



Antimicrobial activity of *Terminalia leiocarpa* Baill. and *Terminalia avicennioides* Guill. & Perr. root bark extracts in resistant clinical isolates

[Actividad antimicrobiana del extracto de corteza de raíz de *Terminalia leiocarpa* Baill. y *Terminalia avicennioides* Guill. & Perr. en aislamientos clínicos resistentes]

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Abstract

Context: The utilisation of plants as a reservoir for potential chemotherapeutic and antimicrobial agents is a common practice in ethnomedicine. However, folkloric use of *Terminalia leiocarpa* and *Terminalia avicennioides* in treatment of microbial infection has prompted various pharmacological investigations, especially antimicrobial studies.

Aims: To evaluate the antimicrobial activity of the bioactive compounds of *T. leiocarpa* and *T. avicennioides* roots bark extracts on clinical isolates.

Methods: Phytochemical screening of the extracts was done following standard procedures. Antimicrobial activities of 500 mg/mL of each extract were performed through the agar well diffusion method on clinical isolates of *E.coli*, *Klebsiella pneumonia*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The methanol extract of *T. leiocarpa* was fractionated into four fractions and tested at concentrations 31.25, 62.5, 125, 250 and 500 mg/mL. The Agar well diffusion method and 96 titre well were employed to ascertain the percentage inhibition of the fractions and determine the minimum inhibitory (MIC) and bactericidal concentrations (MBC). Compounds were identified utilising gas chromatography-mass spectrometry (GC-MS). Microsoft Excel and SPSS 20.0 version were used to analyze experimental data.

Results: Phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, terpenoids, saponins, and cardiac glycosides. The dichloromethane fraction of *T. leiocarpa* revealed the highest activity with MIC of 31.25-125 mg/mL and of MBC 15.63-125 mg/mL while methanol gave MIC of 62.5-250 mg/mL and MBC of 31.25-125 mg/mL respectively. The GC-MS revealed 15 peaks in the dichloromethane fraction.

Conclusions: The findings suggest the antimicrobial activity of the identified compounds, which could be a potential resistance-free template for future antimicrobial drug discovery.

Keywords: antimicrobial; *Klebsiella pneumonia*; *Terminalia avicennioides*; *Terminalia leiocarpa*.

Resumen

Contexto: La utilización de plantas como reservorio de potenciales agentes quimioterapéuticos y antimicrobianos es una práctica común en etnomedicina. Sin embargo, el uso folclórico de *Terminalia leiocarpa* y *Terminalia avicennioides* en el tratamiento de infecciones microbianas ha dado lugar a diversas investigaciones farmacológicas, especialmente estudios antimicrobianos.

Objetivos: Evaluar la actividad antimicrobiana de los compuestos bioactivos de los extractos de corteza de las raíces de *T. leiocarpa* y *T. avicennioides* en aislados clínicos.

Métodos: El cribado fitoquímico de los extractos se realizó siguiendo procedimientos estándar. Las actividades antimicrobianas de 500 mg/mL de cada extracto se realizaron mediante el método de difusión en pocillos de agar en aislados clínicos de *E. coli*, *Klebsiella pneumonia*, *Staphylococcus aureus* y *Pseudomonas aeruginosa*. El extracto metanólico de *T. leiocarpa* se fraccionó en cuatro fracciones y se probó en concentraciones de 31,25, 62,5, 125, 250 y 500 mg/mL. Se empleó el método de difusión en pocillos de agar y en pocillos de 96 títulos para determinar el porcentaje de inhibición de las fracciones y las concentraciones inhibitorias mínimas (CIM) y bactericidas (CBM). Los compuestos se identificaron mediante cromatografía de gases-espectrometría de masas (GC-MS). Para analizar los datos experimentales se utilizaron las versiones Microsoft Excel y SPSS 20.0.

Resultados: El análisis fitoquímico reveló la presencia de alcaloides, flavonoides, taninos, terpenoides, saponinas y glucósidos cardíacos. La fracción diclorometánica de *T. leiocarpa* reveló la mayor actividad con una CMI de 31,25-125 mg/mL y una CBM de 15,63-125 mg/mL, mientras que el metanol dio una CMI de 62,5-250 mg/mL y una CBM de 31,25-125 mg/mL, respectivamente. La GC-MS reveló 15 picos en la fracción de diclorometano.

Conclusiones: Los hallazgos sugieren la actividad antimicrobiana de los compuestos identificados, que podrían ser una plantilla potencial libre de resistencia para el futuro descubrimiento de fármacos antimicrobianos.

Palabras Clave: antimicrobiano; *Klebsiella pneumonia*; *Terminalia avicennioides*; *Terminalia leiocarpa*.

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Abbreviations: AMR: Antimicrobial resistance; CLSI: Clinical and Laboratory Standards Institute; DCM: Dichloromethane; DMSO: Dimethyl sulphoxide; FHI: Forest Herbarium Ibadan; FRIN: Forestry Research Institute of Nigeria; MBC: Minimum bactericidal concentration; MDR: Multidrug resistance; MHB: Mueller Hinton broth; MIC: Minimum inhibitory concentration; MRSA: Methicillin-resistant *Staphylococcus aureus*; MSSA: Methicillin-susceptible *Staphylococcus aureus*; TIC: Total ion count.

INTRODUCTION

Nature is a great source of natural products, and the supply from plants, animals, or microorganisms is unlimited. Plants, on the other hand, have exhibited unmatched usefulness to man from the time of old. The use of plants as therapies in traditional medicine is as old as mankind. Knowing how plants defend themselves is crucial for safeguarding our food supply as well as for the use of their diverse chemical composition, which yields a wide range of therapeutic applications (Borges et al., 2016). According to the World Health Organization, more than 80% of Africans use plant products or herbal medicines to cure a variety of illnesses (Okaiyeto and Oguntibeju, 2021). In Africa, the utilisation of various plants from the *Combretaceae* family has been a traditional practice to remedy respiratory conditions and coughs, notably persistent coughs (Masoko and Nxumalo, 2013; Salih, 2019). An example of these plants is *Anogeissus leiocarpus* (DC.) Guill. & Perr., the African birch (Salih et al., 2020). *A. leiocarpus* decoctions and macerations play a crucial role in African traditional medicine, demonstrating efficacy against a wide spectrum of infectious diseases, including bacterial and fungal infections. Moreover, these traditional remedies prove beneficial in managing symptoms associated with these infections, such as diarrhea and skin rashes (Dahiru et al., 2021; Salih et al., 2017).

The microbial infection is among the major cause of death around the world due to the capability of creating resistance to the market drugs. Health officials have warned that antimicrobial resistance poses a serious threat to humanity since it has emerged as a main cause of death globally, killing roughly 3,500 people daily, which resulted in over 1.2 million deaths only in 2019 as a direct consequence of antibiotic resistance and indirectly it has been linked to around 4.95 million deaths, during the same period (Kehinde and Dada-Adegbola, 2013). The prevalence of multidrug-resistant (MDR) pathogens has amplified the occurrences of infectious diseases, becoming the primary cause of mortality worldwide (Kalupahana et al., 2017). The widespread misapplication and overuse of antibiotics are fueling instances of AMR within microbial populations (Tariq et al., 2020).

In ethnomedicine, promising antimicrobial and chemotherapeutic drugs are derived from plants. Phytochemicals, also known as metabolites, are naturally occurring substances derived from fruits, herbs, spices, and plants. Plant bioactive chemicals represent

a different approach to lowering the occurrence of pathogenic diseases. In the realm of pharmaceuticals, substances originating from plants are regarded as an initial platform for research and the development of drugs (Hiremath et al., 2014).

According to recent research, antimicrobial chemicals have a greater therapeutic impact when they are transported via colloidal systems (Ghadi and Ebrahimnejad, 2019). The use of medicinal herbs is becoming more and more common, and demand for them is currently high. Generally, plants have been contributing significantly to ecosystems by giving humans and other animals the vital services they need to maintain their health (Ajayi, 2019a; 2019b).

Recent research has shown that one of the most crucial factors in the fight against infectious diseases is the antimicrobial resistance (AMR) phenotype, with some of the newer drugs of some known and easy-to-treat bacterial infectious agents, such as *E. coli*, *Klebsiella spp.*, and some other species of *Enterobacteriaceae* (Huttner et al., 2013; World Health Organization, 2014). This brings about the need to offer alternatives or solutions to the AMR crisis (Huttner et al., 2013).

According to Elegami et al. (2002), the antibacterial potential of 48 extracts obtained from four *Combretaceae* family plants was evaluated against standard organisms and clinical isolates. Notably, the extracts, utilising different solvent systems, displayed significant activity against both standard organisms and clinical isolates. This information suggests that *Combretaceae* family may be particularly promising as sources for antimicrobial extracts.

Various morphological parts of *Terminalia leiocarpa* (DC.) Baill. and *Terminalia avicennioides*, both from the *Combretaceae* family, have been investigated for their antimicrobial activity (Aliyu et al., 2018; Aliyu-Amoo and Isa, 2023), but for the two plants, there is a paucity of knowledge on antimicrobial activity on their roots bark, and so is with their bioactive compounds. In addition, there is no proper study in which the antimicrobial activity of these plants has been demonstrated against methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii*.

This study focused on screening phytochemical constituents and evaluating the *in vitro* antimicrobial activity of the methanolic extracts of *Terminalia leiocarpa* and *Terminalia avicennioides* root barks against clinical isolates of methicillin-resistant *Staphylococcus aureus*, methicillin-susceptible *Staphylococcus aureus*,

Acinetobacter baumannii, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*.

MATERIAL AND METHODS

Equipment, reagents, and chemicals

Equipment used were Gas chromatography-Mass spectrometry (Agilent QP2010 PLUS SHIMADZU, JAPAN), rotary evaporator (BIOBASE, China), beakers, conical flasks, electronic balance, filter papers (No.1 Whatman), measuring cylinders, micropipettes (Gilson France), retort stand, cotton wools, hand gloves, bottles, capillary tubes, oven (Heraeus, Buchschlag-Frankfurt, Germany), Petri dishes, aluminum foil, droppers, sterile containers, metallic spatula, sample bottles, water bath (Gallenkamp, England), cooking gas, Bunsen burner, swab stick, inoculation loop, justers, sterile tips, 96-well microplates (Sterilin, Teddington Middlesex England), Eppendorf tubes, incubator (Baird and Tatlock Limited, England), separatory funnel, 2 mL cryovials bottles, autoclave (Yamato, China), cork borer, microscope (Olympus, China), spectrophotometer (Perkin Elmer, USA).

Reagents and chemicals used were dichloromethane (DCM), methanol, n-hexane, distilled water, normal saline, peptone water, MacConkey agar, Mueller Hinton agar, Mueller Hinton broth, Dragen-dorff's reagent, Mayer's reagent, Wagner's reagent, ammonium sulfate, dilute ammonia, ferric chloride solution, sulphuric acid, sulfate, 20% potassium hydroxide sodium, chloroform, acetic anhydride, 0.1% ferric chloride, dimethyl sulphoxide, Folin-Ciocalteu phenol reagent, ethyl acetate, gallic acid, atropine solution, sodium carbonate, hydrochloric acid, quercetin solution, and sodium hydroxide were purchased from HIMEDIA, India.

Plants material

The matured root bark of *Terminalia leiocarpa* and *Terminalia avicennioides* were collected from the wild Obbo-Ile, Ekiti local government, Kwara state, Nigeria in April 2023 (Lat 8° 4' 59" N; Long 5° 19' 59" E) (Lat/Long (dec) 8.08333,5.33333).

The identification and authentication of the plants were done by the Forest Research Institute of Nigeria, Ibadan (FRIN), with the voucher specimen numbers assigned FHI-109925 and FHI-112835 for *Terminalia leiocarpa* and *Terminalia avicennioides*, respectively.

Preparation of plant extracts

The roots barks of each plant were air-dried and after dried in the dryer machine at 50°C for 72 h until completely dried. The dried materials were then pulverized using a local milling machine, and the pow-

dered samples obtained were stored in an air-tight container, away from light until further use.

Extraction procedures

About 900 g of each powdered plant material of *Terminalia leiocarpa* and *Terminalia avicennioides* was placed into different glass containers for maceration with methanol (4.5 L). The containers were left at room temperature for 72 h and were time to time shaken. After that, the samples were filtered using a sieve cloth and then filtered with Whatman No.1 filter paper for complete separation of liquid from other big-size particles. The volume of methanol filtrate was concentrated at a reduced pressure of 100 mbar at 40°C using a rotary evaporator. After that, the percentage yield of each plant extract was kept at -5°C in screw-capped small bottles for subsequent use. The extraction yield was a metric for how effectively a solvent extracts a particular component from the starting material. Then, the crude extract yield as a percentage was calculated.

Phytochemical screening

The qualitative phytochemical screening procedures for various classes of secondary metabolites in different extracts were carried out based on the standard procedures employed by Ayoola et al. (2008) and Trease and Evans (1996) to test for steroids, anthraquinone, terpenoids, flavonoids, saponins, alkaloids, and cardiac glycosides.

Test for steroids (Liebermann-Burchard)

A volume of 1 mL of concentrated sulfuric acid was added along the test tube's sides after 0.5 mg of the extract was dissolved in acetic anhydride, heated to boiling, and cooled. The presence of steroids was shown by the formation of green colour.

Test for anthraquinones

Boiling 0.5 g of the extract with 10 mL of sulphuric acid (H₂SO₄), followed by hot filtration. The resulting filtrate was then subjected to shaking with 5 mL of chloroform. The chloroform layer was separated and transferred to a separate test tube, where 1 mL of diluted ammonia was added. Colour changes in the resulting solution were observed.

Test for terpenoids (Salkowski test)

A 2 mL of chloroform was added to 0.5 g of each extract. A layer was precisely formed by adding 3 mL of concentrated H₂SO₄. The presence of terpenoids was indicated by the appearance of a reddish-brown colour at the interface.

Test for flavonoids

Three methods were used to test for flavonoids. First, a portion of the extract's aqueous filtrate was mixed with 5 mL of diluted ammonia. 1 mL of concentrated sulfuric acid was added. The presence of flavonoids is indicated by a yellow colouration that disappears on standing. Secondly, a small amount of the filtrate was mixed with a few drops of 1% aluminum solution. Flavonoids were indicated by a yellow colouring. Third, 10 mL of ethyl acetate was used to heat a portion of the extract for three minutes over a steam bath. After the mixture was filtered, 1 mL of diluted ammonia solution was mixed with 4 mL of the filtrate. Flavonoids were indicated by a yellow colouring.

Test for saponins

A 5 mL of distilled water was added to 0.5 g of extract in a test tube. The mixture was vigorously shaken, and the appearance of a consistent froth was monitored. After mixing the frothing with three drops of olive oil and giving it a good shake, the formation of an emulsion was observed.

Test for phenolic compounds and tannins

A quantity of 0.5 g of the extract was subjected to heating in a test tube filled with 10 mL of water, followed by filtration. Subsequently, a few drops of 0.1% ferric chloride were incorporated into the mixture, and the colour of the mixture was observed to be brownish-green or blue-black.

Test for alkaloids

After boiling, filtering, and diluting 0.5 g of extract with acid alcohol to reach a volume of 10 mL, 2 mL of diluted ammonia were added to 5 mL of the filtrate. The alkaloidal base was then extracted by gently shaking the mixture with 5 mL of chloroform. 10 mL of acetic acid were used to remove the chloroform layer. This was utilized in the following ways, separated into three portions, each containing 2 mL of the filtrate:

- i. To the first portion, 2-3 drops of Dragendorff's reagent (a solution of bismuth iodide in potassium iodide) were added and shaken. The presence of alkaloids was indicated by a brick-red precipitate.
- ii. To the second portion, 2 drops of Wagner's reagent (a solution of iodine in potassium iodide) were added and swirled for a few seconds. The presence of alkaloids was indicated by a brownish-red precipitate.
- iii. Mayer's reagent, a mercury iodide in potassium iodide solution, was added to the third portion and shaken. Two drops were added. A creamy or dirty white precipitate indicated the presence of alkaloids.

Test for cardiac glycosides (Keller-Killiani test)

Upon combining a 0.5 g extract with 5 mL of water, 2 mL of glacial acetic acid was introduced, and a ferric chloride solution drop was added to the mixture. 1 mL of concentrated sulfuric acid was added underneath this layer. The presence of a deoxy sugar, a feature common to cardenolides, was shown by a brown ring at the interface. While a greenish ring may grow in the acetic acid layer just above the brown ring and progressively expand across this layer, a violet ring may appear below the brown ring.

The quantitative phytochemical analysis was conducted using established phytochemical methods as outlined by Hussein et al. (2011) and Tambe and Bhambar (2014).

Alkaloids, tannins, phenols, flavonoids, saponins, and terpenoids were quantitatively determined using the standard analytical method, which involved measuring the absorbance of each test and standard solutions against a blank using the UV/Visible spectrophotometer and expressing the content of the metabolites as follows: Alkaloid content in mg of AE/g of extract (Ajanal et al., 2012); tannin and total phenol contents were expressed as mg of GAE/g of extract (Afify et al., 2012; Ghasemzadeh et al., 2010; Singh et al., 2012; Xu and Chang, 2008); flavonoid content was expressed as mg of QE/g of extract (Hidayati et al., 2017; Kaviarasan et al., 2007; Xu and Chang, 2008). Standard curve equations obtained from the calibration graph were used to quantify saponins and terpenoids. The results were presented as % composition of the mean $\pm$ standard error, as shown in Fig. 1.

The calculated yield obtained from the standard calibration curve for each secondary metabolite (tannin, flavonoids, and phenol) was used to calculate the percentage yield with respect to the amount of extract used following formula [1].

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant sample}} \times 100 \quad [1]$$

For alkaloids, terpenoids, and saponins, where the extraction method was used to quantify the secondary metabolites, their percentage yields were calculated based on the yield obtained with respect to the amount of extract used.

Liquid-liquid partitioning of methanol extract of *Terminalia leiocarpa*

About 100 g of the crude methanol extract of *Terminalia leiocarpa* was dissolved in 650 mL of methanol and put into the separating funnel. In order to achieve the desired fractionation, the chosen solvent is added in increasing polarity order, beginning with the least polar solvent, n-hexane, and ending with the most polar solvent, water. The solvent was gently mixed with an equivalent volume of solvent to ensure a good mixture and adequate interaction of the two solvents; after that, using a flashlight, we identified the separating layer, and then we started collecting till all the layers of interest were pulled out. This procedure was repeated till the fresh solvent that was being added became clear. The procedure was repeated for N-hexane, DCM, and water, and the different fractions were collected, labelled, and then poured inside bowls and was concentrated using a rotary evaporator (Sasidharan et al., 2011).

Test organisms

Clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, methicillin-susceptible *Staphylococcus aureus* (MSSA), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* were obtained from the Department of Medical Microbiology and Parasitology of University College Hospital, Ibadan, Nigeria.

Determination of antimicrobial activity of *Terminalia leiocarpa* and *Terminalia avicennioides*

The agar well diffusion method was used to investigate the antimicrobial activities of 500 mg/mL of each extract. Each Mueller Hinton (susceptibility) agar plate used had six wells, corresponding to the number of samples to be tested. The methanol crude extracts for the two plants were tested, and the most active crude extracts were fractionated; the fractions used for this assay were n-hexane, dichloromethane, aqueous+ methanol, and methanol. The determination of isolates' susceptibility was conducted to identify the most effective fraction exhibiting antimicrobial properties. Furthermore, all fractions which showed a zone of inhibition were used to determine MIC and MBC (Mills-Robertson et al., 2012).

Mueller Hinton agar was prepared in a 25 mm Petri dish, featuring evenly spaced wells around the periphery and a singular central well, with a sterilised cork borer by employing a Bunsen burner flame to guarantee the absence of microbial contamination. Space was intentionally reserved for the inclusion of two standard antibiotic discs serving as controls, and appropriate labeling was applied to the wells (Mills-Robertson et al., 2012).

The isolate was prepared in peptone water and diluted to 0.5 MacFarland standard. A sterile cotton swab was immersed in the broth, excess liquid was gently drained, and the swab was utilized to evenly inoculate the surface of the agar plate by swabbing. After allowing approximately thirty minutes for the media surface to dry, 100 μ L of distinct pre-determined extract concentrations were meticulously added to the wells. Depending on the inoculated organism, triplicate samples were prepared for each organism to verify the inhibition zones caused by the extracts and standard antibiotic discs.

The plates were either incubated for 18 to 24 h or overnight at 37°C. After the incubation phase, the diameter of the clear zone around the antibiotic disc and wells was gauged using a ruler or a divider against a ruler. The three distinct readings of the inhibition zones on the respective plates were documented and employed in the final analysis to determine the mean and standard deviation.

Determination of Minimum Inhibitory Concentration (MIC)

The antibacterial activity of the agar dilution method and disc diffusion method were both put through the serial dilution procedure to measure the extracts MIC in line with the CLSI (2015). When the fractions of the same plant were tested for susceptibility, the extract of crude methanol showed a zone. The fraction with the best activity was then used in subsequent tests to establish the MIC. Plant extract values in relation to test organisms were established in order to evaluate the range of MIC values. As a result, the extract was diluted serially to produce the following specified concentrations: 31.25, 62.5, 125, 250, and 500 mg/mL.

The bacterial suspension was created in accordance with Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2015) by dilution of the 0.5 MacFarland standard turbidity equivalents to bacterial suspensions in the Muller Hinton Broth (MHB) at a ratio of 1:150, yielding a bacterial concentration of approximately 1×10^6 CFU/mL. Briefly, each test tube was added with 1 mL of a diluted bacterial sample.

After being infected, the test tubes contained approximately 10^5 CFU/mL. Both the test tube containing only MHB and the test tube containing broth inoculum (without extract) were utilized as a negative and positive control. The cultures were then incubated for 18 to 24 hours at 37°C. At the end of the incubation phase, turbidity was assessed to indicate bacterial growth; a clear solution signified the absence of growth. To quantify turbidity, absorbance measure-

ments were taken using a spectrophotometer set at 600 nm, allowing for a precise correlation between absorbance values and bacterial concentration. The extract concentration at which there was no visible growth, as indicated by an absorbance reading comparable to the negative control, was defined as the MIC. The extract concentration at which there was no visible growth was known as the MIC. Each bacterium underwent each experiment three times in order to determine the average value.

Determination of Minimum Bactericidal Concentration (MBC)

After 18 to 24 hours of incubation at 37°C, the antimicrobial agent's lowest effective concentration killed 99.9% of the final inoculum, known as the minimum bactericidal concentration (MBC). According to a number of studies, it was computed by aseptically subculturing the contents of test tubes from the MIC findings for each bacterium to antimicrobial-free agar (Nikolić et al., 2014). Using a sterile wire loop and each test tube from each plate that had a concentration of the test material higher than the MIC value, MHA was aseptically streaked. At 37°C, the incubation period lasted for 24 hours. The extract with the minimal concentration and no bacterial growth during incubation was the MBC.

Gas chromatography-mass spectrometry analysis of the DCM fraction

This GC-MS analysis of the DCM fraction was done by the methodology outlined by Karimi and Jaafar (2011) and Neelamegam and Ezhilan (2012) using GCMS-QP2010 PLUS SHIMADZU Perkin Elmer Elite - 5 capillary columns (30 m in length and 0.25 mm in diameter, with a film thickness of 0.25 mm), which composed primarily of 95% dimethyl polysiloxane. Helium served as the carrier gas at a flow rate of 0.5 mL/min, with a sample injection volume of 1 µL. The inlet temperature was consistently maintained at 250°C. The oven temperature was programmed to start at 80°C for 4 min, followed by a gradual increase to 200°C, and then further programmed to reach 280°C at a rate of 20°C, concluding with a 5-minute hold. The total runtime of the analysis was 25 minutes, and the MS transfer line was held at a temperature of 200°C. The source temperature was maintained at 180°C. Total Ion Count (TIC) was employed in the analysis of GC-MS data to identify and quantify compounds with electron impact ionization at 70 eV. A database including the spectrums of known components kept in the GC-MS library was compared to the component spectrums.

Statistical analysis

Analysis of the data was performed using Microsoft Excel and SPSS version 20. The mean and standard deviation were used to express experimental data, and a p-value of 0.05 was used to determine statistical significance. One-way analysis of variance was utilised to compare the statistical difference in the mean zone of inhibition of dichloromethane, methanol, and aqueous+MeOH fractions of the root bark of *T. leiocarpa* and *T. avicennioides* for clinical microorganisms.

RESULTS

Phytochemical screening

Table 1 shows the qualitative phytochemical screening of the root bark extracts of *T. leiocarpa* (TL) and *T. avicennioides* (TA) and the dichloromethane fraction (DCM_f) of *T. leiocarpa*, which reveals the presence of saponins, tannins, flavonoids, cardiac glycosides, terpenoids, steroids, alkaloids, and phenols in all the three extracts, except for cardiac glycosides which is absent in DCM_f. The methanol extracts of *T. leiocarpa* and *T. avicennioides* yielded 12.92 and 12.01%, respectively. The DCM fraction gave a yield of 19.72%. Fig. 1 represents the quantitative analysis of the phytochemical composition of TL, TA and DCM_f in % w/w as follows: 0.6 of alkaloids, 0.9 of flavonoids, 2.21 of saponins, 1 of tannins, 0.5 of terpenoids and 0.62 of total phenols, for TL; 1.7 of alkaloids, 1.4 of flavonoids, 0.65 of saponins, 1.14 of tannins, 0.65 of terpenoids and 0.64 of total phenols, for TA; 2.45 of alkaloids, 4.27 of flavonoids, 0.35 of saponins, 1.06 of tannins, 0.8 of terpenoids and 0.65 of total phenols for DCM.

Antimicrobial activity

The antimicrobial assay presented in Table 2 illustrates the inhibitory zone recorded (mm) in mean ± SD of methanol crude extract of *T. leiocarpa* (TL) and *T. avicennioides* (TA) and *T. leiocarpa* fractions as well as control standard antibiotics against six clinical isolates and five control organisms.

From the result, the methanol crude extract of the two plants, as well as fractions, have shown antimicrobial activity against all the selected organisms. For the methanol crude extract of *T. leiocarpa*, the clinical isolates *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were shown to be more susceptible, both at 21 ± 0.8 mm, and for *T. avicennioides*, the *Acinetobacter baumannii* was the most susceptible (24 ± 0.5 mm) among the clinical isolates.

Table 1. Qualitative phytochemical screening of crude root bark extracts of *T. leiocarpa*, *T. avicennioides* and DCM_f.

Secondary metabolites	TL	TA	DCM _f
Saponins	++	++	++
Tannins	++	++	++
Flavonoids	++	++	++
Cardiac glycosides	++	++	-
Antraquinones	++	+	+
Terpenoids	+	+	++
Steroids	+	+	+
Alkaloids	+	++	++
Phenols	++	++	++

TL: *Terminalia leiocarpa*; TA: *Terminalia avicennioides*; DCM_f: Dichloromethane fraction; (-): absent; (+): present; (++) abundant.

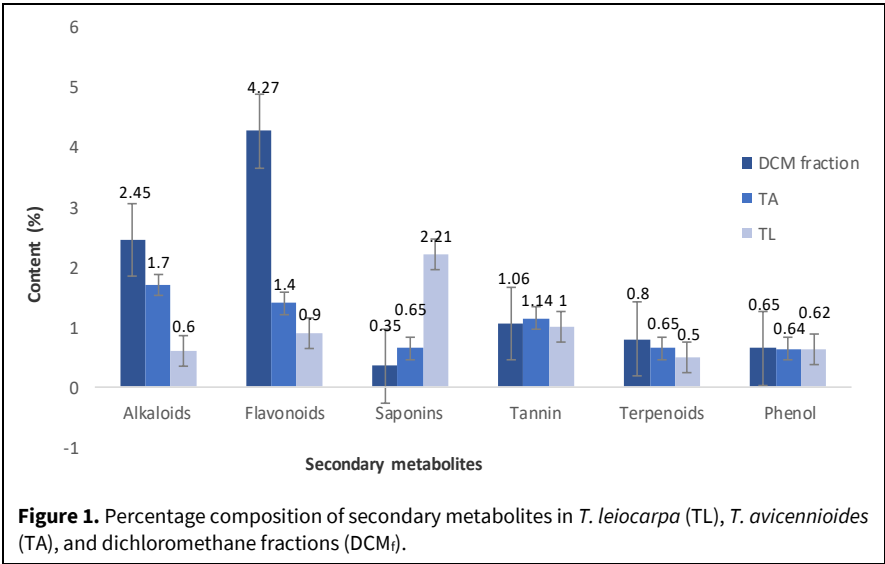


Figure 1. Percentage composition of secondary metabolites in *T. leiocarpa* (TL), *T. avicennioides* (TA), and dichloromethane fractions (DCM_f).

Pseudomonas aeruginosa was the most susceptible (25 ± 0.5 mm) and (22 ± 0.8 mm) among the control organisms for both TL and TA, respectively. In the *T. leiocarpa* fractions, DCM fraction showed the strongest antimicrobial activity with *Acinetobacter baumannii* (27 ± 1.0 mm), which is the most susceptible clinical isolates at 500 mg/mL, among the control organisms, *Pseudomonas aeruginosa* is the most susceptible to MeOH fraction (27 ± 0.5 mm) at 500 mg/mL and all the organisms were least susceptible to the Aq+MeOH fraction across all the five concentrations (500-31.25 mg/mL). However, all the clinical isolates were resistant to the standard drugs (ceftriaxone and amoxicillin) except for MSSA (methicillin-susceptible *Staphylococcus aureus*) and MRSA (methicillin-resistant *Staphylococcus aureus*), which are susceptible to amoxicillin with 30 ± 0.0 mm and 25 ± 0.6 mm, respectively.

Minimum inhibitory concentration (MIC)

The determination of the MIC for fractions of *T. leiocarpa* revealed the lowest concentration, which inhibits the visible growth of microorganisms. DCM fraction revealed a MIC of 31.25-125 mg/mL for clinical isolates and 125-250 mg/mL for control organisms, while methanol fraction 62.5-250 mg/mL for clinical isolates and 62.5-250 mg/mL for control organisms. Aqueous fraction inhibited at a higher concentration of 250-500 mg/mL for both clinical isolates and control organisms. The MIC for the reference drug ceftriaxone on *E. coli*, *K. pneumoniae*, and *A. baumannii* was 12.5 µg, *P. aeruginosa* was 50 µg, *S. aureus* was 6.25 µg, and MRSA was 25 µg. The MIC for the reference drug amoxicillin/clavulanic acid on *E. coli* was 50 µg, *K. pneumonia* was 25 µg, *S. aureus* was 12.5 µg, *A. baumannii* was 25 µg, and MRSA was 6.25 µg, *P. aeruginosa* showed no MIC because it was resistance to amoxicillin/clavulanic acid (Table 3).

Table 2. Zone of inhibition of crude MeOH extracts of the two plants and the fractions of *Terminalia leiocarpa*.

Clinical isolates/ control organisms	Zone of inhibition produced by different concentrations of methanol extracts/ fractions/ standard antibiotics in mm (SD)																		
	Methanol crude extracts		<i>T. leiocarpa</i> fractions (mg/mL)															Standard antibiotics	
	TL	TA	Aq+MeOH					DCM					MeOH					CRO	AMC
			500	250	125	62.5	31.25	500	250	125	62.5	31.25	500	250	125	62.5	31.25		
<i>P. aeruginosa</i>	22 ± 0.5	21 ± 0.8	10 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	23 ± 0.6	20 ± 0.0	16 ± 0.0	11 ± 0.6	0 ± 0.0	24 ± 0.0	22 ± 1.2	16 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
<i>E. coli</i>	19 ± 0.5	10 ± 0.0	12 ± 0.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	22 ± 0.5	19 ± 1.2	16 ± 1.0	14 ± 0.0	0 ± 0.0	17 ± 0.0	14 ± 0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
<i>K. pneumoniae</i>	15 ± 0.9	18 ± 0.5	9.3 ± 0.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	24 ± 0.0	20 ± 0.6	18 ± 0.0	15 ± 0.0	10 ± 1.2	20 ± 0.5	10 ± 0.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
MSSA	20 ± 0	17 ± 0.5	11 ± 0.5	9 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	21 ± 0.0	14 ± 0.5	11 ± 1.0	0 ± 0.0	0 ± 0.0	20 ± 0.5	18 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	30 ± 0.0
<i>A. baumannii</i>	24 ± 0.5	21 ± 0.8	10 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	27 ± 1.0	22 ± 0.0	18 ± 0.6	0 ± 0.0	0 ± 0.0	24 ± 0.0	21 ± 0.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
MRSA	18 ± 0.8	14 ± 0.5	10 ± 0.5	8 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	22 ± 0.0	20 ± 0.0	16 ± 1.2	10 ± 0.6	0 ± 0.0	18 ± 0.0	13 ± 0.6	10 ± 0.0	8 ± 0.0	0 ± 0.0	0 ± 0.0	25 ± 0.6
<i>P. aeruginosa</i> ATCC 29523	25 ± 0.5	22 ± 0.8	12 ± 0.5	10 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	25 ± 0.0	15 ± 0.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	27 ± 0.5	25 ± 1.0	23 ± 0.0	20 ± 0.0	0 ± 0.0	ND	ND
<i>E. coli</i> ETCC 35218	21.3 ± 1.5	19 ± 0.0	14 ± 0.5	12 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	23 ± 0.5	11 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	16 ± 0.5	14 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	ND	ND
<i>K. pneumoniae</i> ATCC 27853	20 ± 0	20 ± 1.0	12 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	19 ± 0.6	13 ± 0.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	14 ± 1.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	ND	ND
<i>S. aureus</i> ATCC 700603	22 ± 3.5	19 ± 0.0	14 ± 0.0	11 ± 0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	20 ± 0.0	15 ± 0.0	10 ± 0.0	0 ± 0.0	0 ± 0.0	21 ± 0.5	16 ± 0.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	ND	ND
<i>A. baumannii</i> NCTC 7363	22 ± 0	20 ± 0.6	13 ± 0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	25 ± 1.2	18 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	22 ± 0.6	15 ± 1.2	0 ± 0.0	0 ± 0.0	0 ± 0.0	ND	ND

Data represent mean ± SD (n = 3). TL: *Terminalia leiocarpa*; TA: *Terminalia avicennioides*; CRO: Ceftriaxone; AMC: Amoxicillin/clavulanic acid; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*; MSSA: Methicillin-susceptible *Staphylococcus aureus*; *A. baumannii*: *Acinetobacter baumannii*; MRSA: Methicillin-resistant *Staphylococcus aureus*; ND: not determined.

Table 3. Minimum inhibitory concentration (MIC) of *T. leiocarpa* fractions on selected isolates.

N°	Isolates	Aq+MeOH (mg/mL)	DCM (mg/mL)	MeOH (mg/mL)	Standard antibiotics	
					CRO (µg/mL)	AMC (µg/mL)
1.	MSSA	250	125.00	250.0	6.25	12.50
2.	MRSA	250	62.50	62.5	25.00	6.25
3.	<i>Escherichia coli</i>	500	62.50	250.0	12.50	50.00
4.	<i>Pseudomonas aeruginosa</i>	500	62.50	125.0	50.00	R
5.	<i>Klebsiella pneumoniae</i>	500	31.25	250.0	12.50	25.00
6.	<i>Acinetobacter baumannii</i>	500	125.00	250.0	12.50	25.00
7.	<i>Staphylococcus aureus</i> ATCC 29523	250	125.00	250.0	6.25	6.25
8.	<i>Escherichia coli</i> ETCC 35218	250	250.00	250.0	6.25	6.25
9.	<i>Pseudomonas aeruginosa</i> ATCC 27853	250	250.00	62.5	50.00	50.00
10.	<i>Klebsiella pneumoniae</i> ATCC 700603	500	125.00	250.0	12.50	25.00
11.	<i>Acinetobacter baumannii</i> NCTC 7363	250	250.00	250.0	6.25	12.50

MSSA: Methicillin-susceptible *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphylococcus aureus*; Aq+MeOH: Aqueous methanol; DCM: Dichloromethane; MeOH: Methanol; CRO: Ceftriaxone; AMC: Amoxicillin/clavulanic acid; R: Resistant.

Minimum bactericidal concentration (MBC)

The concentration range required to inhibit the physical growth and kill the organism was determined as 15.63-125 mg/mL, 31.25-125 mg/mL, and 125-250 mg/mL for DCM, methanol, and aqueous methanol, respectively. The MBC is 100 µg for all the organisms that are sensitive to both reference drugs ceftriaxone (CRO) and amoxicillin/clavulanic acid (AMC) (Table 4).

Gas chromatography-mass spectrometry analysis of the DCM fraction

The mass spectrophotometer determines the nature and structure of the chemicals by analyzing the eluted samples at various times. The GC-MS of the DCM fraction of the root bark of *Terminalia leiocarpa* showed the presence of compounds in the class of hydrocarbons, ketones, esters, cyclic compounds, and fatty acids (Table 5). Peaks relating to the retention time were represented in the chromatogram, as illustrated in Fig. 2.

DISCUSSION

Phytochemical screening confirms the presence of secondary metabolites in plants at the collection time (Wachira et al., 2024). The presence of these secondary metabolites can justify the antimicrobial activity and the folkloric uses for the management of various illnesses such as infections, malaria, jaundice, and an alternative as a pain killer (Kuisseu et al., 2021). As shown in Table 1, qualitatively, all three extracts in

this study; *T. leiocarpa* (TL) and *T. avicennnioides* (TA) and the dichloromethane fraction (DCM_f) of *T. leiocarpa* were tested to contain saponins, tannins, flavonoids, cardiac glycosides, terpenoids, steroids, alkaloids, and phenols except for cardiac glycosides which is absent in DCM_f. Furthermore, as shown in Fig. 1, quantitative analysis was carried out to ascertain the exact amount of secondary metabolites in % w/w, with saponins (2.21% w/w), alkaloids (1.7% w/w), and flavonoids (4.27% w/w) as the most abundant secondary metabolites in the root barks extracts of *T. leiocarpa*, *T. avicennnioides* and dichloromethane (DCM) of *T. leiocarpa* respectively. This quantification of phytochemicals is helpful when extracting, purifying, and identifying the bioactive compounds for drug discovery to aid human use (Santhi and Sengottuvel, 2016). This result agrees with that of Salau et al. (2013), which also revealed the presence of alkaloids, tannins, flavonoids, and phenolics in root bars of *T. leiocarpa* and *T. avicennnioides*. Overall, in all the extracts, the DCM fraction of *T. leiocarpa* has the highest amount of alkaloids (2.45% w/w), flavonoids (4.27% w/w), terpenoids (0.8% w/w), and phenols (0.65% w/w).

The abundance of these phytochemicals could be responsible for their high antibacterial activity amongst other fractions. In addition, Wintola and Afolayan (2015) established the biological activity of phytochemical compounds like alkaloids, saponins, tannins, flavonoids, and steroids in promoting the antimicrobial efficacy of plants.

Table 4. Minimum bactericidal concentration (MBC) of *T. leiocarpa* fractions on selected isolates.

N°	Isolates	Aq+MeOH (mg/mL)	DCM (mg/mL)	MeOH (mg/mL)	Standard antibiotics	
					CRO (µg/mL)	AMC (µg/mL)
1.	MSSA	125	15.63	125.00	100	100
2.	MRSA	125	31.25	31.25	100	100
3.	<i>Escherichia coli</i>	250	125.00	125.00	100	100
4..	<i>Pseudomonas aeruginosa</i>	250	15.63	62.50	100	R
5.	<i>Klebsiella pneumoniae</i>	250	31.25	125.00	100	100
6.	<i>Acinetobacter baumannii</i>	250	15.63	125.00	100	100
7.	<i>Staphylococcus aureus</i> ATCC 29523	250	31.25	125.00	100	100
8.	<i>Escherichia coli</i> ETCC 35218	125	125.00	125.00	100	100
9.	<i>Pseudomonas aeruginosa</i> ATCC 27853	125	31.25	31.25	100	100
10.	<i>Klebsiella pneumoniae</i> ATCC 700603	250	31.25	125.00	100	100
11.	<i>Acinetobacter baumannii</i> NCTC 7363	125	15.63	62.50	100	100

MSSA: Methicillin-susceptible *Staphylococcus aureus*; MRSA: Methicillin-resistant *Staphylococcus aureus*; Aq+MeOH: Aqueous methanol; DCM: Dichloromethane; MeOH: Methanol; CRO: Ceftriaxone; AMC: Amoxicillin/clavulanic acid; R: Resistant.

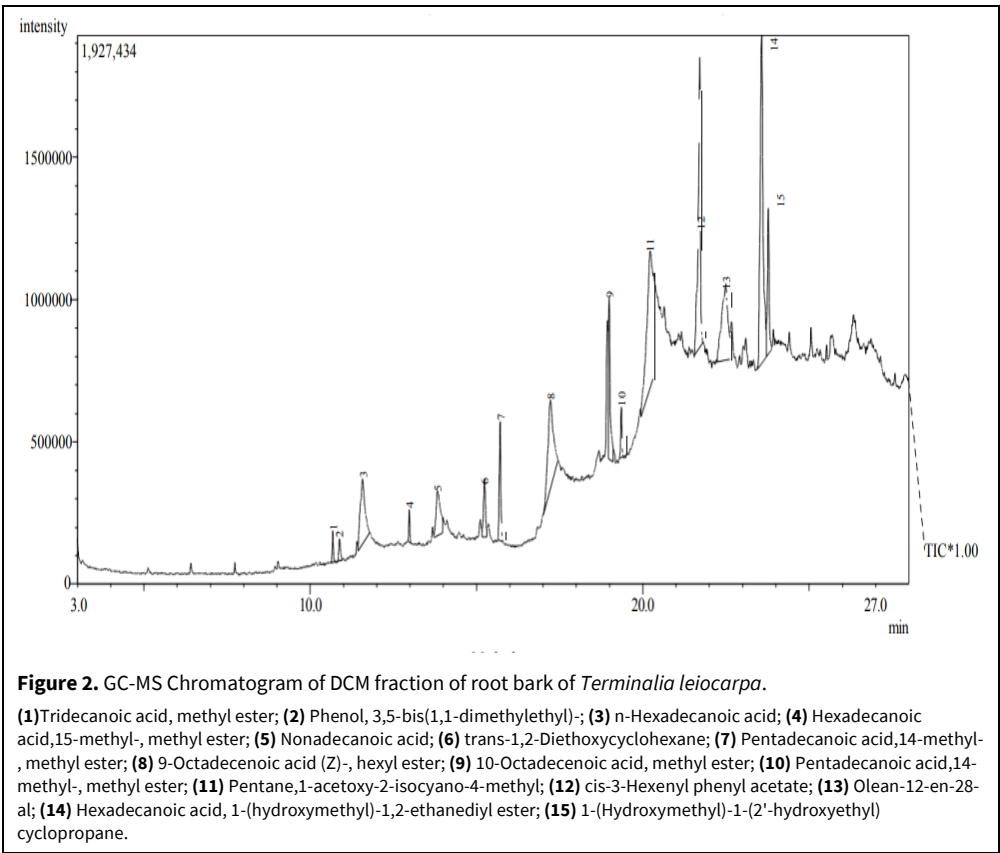


Table 5. List of the 15 compounds present in the DCM fraction of root bark of *Terminalia leiocarpa*.

Peak	Retention time	Area%	Name of the compounds	Molecular weight	Molecular formula
1	10.678	0.69	Tridecanoic acid, methyl ester	228	C ₁₄ H ₂₈ O ₂
2	10.877	0.72	Phenol, 3,5-bis(1,1-dimethylethyl)-	206	C ₁₄ H ₂₂ O
3	11.571	6.39	n-Hexadecanoic acid	256	C ₁₆ H ₃₂ O ₂
4	12.975	0.70	Hexadecanoic acid,15-methyl-, methyl ester	284	C ₁₈ H ₃₆ O ₂
5	13.832	3.99	Nonadecanoic acid	298	C ₁₉ H ₃₈ O ₂
6	15.237	2.44	trans-1,2-Diethoxycyclohexane	172	C ₁₀ H ₂₀ O ₂
7	15.707	3.77	Pentadecanoic acid,14-methyl-, methyl ester	270	C ₁₇ H ₃₄ O ₂
8	17.223	10.18	9-Octadecenoic acid(Z)-, hexyl ester	366	C ₂₄ H ₄₆ O ₂
9	18.986	5.96	10-Octadecenoic acid, methyl ester	296	C ₁₉ H ₃₆ O ₂
10	19.353	1.31	Pentadecanoic acid,14-methyl-, methyl ester	270	C ₁₇ H ₃₄ O ₂
11	20.217	16.38	Pentane,1-acetoxy-2-isocyano-4-methyl	169	C ₉ H ₁₅ NO ₂
12	21.706	13.10	cis-3-Hexenyl phenyl acetate	218	C ₁₄ H ₁₈ O ₂
13	22.491	9.67	Olean-12-en-28-al	424	C ₃₀ H ₄₈ O
14	23.571	19.69	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	568	C ₃₅ H ₆₈ O ₅
15	23.771	5.01	1-(Hydroxymethyl)-1-(2'-hydroxyethyl) cyclopropane	116	C ₆ H ₁₂ O ₂

The antimicrobial activity overall results, as shown in Table 2, revealed that both gram-positive (*S. aureus* ATCC 700603) and gram-negative (*P. aeruginosa* ATCC 29523) bacteria are susceptible to *T. leiocarpa* and *T. avicennnioides* extracts with the higher zone of inhibition against the isolates exhibited by *T. leiocarpa*; therefore, making *T. leiocarpa* the most active extract. These results are similar to a study done by Chomini et al. (2021) whereby ethanolic leaf extract of *T. leiocarpa* inhibited the growth of *Escherichia coli* and *Salmonella typhimurium*. Hence, the crude extract of *T. leiocarpa* was fractionated, and an antimicrobial assay was carried out on its fractions.

The aqueous-methanol, DCM, and methanol fractions showed activity against all the isolates, but the antimicrobial activity decreased as concentration decreased from 500-31.25 mg/mL, as reported in Table 2. Except for *P. aeruginosa*, *P. aeruginosa* ATCC 29523, and *S. aureus* ATCC 700603, which were more susceptible to the methanol fraction in the *clinical* isolates and control organisms, DCMf showed the highest inhibitory activity against all the other eight clinical isolates and control organisms, while the aqueous methanol fraction showed the least zone of inhibition for all the organisms as revealed in Table 2.

The determination of the minimum inhibitory concentration (MIC) for fractions of *T. leiocarpa* revealed the lowest concentration that inhibits the visible growth of microorganisms, as shown in Table 3. The DCM fraction gave MIC of 31.25-125 mg/mL for clinical

isolates and 125-250 mg/mL for control organisms, while methanol gave 62.5-250 mg/mL for clinical isolates and 62.5-250 mg/mL for control organisms. Aqueous methanol was inhibited at a higher concentration range of 250-500 mg/mL for both clinical isolates and control organisms. The lower the MIC value, the higher the antimicrobial activity (Cushnie et al., 2020); therefore, amongst the fractions, the DCM fraction demonstrates superior overall antimicrobial efficacy against both clinical isolates and control organisms, with *Klebsiella pneumonia* was most susceptible to the fraction with the lowest MIC value of 31.25 mg/mL. The high antibacterial activity of DCM fraction over others can be attributed to its relatively high composition of alkaloids (2.45% w/w) and flavonoids (4.27% w/w), which are phytoconstituents that have been widely reported for their antimicrobial activity (Odongo et al., 2023).

The concentration range required to inhibit the physical growth and kill more than 99.9% of the bacteria strains is the MBC (Rodríguez-Melcón et al., 2021), which is shown in Table 4 and was determined as 15.63-125 mg/mL, 31.25-125 mg/mL, and 125-250 mg/mL for DCM, methanol, and aqueous methanol, respectively. These results indicate that DCM and methanol fractions demonstrate a superior ability to kill all the organisms at a lower concentration range of (15.63 and 31.25 mg/mL) compared to the aqueous methanol fraction except for *Escherichia coli* (both clinical and standard strain), which was most resistant to both fractions with high MBC values of 125 mg/mL

respectively. This particular observation is in line with the result of Mwangi et al. (2024), whereby crude methanol extract of *Terminalia catappa* had zero MBC against *Escherichia coli*. The positive antibacterial activity of *T. leiocarpa* extract and fractions was not unsurprising, as the *Terminalia* genus is well known for its antibacterial activity. In fact, a study carried out on the leaves of five (5) *Terminalia* spp, *Terminalia alata*, *Terminalia arjuna*, *Terminalia bellerica*, *Terminalia catappa* and *Terminalia chebula*, showed they were effective against tested bacterial strains although at varying degrees (Shinde et al., 2009). Also supporting these results is a similar study carried out by Mann (2012), whereby methanol extract of *T. leiocarpa* stem bark demonstrated antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Bacillus subtilis*.

As in Fig. 2, the GC-MS analysis of the DCM fraction of the root bark of *Terminalia leiocarpa* revealed 15 peaks representing various compounds. Compounds at peak 14 constitute the highest content of the DCM fraction, including n-hexadecanoic acid and 9-octadecenoic acid, which in previous studies were quantitatively analysed for their antimicrobial activity (Pu et al., 2010; Sehim et al., 2023; Shaaban et al., 2021; Siswadi and Saragih, 2021). This proved the highest antimicrobial activity of the DCM fraction. The presence of bioactive compounds supports the ethnomedicinal use of *Terminalia leiocarpa*.

CONCLUSION

This study has demonstrated that methanol extract of root bark of *Terminalia leiocarpa* Baill. and *Terminalia avicennioides* Guill. & Perr. possess varying degrees of antimicrobial activities, which confirms the ethnomedicinal claim in folk medicine. The dichloromethane fraction of *Terminalia leiocarpa* revealed the presence of secondary metabolites believed to be responsible for its highest antimicrobial activity and may be a potential resistance-free template in future antimicrobial drug discovery.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Amou B	Ajayi TO	Dada-Adegbola HO
Concepts or ideas		x	x
Design		x	x
Definition of intellectual content		x	x
Literature search	x		
Experimental studies	x		
Data acquisition	x		
Data analysis		x	x
Statistical analysis	x		
Manuscript preparation	x	x	
Manuscript editing		x	x
Manuscript review	x	x	x

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