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Full Length Research Article

ANTIBACTERIAL ACTIVITIES OF ETHANOL LEAF AND BARK EXTRACTS OF TERMINALIA AVICENNIOIDES AGAINST METHICILLIN RESISTANT STAPHYLOCCOCUS AUREUS

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ABSTRACT
The study was undertaken with the aim to determine the Antibacterial activities of ethanol leaf and bark extracts of *Terminalia avicennioides* against Methicillin resistant *Staphyloccocus aureus* (MRSA). Air-dried leaves and barks of *Terminalia avicennioides,* were powdered and each extracted with 70% ethanol by cold maceration method. Each extract was screened for the presence of some secondary metabolites using qualitative methods. MRSA clinical isolates from infected wounds of patients were reconfirmed using standard microbiological methods. The antibacterial activities of extracts against bacteria were determined by agar well diffusion and broth dilution methods. The results of phytochemical screening of extracts revealed the presence of carbohydrates, alkaloids, tannins, flavonoids, saponins, steroids, triterpens, glycosides and phenols. All the extracts exhibited significant inhibitory effects ($P < 0.05$) against isolates of bacteria at varied concentrations of 100, 50, 25 and 12.5 and 6.25mg/mL and the activity of each extract was found to be concentration dependent. The mean zone of inhibition of the leaf extract against bacteria ranged between 12.52 ± 1.86 mm -17.12 ± 1.89 mm while the mean zone of inhibition of the bark extract ranged between 10.26 ± 1.37 mm - 14.45 ± 1.47 mm. The leaf extract was more effective with MIC and MBC of 6.25mg/mL and 25mg/mL. The results of this study show that the leaf and bark extracts of *T. avicennioides* contain compounds with antibacterial activities against MRSA which provide basis for further studies to isolate, identify and standardize the active compounds for drug development

Keywords: *Terminalia avicennioides,* antibacterial, methicillin resistant *Staphylococcus aureus*

INTRODUCTION

Methicillin-Resistant *Staphylococcus aureus* (MRSA) has long been regarded as a public health problem worldwide causing a wide range of infection including, bacteremia, pneumonia, meningitis, endocarditis, skin and soft tissue, surgical site, urinary tract infection, bone and joint infections and toxic shock syndrome (Motayo *et al.*, 2013). Approximately 94,000 invasive infections are reported yearly in many developed and developing countries (Usman and Syed, 2018).

Wound infection caused by MRSA is a major concern among healthcare practitioners with increased morbidity and its burden on financial resources and the increasing requirement for cost effective management within the healthcare system (Motayo *et al.*, 2013). The expanding bacterial resistance to currently available antibiotics prompted many researchers to look for more potent therapeutic agents from plants (Das and Tiwari, 2010; Tiwari *et al.*,2011; Suhaili *et al*., 2011; Wendakoon *et al.,* 2012; Musa *et al*., 2016)

Terminalia avicennioides commonly called "Indian laurel" and locally referred to as "Baushe" in hausa, belongs to the family Combretaceae. The genus is made of 200-250 species of medium to large flowering trees with thirty (30) species found in the savanna region of Africa and in the northwest vegetation of Nigeria, with the majority occurring in the southern parts of the continent (Mann *et al*., 2008). The plant extract was reported to have some antibacterial effects against many species of bacteria (Musa *et al*., 2016; Mann *et al*., 2008). However, the present study was undertaken to determine the antibacterial effects of ethanol leaf and bark extracts of *Terminalia avicennioides* against Methicillin resistant *Staphylococcus aureus* isolated from infected wounds.

MATERIALS AND METHODS

Collection, Authentication and Preparation of Plant Materials

Terminalia avicennioides plant was collected from Karaukarau village in Zaria Local Government of Kaduna state, Nigeria. The plant was identified and authenticated in the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria with a voucher number (104) deposited at the herbarium section. The fresh plant material was thoroughly washed with water separated in to three portions and air dried at ambient temperature under shade for three weeks. Each dried plant part (leaves and barks) was separately and ground to coarse powder using sterile mortar and pestle, and then put in labeled sterile containers.

Extraction of Plant Materials

Extraction of plant material was done according to the method adopted by Tiwari *et al.* (2011).

Eight hundred grams (800 g) of each powdered plant part was suspended in 100 mL of 70 % ethanol in 250 mL conical flask. The flasks were left to stand for 72 h with constant shaking. At the end of 3 days, each extract was filtered with a muslin cloth and then with Whatman number1 filter paper and put in crucibles. Each filtrate was concentrated in a water bath and evaporated at 40 ^{0}C for two (2) days. Each dried crude extract was kept in a sterile container until required.

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Phytochemical Analysis
The following screening procedure was adapted from Tiwari *et al.* (2011) to ascertain the presence of carbohydrates, cardiac glycosides, saponins, flavonoides, Tannins, alkaloids anthraquinones, phenols, steroids, and triterpenes.

Detection of Carbohydrates: To 5mL of distilled water, 0.2 g of an extract was dissolved and filtered. The filtrate was used to test for the presence of carbohydrates as follows:

Molisch's Test: Filtrate was treated with drops of alcoholic a-naphthol solution in a test tube. The formation or non formation of a violet ring at a junction indicated the presence or absence of carbohydrates.

Benedicts Tests: Filtrate was treated with Benedict's reagent and heated gently. Formation or non formation of orange red precipitate indicated the presence or absence of reducing sugars.

Fehlings Test: Filtrate was hydrolyzed with diluted HCl, neutralized with alkali and heated with fehling's A and B solutions. Formation or non formation of red precipitate indicated the presence or absence of reducing sugars.

Detection of Cardiac glycosides: (Keller-Kiliani Test). Five milliliters (5 ml) of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under layed with 1ml of concentrated sulphuric acid. Formation or non formation of brown ring at the interface,characteristic of cardenolides, indicated the presence or absence of a deoxysugar. A violet ring (may or may not appear below the brown ring, while in the acetic acid layer, a greenish ring may or may not form gradually), indicated the presence or absence of cardiac glycosides

Detection of Saponins

Froth Test: To 1 g of extract, 20 ml of distilled water was added and shaken in a graduated cylinder for 15 minutes. Formation or non formation of 1cm layer of foam indicated the presence or absence of saponins.

Foam Test: Zero point five gram (0.5 g) of extract was shaken with 2 ml of water. Persistent production or non production of foam for 10 minutes indicated the presence or absence of saponins

Detection of Flavonoids

Alkaline Reagent Test: To 2.0 mL of water 0.2 g of extract was dissolved and treated with few drops of sodium hydroxide solution formation of intense yellow colour, which became colourless on addition of dilute acid or non formation ofyellow colour indicated the presence or absence of flavonoid.

Lead Acetate Test: To 2 mL of water 0.2 g of extract was dissolved and treated with 3 drops of lead acetate solution. Formation or non formation of yellow colour precipitate indicated the presence or absence of flavonoids.

Detection of Tannins
To 2.0 mL of water 0.2 g of extract was dissolved. About 1.0 mL of 1 % gelatin solution containing sodium chloride was added. Formation or non formation of a white precipitate indicated the presence or absence of tannins.

Detection of alkaloids

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium mercuric Iodide). Formation or non fomation of a yellow colour precipitate indicated the presence or absence of alkaloids.

Dragendoff's Test: Filtrates were treated with dragendroff's reagent (solution of potassium bismuth iodide). Formationor non formation of a red precipitate indicated the presence or absence of alkaloids.

Detection of Anthraquinones:
Five grams (5.0 g) of each extract was stirred with 10.0 mL of aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5 mL of benzene. The benzene layer was then separated and 10% ammonia solution was added to half of its volume. Formation or non formation of a pink red or violet colouration in the ammonia phase (lower layer) indicated the presence or absence of anthraquinonederviatives in the extract

Detection of Phenols

Ferric Chloride Test
To 2.0 mL of water 0.2 g of extract was dissolved and treated with 3 drops of ferric chloride solution. Formation or non formtion of bluish black colour indicated the presence or absence of phenols.

Detection of Phytosterols and Triterpenes

Salkowski's Test. To 2.0 mL of water 0.2 g of extract was dissolved and treated with chloroform and filtered. The filtrate was treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance or non appearance of golden yellow colour indicated the presence or absence of triterpenes.

LibermannBurchard'sTest. To 2.0 mL of water 0.2 g of extract was dissolved and treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was added. Formation or non formation of brown ring at the junction indicated the presence or absence of phytosterols

Collection of Clinical Isolates of Bacteria
Methicillin Resistant *Staphyloccocus aureus* (MRSA) clinical isolates were collected from the Department of Microbiology Laboratory, Kaduna State University for bacteriological analysis.

Reconfirmation of MRSA Clinical Isolates
Isolates of MRSA were inoculated on nutrient broth and allowed to stand for 6 hours. A loop-full of the medium was inoculated on Mannitol Salt Agar (MSA) and incubated at 37^0C for 24 h. Colonies showing yellowish pigmentation were Gram stained and sub-cultured on MSA and blood agar plates. Pure cultures were

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further confirmed by Catalase and Coagulase tests as well as Oxacillindisc test with Oxacillin media. Confirmed MRSA isolates were kept on agar slants and stored in a refrigerator at 4^0C until required (Cheesbrough, 2006).

Preparation of Extract Concentration
One gram (1.0 g) each of leaf and bark extracts of *T. avicennioides* was weighed and added to 10 ml each of 10 % dimethylsulfuroxide (DMSO) to obtain 100 mg/mL stock solutions of each extract. Using two-fold serial dilution, concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL were prepared from each stock solution. The different concentrations were labeled and kept in bijou bottles for subsequent use (Srinivasan *et al.,* 2009).

Standardization of Bacterial inoculum.
A colony of cells from an overnight growth culture of a test bacterium was added to 2.0 mL of sterile physiological saline as suspension medium using a sterile wire loop. The bacterial suspension was compared to 0.5 McFarland standards (1.5×10^8 CFU/ mL) under a white background with contrasting black lines (Cheesbrough, 2006).

Antibacterial Activities of Crude Extracts of *T. avicenniodes* against Bacteria
Agar well diffusion method, described by Srinivasan *et al*. (2009) was used to determine the antibacterial activity of the various different concentrations of *Terminalia avicennioides* extracts against MRSA isolates. Using a micropipette, about 100 µl of standardized inoculum of a bacterial suspension was inoculated into Mueller Hinton agar plates (in triplicates) and spread evenly over the entire surface of the plates using a sterile swab. The plates were left for 10 minutes before wells were dug in the agar using 9 mm sterile cork borer. One hundred microliter (100 µl) volume of the different concentrations of extracts (100, 50, 25, 12.5 and 6.25mg/mL) were each filled in to the wells. Additional wells were filled with dimethylsulfuroxide to serve as negative control. Ciprofloxacin (5 µg) was used as a positive control. The plates were left for 10 minutes at room temperature for diffusion of extracts into the agar to take place and then incubated at 37^0C for 24 h. The cultures were examined for zones of growth and the diameter of each zone was measured in millimeters using a meter rule. The means were calculated to the nearest whole number.

Determination of Minimum Inhibitory Concentration (MIC)
The MICs of crude extracts against MRSA clinical isolates were determined using broth dilution method described by Andrews (2001). Varied concentration of extracts (100, 50, 25, 12.5 and 6.25mg/mL) were prepared. One milliliter (1.0 mL) of each extract concentration was added to a test tube containing 9 ml of Mueller Hinton broth. One hundred micro liters (100 µl) each of a standardized inoculum of a test bacterium was added to each tube containing a mixture of an extract concentration with Mueller Hinton broth. All test tubes were incubated at 37^0C for 24 h. The growth of bacteria in the broth were examined which was indicated by the turbidity of the broth. However, the lowest concentration of the extract which inhibited the growth of bacteria was recorded as the MIC.
Negative controls were set up as follows; Mueller Hinton broth only and Mueller Hinton broth with extract. While positive control comprised of Mueller Hinton broth and the test bacteria.

Determination of Minimum Bactericidal Concentration (MBC)
The Minimum Bactericidal Concentration (MBC) was determined from the MIC tube that showed no growth. An inoculum from the tube was sub cultured on to nutrient agar plate and incubated at 37 ^{O}C for 24 h. The lowest concentration of extract that yielded no growth was the Minimum Bactericidal Concentration (MBC). The negative controls were nutrient agar only and nutrient agar with extracts only (Andrews, 2001).

DATA ANALYSIS
Results were expressed as means ± standard deviation. Comparison of mean of 3 determinations; and results adjudged significant at $p < 0.05$.

RESULTS

Phyto-chemical Constituents of Ethanol Leaf and Bark Extracts of *Terminalia avicenioides*
The phytochemical screening carried out on the crude extracts of leaves and stems of *T. avicennioides* using qualitative methods, revealed the presence of compounds shown in Table 1. Carbohydrates, cardiac glycosides, saponins, flavonoides, tannins, alkaloids, phenols and triterpenes were detected in all the extracts. However, anthraquinones and steroids were found to be absent.

Reconfirmation of Clinical Isolate of Methicillin Resistant *Staphylococcus aureus*
Methicillin Resistant *Staphylococcus aureus* showed characteristic circular colonies with yellow pigmentation on Manitol Salt Agar and β-heamolysis on blood agar. Presumptive isolates were both catalase and coagulase positive. Positive isolates were resistant to Oxacillin and confirmed as Methicillin Resistant *Staphylococcus aureus*

Antibacterial Activities of *T. avicenniodes* Extracts
All the extracts exhibited significant inhibitory effects ($P < 0.05$) against Methicillin Resistant *Staphylococcus aureus* at varied concentrations of 100, 50, 25, 12.5 and 6.25mg/mL. The activity of each extract was concentration dependent. The mean zone of inhibition of the leaf extract ranged between 12.52 ± 1.86 mm - 17.12 ± 1.89mm and the bark extracts ranged between 10.26 ± 1.37 mm - 14.45 ± 1.47mm (Table 2). Ciprofloxacin (5 µg), a positive control, showed a zone of inhibition of 26 ± 0.00 mm.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the Leaf and Bark Extracts of *T. avicenniodes*

The results of MIC and MBC of plant extracts presented in Table 3 show that the leaf extract of *Terminalia avicennioides* exhibited a lower MIC of 6.25mg/mL and a corresponding MBC of 12.5 mg/mL. The bark extract was less effective against the test bacteria with MIC of 12.5 mg/ml .and MBC of 25mg/mL.

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Table 1: Phytochemical Constituents of Ethanol Leaf and Bark Extracts of *Terminalia avicennioides*

Constituents	Plant Part Crude Extract	
	Leaf	Stem
Carbohydrates	+	+
Cardiac glycocides		
Saponins	+	+
Flavonoides	+	+
Tannins	+	+
Alkaloids	+	+
Anthraquinones	+	+
Phenols	-	-
Stroides	+	+
Triterpenes	-	-
	+	+

Key: + = Detected, - = Not detected

Table 2: Antibacterial Activities of Ethanol Leaf and Bark Extracts of *Terminalia avicenioides*

Concentration (mg/mL)	Zone of inhibition of Plant Extracts (mm)	
	Leaf	Bark
100	17.12 ± 1.89	14.45 ± 1.47
50	16.38 ± 1.49	13.92 ± 1.41
25	15.46 ± 1.23	13.45 ± 1.39
12.5	13.43 ± 1.10	12.53 ± 1.43
6.25	12.52 ± 1.86	10.26 ± 1.37
Ciprofloxacin (5 µg)	26.00 ± 0.00	
P-Value	0.0010	0.0001

Table 3: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Ethanol Leaf and Bark Extracts of *Terminalia avicenioides* against Bacteria

Plant parts	MIC (mg/mL)	MBC (mg/mL)
Leaf	6.25	12.5
Bark	12.5	25

Key: MIC= Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration

DISCUSSION

This research has shown that ethanolic leaf and bark extracts of *Terminalia avicennioides* contain some phytochemicals. The compounds were found to have antibacterial activity against methicillin resistant *Staphyloccocus aureus.*These compounds have also been reported to show some curative effects against several microorganisms. Alkaloids for example, being one of the compounds present in *T.avicenniodes* is one of the largest groups of phytochemicals in plants, with amazing effects in humans (Abdul *et al*., 2013; Misonge *et al*., 2015). Flavonoids and tannins compounds in general have been known to possess antioxidant, astringency, bitterness and colour properties (Mamta *et al*., 2013). Saponins protect plants against attack by potential pathogens. Triterpenes possess analgestic, anti-inflammatory, anti-cancer, anti-malaria, anti-viral, anti-bacterial activities and inhibition of cholesterol synthesis (Abdelmohsen *et al*., 2014). Cardiac glycosides have direct action on the heart thereby helping to support its strength and rate of contraction when it is failing (Yadav and Munnin, 2011). Carbohydrates are the main components of cell wall and protoplasm and have been widely recognized to play important roles in diverse biological processes, including viral and bacterial infections, cell growth and proliferation, cell-cell communication, as well as immune response (Majaw and Moringthem, 2009). These could be some of the reasons why extracts of *T. avicenniodes*is are widely used for the treatment of many ailments among many tribes in Nigeria hence supporting its traditional use in the management and treatment of different types of ailments caused by resistant microorganisms (Musa *et al*., 2016; Mann *et al*., 2008).

The high activity of the leaf extract could be because the active components were more concentrated in the leaves. Similar findings were reported by Sule and Agbabiaka, (2008), Das and Tiwari (2010), Tiwari *et al*. (2011), Suhaili*et al*. (2011) and Wendakoon*et al. (*2012) who evaluated the antibacterial profile of some medicinal plants against selected human pathogens. The low MICs exhibited by all the extracts show that the plant is bactericidal in action (Table 3). This corresponds to the findings of Mann *et al*. (2008) and Musa *et al*. (2016) who evaluated the antibacterial activities of extracts of *T. avicennioides* against respiratory tract pathogens and diarrhoeal pathogens and to the findings of Wendakoon *et al*. (2012) as well as Mann and Kuta (2014) who evaluated the antibacterial activities of selected medicinal plants and crude extracts of *T. avicennioides* against humans and fish pathogenic bacteria.

Conclusion

Important secondary metabolites were detected in the leaf and bark extracts of *Terminalia avicennioides*. The leaf and bark extracts exhibited significant antibacterial activities against Methicillin Resistant *Staphylococcus aureus* The leaf extract was more effective against Methicillin Resistant *Staphylococcus aureus* with MIC and MBC of 6.25 and 12.5 mg/ml. The observed activities of extracts could be due to the presence of secondary metabolites. This implies that the leaf extracts of *Terminalia avicennioides* can be used to treat infections caused by Methicillin Resistant *Staphylococcus aureus*. Isolation and purification of the active components of the plant should be carried out to unravel the identity of the active ingredients that could be responsible for the antibacterial activity.

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