that may contribute to the observed antibacterial activity. The detection of phytochemicals such as alkaloids, flavonoids, tannins, saponins, and steroids can provide a basis for interpreting the antimicrobial effects observed in

laboratory assays. Although qualitative phytochemical screening does not identify individual active compounds or determine their concentrations, it provides an important starting point for further studies involving compound isolation, characterization, mechanism-of-action investigations, and antimicrobial synergy.

Despite the reported medicinal and antimicrobial properties of *M. oleifera*, there is still a need for studies specifically examining its activity against vancomycin-resistant *E. faecalis* obtained from clinical sources. Such research is particularly relevant in settings where antimicrobial resistance is increasing and access to newer or more expensive antimicrobial agents may be limited. The widespread availability and relatively low cost of *M. oleifera* further support the need to investigate its potential as a source of antimicrobial compounds (Leone et al., 2015; World Health Organization [WHO], 2019). However, laboratory evidence of antibacterial activity represents only an initial step. Further studies are required to establish the safety, pharmacological properties, effective dosage, mechanisms of action, and in vivo effectiveness of promising plant-derived compounds before their potential clinical application can be determined.

MATERIALS AND METHODS

Study Area

The study was conducted at the Federal Teaching Hospital (FTH), Gombe, located in Gombe State, northeastern Nigeria. The hospital is situated within Gombe metropolis, the administrative capital of the state. Gombe metropolis lies approximately between latitudes 10°N and 11°N of the Equator and is located within the Sudan Savannah ecological zone. Gombe State covers a land area of approximately 18,000 km² and had a population of about 2.5 million people according to the 2006 population census (Figure 1). Federal Teaching Hospital, Gombe, was established in 1996 to provide specialized and tertiary healthcare services to the people of Gombe State and

neighbouring areas. The hospital also serves as a referral centre for patients requiring specialized medical care from surrounding communities and other healthcare facilities. It has an estimated bed capacity of about 500, with an average bed occupancy rate of approximately 90% to 95%. The high bed occupancy reflects the considerable demand for healthcare services at the facility and highlights its role as an important tertiary healthcare centre in the region.

Study Design and Ethical Approval

A laboratory-based cross-sectional research design was used for this study at the Department of Microbiology, Gombe State University, Nigeria. Before the commencement of the study, ethical approval was obtained from the Research and Ethics Committee of the Federal Teaching Hospital, Gombe. This ensured that all aspects of the study were carried out in accordance with the required ethical standards.

Source of Clinical Isolates

Clinical isolates of *Enterococcus faecalis* were collected from the Microbiology and Immunology Laboratory of the Federal Teaching Hospital, Gombe.

Confirmation of *Enterococcus faecalis*

Suspected *E. faecalis* isolates were confirmed using standard microbiological procedures, including Gram staining, the catalase test, salt tolerance testing based on growth in 6.5% NaCl, and bile esculin hydrolysis (Murray et al., 2020).

Gram Staining

A small portion of the bacterial isolate was used to prepare a thin smear on a clean glass slide, which was then heat-fixed. The smear was flooded with crystal violet and allowed to stand for 1 minute, followed by Lugol's iodine for 1 minute. Acetone was used as the decolorizing agent, after which the smear was counterstained with safranin for 2 minutes. Following staining, Gram-positive cocci observed singly, in pairs, or in short chains were presumptively identified as members of the genera *Enterococcus* or *Streptococcus*.

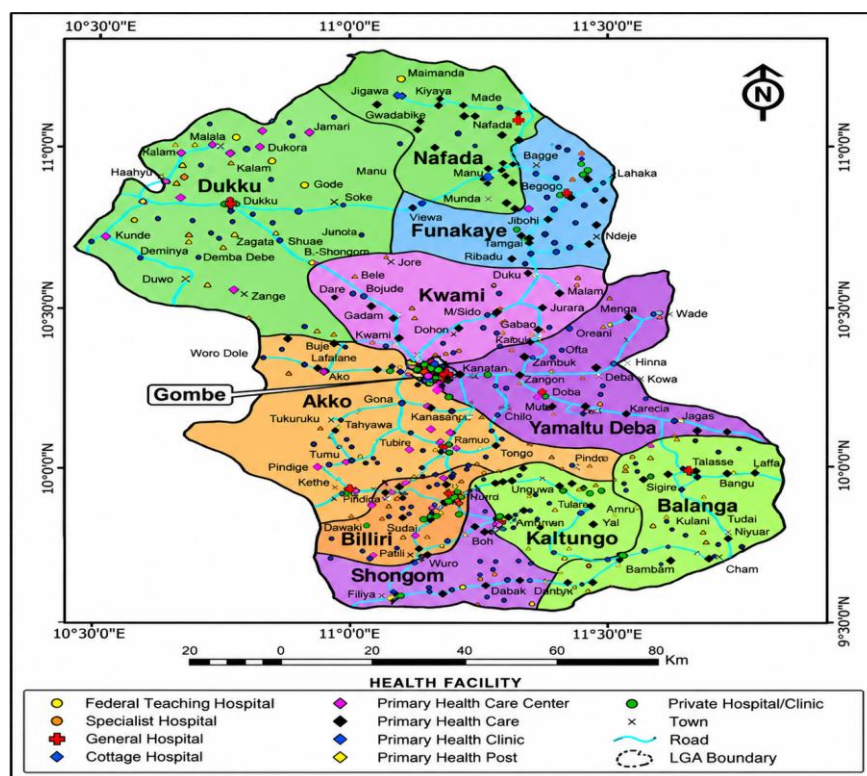


Figure 1: Map of Gombe State indicating the location of Federal Teaching Hospital, Gombe (Oghenebrume, 2021).

Catalase Test

For the catalase test, several well-isolated colonies were picked and emulsified in 3% hydrogen peroxide on a clean glass slide. The preparation was observed immediately for the formation of gas bubbles. The absence of visible effervescence was recorded as a negative catalase reaction. This test helped to differentiate enterococci and streptococci, which are catalase-negative, from staphylococci, which are catalase-positive.

Salt Tolerance Test

Salt tolerance was determined by inoculating the test isolate into Brain Heart Infusion (BHI) broth supplemented with 6.5% sodium chloride (NaCl). The inoculated broth was incubated at 37°C for 24-48 hours and examined for bacterial growth. Visible turbidity in the broth was recorded as a positive result, indicating the ability of the isolate to grow in the presence of the elevated salt concentration and

supporting its presumptive identification as an *Enterococcus* species.

Bile Esculin Hydrolysis Test

The ability of the isolate to hydrolyse esculin in the presence of bile was determined using Bile Esculin Agar. The isolate was inoculated onto the surface of the agar slant and incubated at 37°C for 24 hours. A positive reaction was indicated by the development of a dark brown to black colouration of the medium, indicating esculin hydrolysis in the presence of 40% bile. This reaction further supported the presumptive identification of the isolate as an *Enterococcus* species.

Molecular Confirmation by PCR

DNA Extraction: Genomic DNA was extracted from overnight cultures of presumptive *E. faecalis* isolates using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for Gram-positive bacteria. The concentration and purity

of the extracted DNA were determined using a NanoDrop™ spectrophotometer. DNA samples with A260/A280 ratios between 1.7 and 2.0 were considered of acceptable purity for PCR analysis.

PCR Amplification: The species-specific *ddl* gene, which encodes D-alanine-D-alanine ligase, was amplified using previously validated primers described by Kariyama et al. (2000). The primer sequences used were as follows:

Forward primer: 5'-

ATCAAGTACAGTTAGTCTTTATTAG-3'

Reverse primer: 5'-

ACGATTCAAAGCTAACTGAATCAGT-3'

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2025).

Preparation of Inoculum: Three to five morphologically similar colonies from an overnight pure culture were selected and suspended in sterile normal saline. The turbidity of the suspension was adjusted to the 0.5 McFarland standard, corresponding to approximately 1.5×10^8 colony-forming units per millilitre (CFU/mL). The standardized suspension was used within 15 minutes of preparation.

Inoculation: A sterile cotton swab was dipped into the standardized bacterial suspension, and excess fluid was removed by pressing the swab against the inner wall of the tube. The swab was then used to evenly inoculate the entire surface of a Mueller-Hinton agar plate by streaking in three directions to obtain a uniform lawn of bacterial growth. The inoculated plates were allowed to stand for 5-10 minutes to allow excess moisture to dry.

Antibiotic Disk Application: The following antibiotic disks (Oxoid, UK) were aseptically placed on the surface of the inoculated plates using sterile forceps: amoxicillin (30 µg), erythromycin (5 µg), ceftriaxone

(30 µg), levofloxacin (5 µg), ciprofloxacin (10 µg), doxycycline (30 µg), rifampicin (5 µg), cefepime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), and clindamycin (2 µg). The disks were positioned at least 24 mm apart to prevent overlapping of inhibition zones. The plates were incubated aerobically at 37°C for 24 hours. Following incubation, the zones of complete inhibition around each antibiotic disk were measured to the nearest millimetre using a calibrated ruler.

Detection of Vancomycin Resistance Genes (*vanA* and *vanB*)

Genomic DNA was extracted using the boiling lysis method described by Sambrook and Russell (2001). PCR amplification was performed using primers targeting the *vanA* and *vanB* genes, producing expected amplicon sizes of 1030 bp and 433 bp, respectively, following the method described by Dutka-Malen et al. (1995). The PCR products were separated and visualized using 1.5% agarose gel electrophoresis.

Plant Material Collection and Extract Preparation

Fresh leaves of *Moringa oleifera* were collected, shade-dried, and ground into a fine powder. One hundred grams of the powdered leaves were macerated in 70% ethanol at a ratio of 1:10 (w/v) for 72 hours at room temperature. The mixture was filtered, and the resulting filtrate was concentrated using a rotary evaporator at 40°C. The concentrated extract was then used to prepare stock solutions in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL (Leone et al., 2015).

Phytochemical Screening

Qualitative phytochemical screening was carried out to detect the presence of alkaloids, tannins, steroids, flavonoids, and saponins. Alkaloids were tested using Meyer's reagent, tannins using ferric chloride, steroids using the Salkowski test, flavonoids using sodium hydroxide, and saponins using the froth test (Amabye & Tadesse, 2016; Mukhtar et al., 2017).

Antibacterial Assay

The antibacterial activity of the *M. oleifera* leaf extract was evaluated using the agar well diffusion method. Wells measuring 6 mm in diameter were made in Mueller-Hinton agar plates previously inoculated with a bacterial suspension adjusted to 0.5 McFarland standard. Different concentrations of the extract (200, 100, 50, and 25 mg/mL) were added to the wells at a volume of 100 µL per well. The plates were incubated at 37°C for 24 hours, after which the zones of inhibition were measured in millimetres (Muhammad et al., 2016).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) of the extract was determined using the broth microdilution method with two-fold serial dilutions ranging from 200

mg/mL to 1.56 mg/mL, following CLSI (2023) guidelines. The MIC was recorded as the lowest concentration of the extract that showed no visible bacterial growth. To determine the minimum bactericidal concentration (MBC), samples from wells showing no visible growth were subcultured onto fresh agar plates and incubated. The MBC was defined as the lowest concentration that produced no bacterial growth on the subculture (French, 2006).

RESULTS

Identification of Clinical Isolates

A total of four clinical isolates were confirmed as *Enterococcus faecalis* based on: Gram-positive cocci, catalase-negative, growth in 6.5% NaCl (positive), and bile esculin hydrolysis (positive). One isolate was identified as *Staphylococcus* spp. and excluded. (Table 1.)

Table 1: Biochemical Characteristics and Identification of Clinical Isolates

S/N	GR	Cat	ST	BEH	ORG
1.	+	+	+	-	<i>Staphylococcus Spp.</i>
2.	+	-	+	+	<i>Enterococcus faecalis</i>
3.	+	-	+	+	<i>Enterococcus faecalis</i>
4.	+	-	+	+	<i>Enterococcus faecalis</i>

Key: GR = Gram reaction, Cat = Catalase, ST = Salt tolerance, BEH = Bile esculin hydrolysis; +=Positive, -=Negative

Three of the four isolates (75%) were identified as *E. faecalis*, while one isolate (25%) was identified as *Staphylococcus spp.* All three *E. faecalis* isolates were catalase-negative, capable of growing in 6.5% NaCl, and able to hydrolyze esculin in the presence of bile.

Molecular Confirmation

PCR amplification of the *ddl* gene confirmed the identity of the three isolates that were phenotypically identified as *E. faecalis*. All three isolates produced distinct bands at approximately 1500 bp following electrophoresis on a 1% agarose gel, confirming their molecular identity (Kariyama et al., 2000).

Antibiotic Susceptibility Profile

As presented in Table 2, all four (100%) clinical isolates of *Enterococcus faecalis* were resistant to vancomycin. None of the isolates showed susceptibility to vancomycin, indicating complete resistance among the isolates tested.

Table 2. Antibiotic Susceptibility Profiles of *E. faecalis* Isolate.

S/N	Antibiotic	R	S	I
1.	Vancomycin	+	-	-
2.	Vancomycin	+	-	-
3.	Vancomycin	+	-	-
4.	Vancomycin	+	-	-

KEY: + = Positive, - = Negative, R= Resistance, S= Susceptible, I = Intermediate.

Phytochemical Screening

The phytochemical screening of the extract revealed the presence of all five tested phytochemical groups, namely alkaloids, flavonoids, tannins, saponins, and steroids.

Table 3: Phytochemical constituents of *Moringa oleifera* leaf extract

S/N	Phytochemical	<i>Moringa oleifera</i> leaf extract
1.	Alkaloids	+
2.	Flavonoids	+
3.	Tannin	+
4.	Saponin	+
5.	Steroids	+

KEY: + = Positive (presence of phytochemical constituent), - = Negative (absence of phytochemical constituent)

Antibacterial Activity

The extract demonstrated concentration-dependent antibacterial activity against the VRE isolates, with the zones of inhibition increasing from 9-11 mm at 25 mg/mL to 20-24 mm at 200 mg/mL (Table 3). This indicates that higher concentrations of the extract produced greater inhibition of bacterial growth.

Table 3: Antibacterial Activity of *Moringa oleifera* leaf Extract Against VRE Isolates

S/N	Isolate code	Zone of inhibition (mm) at different concentrations (mg/ml)			
		200	100	50	25
1.	VRE 01	23	20	18	10
2.	VRE 02	24	19	15	9
3.	VRE 03	20	18	15	10
4.	VRE 04	23	19	17	11

Key: VRE= Vancomycin resistant *Enterococcus faecalis*.

Minimum Inhibitory and Bactericidal Concentrations

The minimum inhibitory concentration (MIC) of the extract was 6.25 mg/mL, while the minimum bactericidal concentration (MBC) was 12.5 mg/mL. The resulting MBC/MIC ratio of 2.0 indicates that the extract exhibited bactericidal activity against the VRE isolates (French, 2006).

DISCUSSION

The present study investigated the antibacterial activity of ethanolic *Moringa oleifera* leaf extract against vancomycin-resistant *Enterococcus faecalis* clinical isolates. The findings showed that the three isolates identified as *E. faecalis* were resistant to vancomycin, while the ethanolic leaf extract demonstrated antibacterial activity against all the isolates tested. The activity increased as the concentration of the extract increased. The MIC and MBC results further showed that the extract was capable of producing a bactericidal effect under the conditions of the assay. These findings are relevant because vancomycin resistance can considerably reduce the available treatment options for serious enterococcal infections.

The phenotypic identification of the isolates was supported by their characteristic biochemical reactions. The three isolates were Gram-positive cocci, catalase-negative, able to grow in 6.5% NaCl, and positive for bile esculin hydrolysis. These characteristics are consistent with the established features of enterococci and supported their initial identification. The identification was further confirmed by PCR amplification of the species-specific *ddl* gene, with all three isolates producing the expected molecular bands. The agreement between the phenotypic and molecular results increases confidence in the identity of the organisms used for the antibacterial assays. Enterococci are well adapted to a variety of environmental conditions and possess characteristics that contribute to their persistence and pathogenicity, particularly in healthcare settings (García-Solache & Rice, 2019; Murray et al., 2020).

A major finding of the study was the complete resistance of the tested isolates to vancomycin. All

the isolates showed resistance by disk diffusion, with inhibition zones within the resistant range used in the study. This finding is clinically important because vancomycin has traditionally been an important treatment option for serious infections caused by Gram-positive organisms, including enterococci. The emergence of vancomycin-resistant enterococci has reduced the usefulness of this antibiotic and created additional challenges in the treatment of enterococcal infections (Ahmed & Baptiste, 2018; Miller et al., 2020). The complete resistance observed in this study also highlights the need for continued monitoring of antimicrobial susceptibility patterns in healthcare facilities.

The presence of vancomycin-resistant *E. faecalis* is particularly concerning because resistance to vancomycin may occur together with resistance to other antimicrobial agents. Enterococci have intrinsic resistance characteristics and can acquire additional resistance determinants through genetic exchange and other mechanisms. As a result, infections caused by resistant enterococci may become increasingly difficult to treat, particularly when resistance involves several antimicrobial classes (Miller et al., 2020; Sood et al., 2020). Against this background, the antibacterial activity demonstrated by *M. oleifera* leaf extract provides a useful basis for further investigation of plant-derived compounds as potential sources of antimicrobial agents.

Although phenotypic resistance to vancomycin was clearly demonstrated, PCR detection of the *vanA* and *vanB* resistance genes was unsuccessful in the present study. Consequently, the observed resistance cannot be specifically attributed to either of these genes based on the available results. The failure to detect the target genes may have been associated with factors such as DNA extraction quality, PCR amplification conditions, primer specificity, or the presence of other vancomycin resistance determinants that were not investigated. Previous studies have shown that vancomycin resistance in enterococci can involve different genetic determinants, with *vanA* and *vanB* being among the most clinically important (Ahmed

& Baptiste, 2018; Miller et al., 2020). The present findings should, therefore, be interpreted as evidence of phenotypic vancomycin resistance rather than molecular confirmation of *vanA*- or *vanB*-mediated resistance.

Phytochemical screening of the ethanolic *M. oleifera* leaf extract revealed the presence of alkaloids, flavonoids, tannins, saponins, and steroids. This finding is consistent with previous reports describing the diverse phytochemical composition of *M. oleifera* leaves (Amabye & Tadesse, 2016; Mukhtar et al., 2017). The presence of several classes of secondary metabolites may partly explain the antibacterial activity observed in this study. Plant-derived phytochemicals can affect bacterial cells through different mechanisms, including disruption of cell membrane integrity, interference with enzymes and cellular proteins, changes in membrane permeability, and inhibition of essential metabolic processes (Górniak et al., 2019; Khameneh et al., 2019; Othman et al., 2019).

Flavonoids, for instance, have been widely studied for their antimicrobial properties and may interfere with bacterial cell structures and proteins. Alkaloids have also been associated with antimicrobial effects through their interaction with cellular components, while tannins may bind to proteins and interfere with microbial enzymes. Saponins, in particular, have been associated with effects on bacterial membranes. The combined presence of these phytochemicals may contribute to the antibacterial activity of the extract rather than the activity being due to a single compound (Górniak et al., 2019; Othman et al., 2019). However, because the phytochemical screening in this study was qualitative, it was not possible to determine which specific compounds were responsible for the observed antibacterial activity.

The antibacterial assay showed a clear concentration-dependent response. At 25 mg/mL, the extract produced inhibition zones ranging from 9 to 11 mm, which increased to 15–18 mm at 50 mg/mL. At 100 mg/mL, inhibition zones ranged from 18 to 20 mm,

while the highest concentration tested, 200 mg/mL, produced zones ranging from 20 to 24 mm. The progressive increase in inhibition with increasing extract concentration suggests that higher concentrations provided greater amounts of active antibacterial constituents capable of interacting with the bacterial cells. Similar antibacterial activity has been reported for *M. oleifera* leaf extracts against different pathogenic bacteria, supporting the antimicrobial potential of the plant (Amabye & Tadesse, 2016; Muhammad et al., 2016; Mukhtar et al., 2017).

The concentration-dependent response observed in this study is also biologically reasonable because crude plant extracts contain mixtures of active compounds whose effects may depend on their concentration. At lower concentrations, the amount of active compounds available to interact with bacterial cells may be insufficient to produce extensive inhibition. Increasing the concentration may provide a greater quantity of these compounds, resulting in increased interference with bacterial structures and metabolic processes. The gradual increase in inhibition zones observed across the concentrations tested therefore suggests a concentration-related antibacterial effect. Some differences were observed in the susceptibility of the individual isolates to the extract. For example, at 200 mg/mL, the inhibition zones ranged from 20 to 24 mm, while at 50 mg/mL they ranged from 15 to 18 mm. Such variation is not unexpected among clinical isolates and may be related to differences in cell-envelope characteristics, stress responses, or susceptibility to particular phytochemical constituents. Although all the isolates were identified as *E. faecalis* and were resistant to vancomycin, their responses to the plant extract did not have to be identical. Resistance to vancomycin and susceptibility to plant-derived compounds may involve different cellular targets and mechanisms.

The MIC result provided further evidence of the antibacterial effect of the extract. The MIC was 6.25 mg/mL, indicating that this was the lowest concentration at which visible bacterial growth was

inhibited under the conditions of the broth microdilution assay. MIC determination provides a quantitative measure of the concentration required to inhibit bacterial growth and complements the inhibition-zone results obtained through agar well diffusion. The ability of the extract to inhibit the isolates at this concentration provides additional evidence that its antibacterial constituents remained active under broth dilution conditions.

The MBC of 12.5 mg/mL further indicated that the extract was capable of killing the bacteria rather than simply inhibiting their growth. The MBC/MIC ratio of 2.0 supports the classification of the extract as bactericidal according to the criterion applied in this study (French, 2006). The distinction between bacteriostatic and bactericidal activity is important when evaluating potential antimicrobial agents because the absence of visible growth does not necessarily mean that the organisms have been killed. The combined MIC and MBC findings therefore provide stronger evidence of antibacterial activity than the agar diffusion results alone.

The bactericidal effect observed may be related to the combined action of the phytochemicals detected in the extract. A crude plant extract contains several compounds that may act on different bacterial targets. Some may affect membrane integrity, while others may interfere with enzymes, proteins, or intracellular processes. This combined activity may partly explain why the extract was effective against isolates that were resistant to vancomycin. However, the findings should not be interpreted as evidence that the extract reverses vancomycin resistance. Rather, the results suggest that the extract contains antibacterial constituents that may act through mechanisms different from those of vancomycin.

The findings are comparable with previous studies investigating plant-derived antimicrobial agents against resistant enterococci. Tanaka et al. (2004) reported antibacterial activity of phytochemicals isolated from *Erythrina zeyheri* against vancomycin-resistant enterococci, demonstrating the potential of

plant-derived compounds against organisms resistant to conventional antibiotics. Similarly, Lee et al. (2011) reported antimicrobial activity of *Alpinia officinarum* extracts against VRE and other pathogenic microorganisms. More recently, Romyasamit et al. (2024) reported antibacterial and antibiofilm activity of *Gymnema inodorum* leaf extract against vancomycin-resistant *Enterococcus faecium*. These findings, together with the results of the present study, support continued investigation of medicinal plants as potential sources of antimicrobial compounds against resistant enterococci.

The antibacterial activity observed in this study also agrees with previous research on *M. oleifera*. Amabye and Tadesse (2016) reported the presence of phytochemical constituents and antibacterial activity in *M. oleifera*, while Muhammad et al. (2016) demonstrated in vitro antibacterial activity of *M. oleifera* leaf extracts against pathogenic bacteria. Mukhtar et al. (2017) likewise reported antimicrobial activity of *Moringa* against vancomycin-resistant cocci and other microorganisms. The present study adds to these findings by demonstrating antibacterial activity of ethanolic *M. oleifera* leaf extract against phenotypically confirmed vancomycin-resistant *E. faecalis* clinical isolates.

Differences in antibacterial activity reported between studies may be explained by several factors, including the plant part used, geographical origin, maturity of the plant, environmental conditions, extraction method, solvent, extract concentration, and characteristics of the test organism. The use of 70% ethanol in the present study may have allowed the extraction of a broad range of polar and moderately non-polar phytochemicals. The activity observed in this study may therefore differ from that obtained using aqueous extracts, other organic solvents, or purified compounds from *M. oleifera*.

The findings also provide a basis for future studies investigating combinations of *M. oleifera* extract with conventional antibiotics. Previous research has suggested that *M. oleifera* may have synergistic

effects when combined with selected antibiotics, although such interactions depend on the organism, antibiotic, extract composition, and experimental conditions (Mabrouk et al., 2022). Studies involving other plant-derived compounds have similarly suggested that phytochemicals may enhance the activity of conventional antimicrobial agents against resistant bacteria (Tanaka et al., 2004). Further investigation could therefore determine whether *M. oleifera* extract has synergistic, additive, or antagonistic effects when combined with antibiotics commonly used against enterococcal infections.

Despite the promising laboratory findings, the antibacterial activity observed in this study should not be taken as evidence that *M. oleifera* leaf extract can be used directly as a clinical treatment for VRE infections. The study involved a relatively small number of clinical isolates, and the assays were conducted under controlled laboratory conditions. In addition, the phytochemical analysis was qualitative and did not determine the concentration of individual active compounds. The unsuccessful detection of *vanA* and *vanB* also limits the extent to which the genetic basis of vancomycin resistance can be described. Further studies involving larger numbers of clinical isolates, quantitative phytochemical analysis, isolation and characterization of active compounds, broader molecular testing, and toxicity and pharmacological assessments are needed.

Overall, the findings demonstrate that ethanolic *M. oleifera* leaf extract exhibited measurable antibacterial activity against the phenotypically confirmed VRE isolates examined in this study. The concentration-dependent inhibition, MIC of 6.25 mg/mL, and MBC/MIC ratio of 2.0 provide complementary evidence of bactericidal activity under the experimental conditions. The detection of

alkaloids, flavonoids, tannins, saponins, and steroids also provides a possible chemical basis for the observed antibacterial effect, although the specific compounds responsible could not be established. Given the increasing challenge of antimicrobial resistance and the widespread availability of *M. oleifera*, the plant may serve as a useful source for further antimicrobial research. However, additional studies are required to identify the active compounds, determine their mechanisms of action, evaluate their safety and pharmacological properties, and assess their potential interaction with conventional antibiotics before any clinical application can be considered.

CONCLUSION

Ethanolic *Moringa oleifera* leaf extract demonstrated bactericidal activity against the phenotypically confirmed VRE isolates, with an MIC of 6.25 mg/mL and an MBC of 12.5 mg/mL (MBC/MIC ratio = 2.0). Phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, saponins, and steroids, which may contribute to the antibacterial activity observed (Amabye & Tadesse, 2016). However, molecular confirmation of the *vanA* and *vanB* genes was unsuccessful; therefore, the findings regarding vancomycin resistance are based on phenotypic evidence only.

Despite this limitation, the antibacterial activity observed suggests that *M. oleifera* may have potential as a source of compounds for further investigation against VRE (Rather et al., 2021). Further studies should focus on isolating and characterising the active compounds, evaluating their possible synergy with conventional antibiotics, and assessing their safety and efficacy in vivo before any potential therapeutic application can be considered (Mabrouk et al., 2022; Seukep et al., 2025).

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