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In vitro antibacterial activity of biologically synthesised silver nanoparticles using *Terminalia avicenoides* extracts against multidrug resistant *Staphylococcus aureus* strains

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ABSTRACT

Antimicrobial resistance is currently one of the risen concerns to global healthcare in the 21st century. The search for new phytochemicals that could be developed as drugs for treatment of infectious diseases consequently increased with medicinal plants extracts derived nanoparticles receiving greater attention. This study was carried out to determine invitro antimicrobial activity of biologically synthesized silver nanoparticles using *Terminalia avicenoides* extracts against multidrug resistant *Staphylococcus aureus* strains isolated from wound infections. Isolation and characterization of *Staphylococcus aureus* was carried out using standard phenotypic and genotypic methods. Antimicrobial activity of selected antibiotics, Terminalia avicenoides extracts and biologically synthesized silver nanoparticles against multidrug resistant *Staphylococcus aureus* was carried out using standard procedures. The results of the susceptibility profile showed *Staphylococcus aureus* isolates resistant to 8.18% to 100% conventional antibiotics used, but 100% sensitive to imipenem. Phytochemical analysis of the extracts revealed the presence of tannins, alkaloids, flavonoids, cardiac glycoside, phenols, saponins and terpenoids. The antimicrobial activity of the biologically synthesized silver nanoparticles against multidrug resistant *Staphylococcus aureus* ranged from 28.25±1.90–30.65±2.21 mm and showed significant difference ($p<0.05$). Comparative analysis of *Terminalia avicenoides* extracts and their respective biologically synthesized silver nanoparticles activity showed significant difference ($p<0.05$) with antimicrobial activity of silver nanoparticles having larger zones of growth inhibition (29.60±2.83mm) compared to that of extracts (19.88±13.09mm). Remarkably, *Terminalia avicenoides* extracts derived silver nanoparticles exhibit higher inhibitory effects against the multidrug resistant *Staphylococcus aureus* strains, hence, can further be study and develop for wound infections therapy.

Keywords: *Staphylococcus aureus*, Multidrug Resistance, Nanoparticles, Wound, Antimicrobial, *Terminalia avicenoides*.

INTRODUCTION

Staphylococcus aureus is known to acquire resistance to new drugs and continues to defy attempts to control it. Infections caused by antibiotic resistant strains of *Staphylococcus aureus* have reached epidemic proportions globally and the increasing rates of antimicrobial resistance are resulting in fewer treatment options [1]. Hence, the World Health Organization (WHO) recommended for a focus on discovery and development of new antibiotics specifically active against multidrug and extensively drug-resistant bacteria; and development of new types of antibiotics that lacks cross- and co-resistance to the existing classes of antibiotics [2].

Interestingly, medicinal plants are considered potential sources of new antimicrobial molecules globally, and traditional herbal medicines have been used worldwide to treat various infectious diseases for thousands of years ago [3,4]. Also, because of rising concern to antibacterial resistance, especially multidrug resistance, scientists are now exploring novel compounds including silver nanoparticles (AgNPs) to halt multidrug-resistant microorganisms, and silver nanoparticles have received much attention due to their unique high antibacterial activity against a broad range of bacteria without any toxicity to animal cell [5]. Current trends in enhancing the development of innovative wound care treatments includes the combining use of traditional healing agents and modern products/practices, such as nanofibers containing silver nanoparticles [6]. Hence, many medicinal plants including *Terminalia cuneata* have been used for synthesis of silver nanoparticles [7]. This study focused on evaluating the in vitro antimicrobial activity of *Terminalia avicenoides* extracts as well as the biologically synthesized silver nanoparticles using the plant extracts against multidrug resistant *Staphylococcus aureus* isolated from wounds.

MATERIALS AND METHODS

Ethical Consideration

Permission to collect patients' wound swab samples for isolation of *Staphylococcus aureus* was obtained from the research ethic committee (Reference number: HREC-20-0004) of the Barau Dikko Teaching Hospital, Kaduna State University, Kaduna, Nigeria. Barau Dikko Teaching Hospital, Kaduna State University, Kaduna, Nigeria. Inform consent forms were administered to patients with wound infections for their consent before obtaining relevant data and wound swab samples. Prior to collection of wound swabs from patients, the hospital ethical committee approval and appropriate intended research information were disseminated to the nurses of the selected hospital wards and units. A brief explanation of the aim and objectives of the research was done to enlighten the patients. Patients were also informed of their freedom to consent or decline participation. Guardian or parents of children with wound infection were requested to give assent for the children.

Collection and Transportation of Clinical Wound Swabs

A total of sixty wound swabs samples were collected from in and out patients with wound at Barau Dikko Teaching Hospital Kaduna, Nigeria Exudate or purulent or pus discharge were aseptically swabbed with sterile swab cotton tip and the cotton tip broke immediately into a sterile Brain Heart Infusion (BHI) broth in a universal bottle. The collection of the samples from the patients were carried out with the help of the hospital Nurses. The samples collected were then transported in ice packed thermo flasks to Kaduna State University Postgraduate Medical Microbiology Laboratory for isolation of *Staphylococcus aureus* isolates.

Isolation of Staphylococcus aureus from Wound Swabs

All media were prepared according to manufacturer's instructions. All clinical samples collected were cultured aerobically for isolation of *Staphylococcus aureus* in the laboratory as described by Valli's *et al*. and Chesbrough [8,9]. The swab samples were first cultured aerobically in an enrichment medium (Brain Heart Infusion (BHI) broth) at 370℃ for 24 hours. The broth cultures from the BHI broth were then Mannitol Salt agar (MSA) plates for selective isolation of *Staphylococcus aureus*. Pure culture colonies of presumptive *Staphylococcus aureus* on MSA plates were further subculture aerobically on Bared Parker agar plates at 370℃ for 24 hours for morphological characteristics study of the isolates. Pure single colonies from this medium were subculture on nutrient agar slant and kept at 40℃ for morphological and biochemical characterization.

Morphological and Biochemical Characterization of presumptive Staphylococcus aureus Isolates

Biochemical characterization of the pure isolates obtained was carried out as described by Aneja, Ochi and Kolhatkar, and Chesbrough [9,11]. Motility, catalase, coagulase, hemolysis, citrate utilization, methyl red, Voges-Proskauer, indole, and sugars (lactose, mannitol and sucrose) fermentation test were carried out for identification of *Staphylococcus aureus* isolates.

Molecular Identification of *Staphylococcus aureus*

Chromosomal DNA Extraction

The DNA extraction was carried out using pioneer bacterial extraction kits (Genomic DNA extraction kits) protocols - "Bioneer accuprep genomic DNA extraction kit (K-3032).

Standard inoculumn (a density of $1x10^8$ cells/ml) of *Staphylococcus aureus* were prepared from 24 hours broth culture.

Two milliliters (2ml) of the prepared standard inoculumn were transferred to 5 ml sterile Eppendorf tube and centrifuged for 5 min at 10,000 rpm. The supernatant was carefully discarded without disturbing the pellet. Another two milliliters (2ml) of the standard inoculumn added and centrifuged at 10,000 rpm for 5 minutes., followed by carefully discarding the supernatant, and repeated once again to obtained more quantity of DNA.

The pellets obtained was resuspended in (200µl) of phosphate buffer saline (PBS) in the Eppendorf tube. Twenty microliters (20µl) of proteinase were added to the tube containing the pellet in PBS, followed by addition of 10µl of RNase, then mixed thoroughly by vortexing and incubated at room temperature.

Two hundred microliters (200µl) of GB buffer (lysis buffer) were added to the sample and mixed by vortexing, followed by incubation at 60ºC for 10 minutes using heating block.

Four hundred microliters (400µl) of absolute ethanol (Biological grade) were added and mixed well by pipetting, followed by careful transferred of the lysate into the upper reservoir of the binding or absorption column (fitted in the collection tube) without wetting the rim. The tube was closed and centrifuged at 8,000 rpm for 1 minute. followed by discarding the solution from the collection tube and then reused the collection tube.

Five hundred microliters (500µl) of W2 buffer were added without wetting the rim, followed by closing the tube and then centrifuged at 8,000 rpm for 1 minute. The solution from the collection tube was discarded and then reused the collection tube.

The sample was centrifuged once more at 13,000 rpm for 1 minute to completely removed ethanol, followed by checking to ensured that there were no droplets clinging to the bottom of the binding column tube. The binding column tube was transferred to new 1.5ml tube for elution and (100µl) of EA buffer (elution buffer) was added on to the binding column tube and then kept at room temperature (25ºC) for 1 minute.

Polymerase Chain Reaction (PCR) – Accupower Hotstart PCR premix (Bioneer)

Twenty microliters (20µl) reaction PCR set - up was prepared by adding; 6ul dH_2O, 1µl forward primer- GGACTACAGGGTATCTAAT 16S (RIBOSE-1), 1µl reverse primer - AGAGTTTGATCCTGG 16S (RIBOSE-2), and 2ul template DNA. PCR amplification reaction was performed using PTC 100 thermal cycler with Pre- denaturation at 95ºC for 5 minutes, denaturation at 94ºC for 1 minute, primer annealing at 54ºC 1 minute, extension at 72ºC 1 minute for 25 cycles, and final extension at 72ºC 5 minutes. The PCR products were separated by electrophoresis in 1.5% agarose gel for 35 minutes at 125 volt and then visualized the gel DNA bands using UV lightbox/ gel imaging system (Bio-Rad). Amplified PCR products were sequence and the nucleotides sequences of the 16SrRNA genes were searched for sequences similarities using online BLASTn.

Antimicrobial Susceptibility tests Using Selected Conventional Antimicrobial Agents used for Treatments of Wound Infections

Antimicrobial susceptibility test against *Staphylococcus aureus isolates* was carried out using Kirby-Bauer disc diffusion techniques described by Arora [12]. A loopful of 24 hours growth culture of each isolate in nutrient broth was suspended in 10ml sterile distilled water and then diluted in steps of 1:10 to give turbidity equivalent to the 0.5 McFarland standards (a density of $1x10^8$ cells/ml) before inoculation. Sterile cotton wool swabs were dipped in the suspensions adjusted to $1x10^8$ cells/ml, the excess fluid was removed by pressing and rotating the swabs against the wall of the tubes, and then streaked on the surface of Muller Hinton agar plates. The inoculated plates were allowed to dry for about 5 minutes. Using disc dispenser, single disc

Gram positive antibiotics (Oxoid), Gentamycin (10µg), Amoxicillin-Clavulanic acid (30µg), Nalidixic acid (30µg), Kanamycin (30µg), Ciprofloxacin (5µg), Vancomycin (30µg), Ampicillin (10µg), Oxacillin (1µg), Chloramphenicol (30µg), Imipenem (10µg), Cefoxitin (30µg), and Sulphathiazole (25µg) were dispensed on inoculated plates of *Staphylococcus aureus.* After 30 minutes of applying the discs, the plates were then incubated aerobically at 37°C for 24 hours in an inverted position. Diameter of zone of growth inhibition were measured using a transparent metric ruler and the results were interpreted as either susceptible, intermediate, or resistant according to Clinical and Laboratory Standard Institute guidelines [13].

Collection and Authentication of Terminalia avicenoides Plant Materials

Fresh *Terminalia avicenoides* plant's parts was collected and transported for identification at the Herbarium Unit of Department of Biological Science, Faculty of Life Sciences, Ahmadu Bello University Zaria, Nigeria; where the voucher number (900239) of the plant was obtained. Fresh *Terminalia avicenoides* plant's parts was collected after the authentication of the plant in large quantity and cut into small pieces and dried under shade at 30°C in a clean laboratory cabinet. The dried plant materials were first pounded in a mortar, followed by dry-milling with an electric blender and then sieved to obtained fine powder using 20µm mesh size sieve.

Preparation of Terminalia avicenoides Plant Extracts

Water, acetone and ethanol were used as the extracting solvents. Twenty-five grams (25g), of the processed fine powder sample of plant was soaked in 250 ml of ethanol in clean sterile 500ml conical flask and then covered the mouth of the flask with non-absorbent cotton wool followed by wrapping with aluminum foil paper. The flask was then agitated at 80 rpm for about 48 h. at 28±2 using shaking incubator. The content was filtered first using clean muslin cloths, followed by Whatman's No.1 filter paper. The filtrate was then evaporated using rotary evaporator to concentrate the extracts at 37°C. The same procedure was repeated with water and acetone as the extraction solvents.

Qualitative Phytochemical Screening

The extracts were subjected to qualitative phytochemical tests to determine the presence of saponins, tannins, phenolic compounds, anthraquinones, cardiac glycosides, alkaloids, and flavonoids, using standard procedures described by Trease and Evans [14]. Harborne [15]. and Sofowara [16].

Biosynthesis of Silver Nanoparticles Using Terminalia avicenoides Extracts

The biogenic synthesis of silver nanoparticles was carried out according to Balashanmugam and Kalaichelvan [17]. Henry *et al.* [18] and Suresh *et al.* [19]. Five millimoles (5mMl) of silver nitrate solution was prepared by dissolving 0.0425g in forty-five milliliters (45ml) of sterile distilled water in 100ml conical flask. A magnetic stirrer was used to stir the mixture for 10 minutes. Five milliliters (5ml) of the extract was added to the silver nitrate solution drop-by-drop until an initial color changed was observed. For color shift control, mixture of silver nitrate solution and plant extract was held at 60°C for 60 minutes. The mixture was incubated for 24hours at room temperature in clean dark cupboard. Plant extract solution was also incubated as a negative control. A final color changed to brown, different from the negative control indicating the formation of *Terminalia avicenoides* derived silver nanoparticles (AgNPs) was observed and recorded.

In vitro Determination of the Antibacterial Potency of the Terminalia avicenoides Extracts and (AgNPs) on Multi drug Resistant Staphylococcus aureus Isolates

The antimicrobial potency of the plants extracts and (AgNPs) against all the multi drug resistant *Staphylococcus aureus* isolates was determined using a spread-plate and agar-well diffusion method according to Ochi and Kolhatkar [11]. and Chesbrough [9]. Zero-point eight grams of the extracts of *Terminalia avicenoides* was reconstituted in 2ml of 10% Dimethyl Sulfoxide (DMSO) in water to get a concentration of 400mg/ml. 200mg/ml, 100mg/ml, 50mg/ml, and 25mg/ml concentrations were made from the initial concentration using a standard dilution method. Twenty milliliters (20ml) of Sterile Muller-Hinton agar were poured into each of the petri plate and allowed to solidify on the bench. An overnight broth cultures of each pure isolate was prepared, and 0.1ml of the culture broth was added to 19.9ml sterile distilled water, then adjusted by comparing with 0.5 McFarland turbidity standard (density of 1.0×10^8 cells/ml) against a light background. Sterile cotton wool swab was dipped into the suspension, remove the excess fluid by pressing and rotating the swabs against the wall of the tubes and then streaked uniformly on the surface of Muller-Hinton culture plates. The inoculated plate was allowed to dry for 5minutes. Six millimeters (6mm) diameter corn borer was used to make wells on the inoculated culture plates and 0.2ml each of the reconstituted extracts concentrations was then loaded into the wells using sterile micropipettes. The plates were kept on the laboratory bench for 2 hours to allow the loaded extracts diffused into the culture medium. The plates were then incubated aerobically for 24 hours at 37°C. This was repeated using 1mg/ml of ciprofloxacin as positive control; and also 2% dimethyl Sulphur oxide (DMSO) as negative controls. Zones of growth inhibition form around the wells were measured with a transparent meter rule and the results recorded in millimeter (mm). The antimicrobial activity was expressed as the average diameter of the zones of growth inhibition (mm).

Data Statistical Analysis

Analysis of Variance (one way-ANOVA), Duncan multiple tests, and Independent T-test using SPSS version 23, were used for the data analyses.

RESULTS

Morphological and Biochemical Characteristics of Presumptive Staphylococcus aureus

Presumptive *Staphylococcus aureus* colonies showed by table 1 appeared completely yellowish in color with raised, circular and smooth edges on Mannitol Salt agar (MSA). On Baired Parker agar, the colonies appeared black with shining characteristics and lytic edges. On blood agar, the colonies showed complete lysis of blood cells surrounding the colonies-characteristics of beta-hemolysis. Gram stains cell appeared purple/blueish in color (Gram-positive characteristics) and cocci in shape, arranged in clusters (grape-like) under microscopic examination. The biochemical characteristics showed that the isolates are not motile, but catalase positive, coagulate positive, indole negative, methyl red positive, Voges-Proskauer positive, citrate utilization positive, beta-hemolytic, lactose utilization negative, mannitol utilization positive and sucrose utilization negative.

Molecular Characteristics of Staphylococcus aureus Isolates

Plate 1 showed the Gel electrophoresis of amplified PCR 16SrRNA genes bands of *Staphylococcus aureus* isolates respectively at 800bp of the 100 bp plus DNA marker. The sequences BLAST results (table 2) of the presumptive *Staphylococcus aureus* isolates; S_1, S_2 and S_3 16SrRNA genes revealed the percentage identity and similarity of these isolates from the Gene Bank database as 76.87%, 91.64% and 86.94% respectively, confirming the identity of these isolates as *Staphylococcus aureus* strains.

Table 1: Morphological and Biochemical Characteristics of Presumptive *Staphylococcus aureus* Isolates

Isolate Identification Code	Morphological Characteristics			Biochemical Characteristics											Probable Organism
	Colonial morphology on manitol salt agar (MSA) and baird parker agar and blood agar	Cellular/ microscopic morphology	Gram reaction	Molility	Catalase	coagulase	Indole	Methyl red	Vogas-proskayar	Cirat utilization	Hemolysis	Lactose	manitol	Surcrose	
DR3, DR5, DR11, DR12, DR19, DR21, FSW1, FSW6, MSW2, MSW3, MSW4.	Complete yellow, raised, circular and smooth edges, and moderate colonies on MSA Black shining colonies with lysis at their edge	Cocci appeared in cluster (gape-like) or bundge with fein in singles and pairs	Gram positive	-	+	+	-	-	+	+	+	-	+	-	*Staphylococcus aureus*

Keys: + = positive, - = negative, DR = dressing room wound isolates, FSW=female surgical wound isolates, and MSW= male surgical wound isolates Conflict of Interest

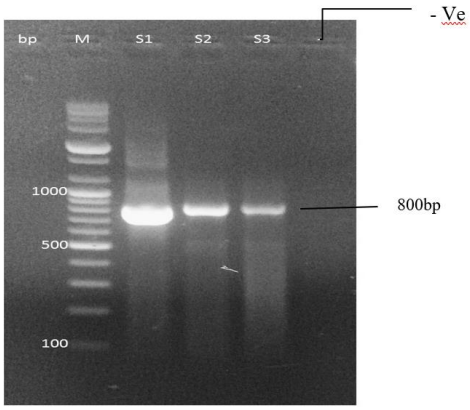


Plate 1: Gel electrophoresis of amplified PCR 16SrRNA genes bands of *Staphylococcus aureus* isolate at 800bp of the 100 bp plus DNA marker. Key: M = 100bp DNA marker, S = *Staphylococcus aureus*, bp = base pair, - Ve = Negative control, S1 = DR12, S2 = FSW1, S3 = DR11

Table 2: BLAST Characteristics of *Staphylococcus aureus* Strains

S/N	Sample Code	Organism	Sequence Searched Gene	Total Scores	Identity and Similarity (%)	E-Value	Query cover (%)	Sequence Searched Accession No
1.	S1	*Staphylococcus aureus*	16SrRNA	134	76.87	8e-29	44	LT6805131
2.	S2	*Staphylococcus aureus*	16SrRNA	878	91.64	0.0	99	LC429749.1
3.	S3	*Staphylococcus aureus*	16SrRNA	360	86.94	9e-94	43	LC57519.1

S1 = DR_{12}, S2 = FSW_1, S3 = DR_{11}

Antimicrobial Activity of Selected Conventional Antibiotics Against Staphylococcus aureus Strains

Figure 1 showed that all *Staphylococcus aureus* strains are multi-drug resistant isolates. Out of eleven *Staphylococcus aureus* isolates screened using twelve selected conventional antibiotics, 2(18.18%) were resistant to gentamycin, 3(27.27%) resistant to kanamycin, 5(45.45%) resistant to ciprofloxacin, 7(63.64%) resistant to chloramphenicol and vancomycin, 10(90.91%) resistant to amoxicillin-clavulanic acid and sulphathiazole, and 11(100.00%) resistant to ceftazidime, ampicillin, oxacillin and cefoxitin. All 11(100.00%) isolates were sensitive to imipenem. The resistant pattern of *Staphylococcus aureus* isolates showed by figure 2 indicated that four isolates (DR_{19}, DR_{21}, FSW_1 and FSW_6) were resistant each to 7 (58.33%) antibiotics used, five isolates (DR_3, DR_5, DR_{11}, MSW_3 and MSW_4) were resistant each to 8(66.64%) antibiotics used, and two isolates (DR_{12}, and MSW_2) were resistant each to 9(75.00%) antibiotics. According to the results; imipenem, gentamycin and kanamycin were the most effective antibiotics against all the *Staphylococcus aureus* strains.

Qualitative Phytochemical Characteristics of Root Barks, Stem Bark and Leaves Extract of Terminalia avicenoides

Table 3 showed the presence of flavonoids, tannins, saponins and phenol in all the root bark, stem bark and leaves extracts obtained using both ethanol, acetone and water solvents. Alkaloids was detected only in ethanolic extracts of root bark, stem bark and also acetone aqueous stem bark extracts. Cardiac glycoside was detected only in all stem bark, ethanolic and aqueous root bark extracts and also ethanolic leaves extracts. Terpenoids was present in all leave extracts acetone stem bark and ethanol root bark extracts. Anthraquinone was not detected in all the extracts.

Figure 1: Susceptibility Profile of *Staphylococcus aureus* Strains against selected antibiotics

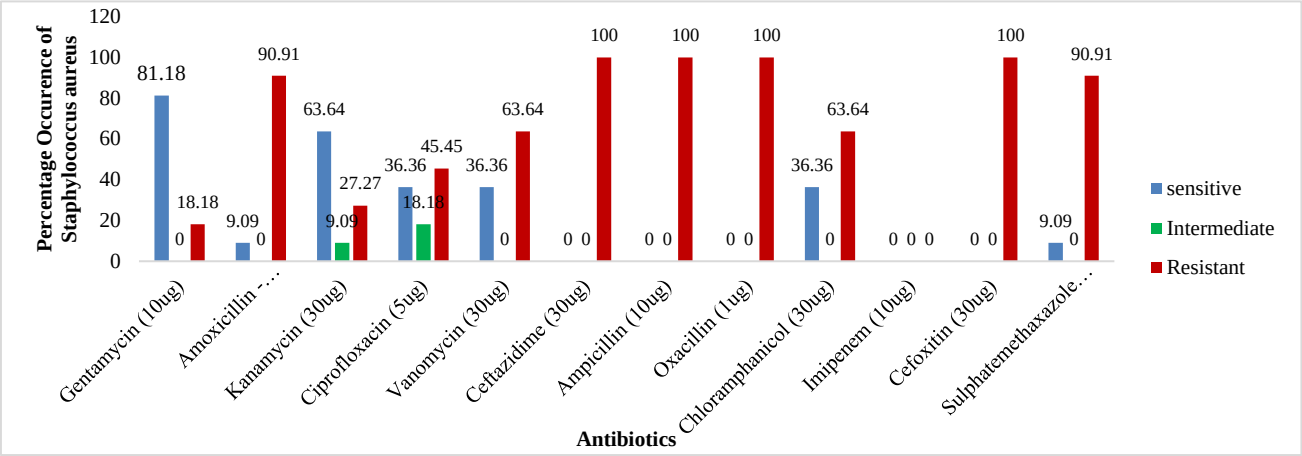


Figure 2: Susceptibility Pattern of Selected Antibiotics Tested against *Staphylococcus aureus* Strain

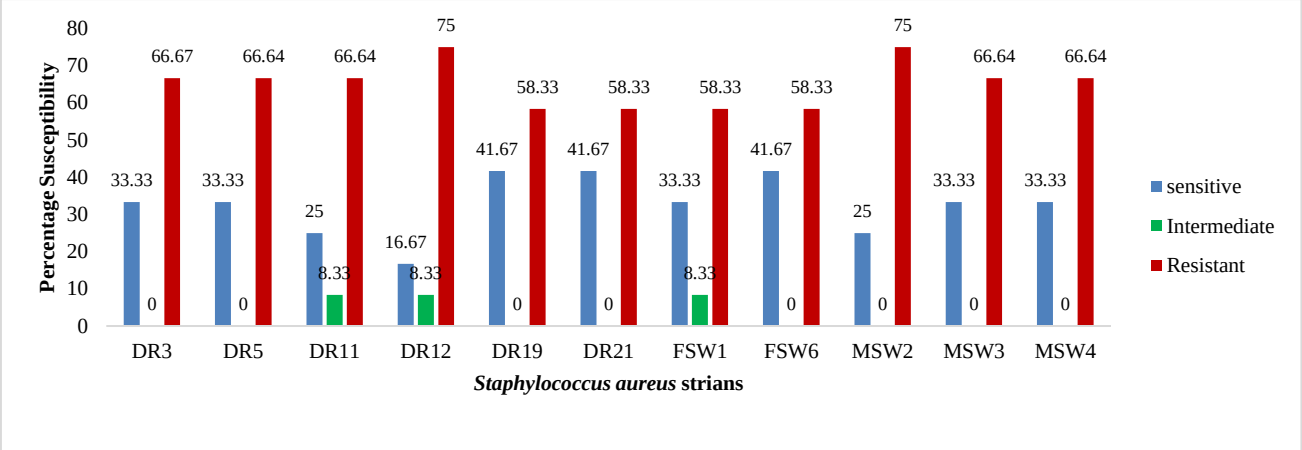


Table 3: Phytochemical Characteristics of Root Barks, Stem Barks and Leave Extracts of *Terminalia avicenoides.*

S/ No	*Terminalia avicenoides* Plant Part	Type of Solvent Extract	Phytochemical Characteristics							
			Alkaloids	Flavonoids	Tannins	Saponins	Cardiac glycosides	Phenols	Anthraquinones	Terpenoids
1.	Root Barks	Ethanol	+	+	+	+	+	+	-	+
		Acetone	-	+	+	+	-	+	-	-
		Aqueous	-	+	+	+	+	+	-	-
2.	Stem Barks	Ethanol	+	+	+	+	+	+	-	-
		Acetone	+	+	+	+	+	+	-	+
		Aqueous	+	+	+	+	+	+	-	-
3.	Leaves	Ethanol	-	+	+	+	-	+	-	+
		Acetone	-	+	+	+	-	+	-	+
		Ethanol	-	+	+	+	+	+	-	+

Key: + = Positive; = Negative

Visual Characteristics of Biologically Synthesised Terminalia avicenoides Extracts Derived Silver Nanoparticles (AgNPs)

Table 4 showed brown - like colors of the biologically synthesised silver nanoparticles using the leave, stem bark, and root bark extracts of Terminalia avicenoides. The final color of (AgNPs) solution; NP_1, NP_2 and NP_3 were light-brown, greenish-brown and light brown respectively. NP_4, NP_5 and NP_6 were radish- brown, coffee brown and radish-brown respectively. NP_7, NP_8 and NP_9 were Dark brown, coffee-brown and dark-brown respectively.

Antibacterial Activity of Biologically Synthesized Silver Nanoparticles (AgNPs) from Terminalia avicenoides Extracts against Multi drug Resistant Staphylococcus aureus isolates.

Table 5 showed antibacterial activity of biologically synthesized silver nanoparticles (AgNPs) of *Terminalia avicenoides* extracts against multidrug resistant *Staphylococcus aureus* isolate strains.

Generally, the zone of growth inhibition produced by the biologically synthesised silver nanoparticles tested against *Staphylococcus aureus* isolate strains ranged from 28.25±1.90–30.65±2.21 mm and showed significant difference ($P<0.05$). However, ciprofloxacin activity showed larger zone of growth inhibition (40.55±1.01 mm) compare to the AgNPs zone of growth inhibition.

Comparative Antibacterial Activity of Terminalia avicenoides Extracts and their Biologically Synthesised Silver Nanoparticles (AgNPs) Tested Against Staphylococcus aureus Strains.

Plate 2 showed the zones of growth inhibition produced by *Terminalia avicenoides* extracts alone, biologically synthesised nanoparticles, and standard antibiotic (Ciprofloxacin). Table 6 showed antimicrobial activity of *Terminalia avicenoides* extracts and their biologically synthesised silver nanoparticles (AgNPs) tested against *Staphylococcus aureus* strains. Generally, the zone of growth inhibition between the extracts and their derived (AgNPs) showed significant difference ($P<0.05$), with the antimicrobial activity of the extracts having lower zone of growth inhibition (19.88±13.09 mm) compared to the AgNPs which showed lager zone of growth inhibition (29.60±2.83 mm). Remarkable, the plant extracts derived AgNPs exhibit higher inhibitory effect with the root bark extracts derived AgNPs producing the largest zone of growth inhibition (29.70±3.49 mm) against the *Staphylococcus aureus* strains.

Table 4: Visual Characteristics of Biologically Synthesised *Terminalia avicenoides* Extracts Derived Silver Nanoparticles.

S/N	Plant extract code	Silver nanoparticle code	Extracts and Silver Nitrate Solution Held at 60°C for 1hour	Plant Extracts Derived Silver Nanoparticle after 24 hours Incubation
1	AETL	NP_1	Greenish brown	Dark brown
2	EETL	NP_2	Greenish brown	Dark brown
3	AQTL	NP_3	Greenish brown	Light Brown
4	AETSB	NP_4	Reddish brown	Reddish brown
5	EETSB	NP_5	Light brown	coffee brown
6	AQTSB	NP_6	Reddish brown	Reddish brown
7	AETRB	NP_7	Light brown	Dark brown
8	EETRB	NP_8	Light brown	coffee brown
9	AQTRB	NP_9	Light brown	Dark brown

Key: NP1 to NP9 = Synthesised Nanoparticles 1 to 9, EETL = ethanol Terminalia avicenoides Leave extract, AETL = acetone Terminalia avicenoides Leave extract, AQTL =AquoeusTerminalia avicenoides Leave extract, EETSB = ethanol Terminalia avicenoides stem bark extract, AETSB acetone Terminalia avicenoides stem bark extract, AQTSB = AquoeusTerminalia avicenoides stem bark extract, EETRB = ethanol Terminalia avicenoides root bark extract, AETRB = acetone Terminalia avicenoides root bark extract, and AQTRB = aqueous Terminalia avicenoides root bark extract

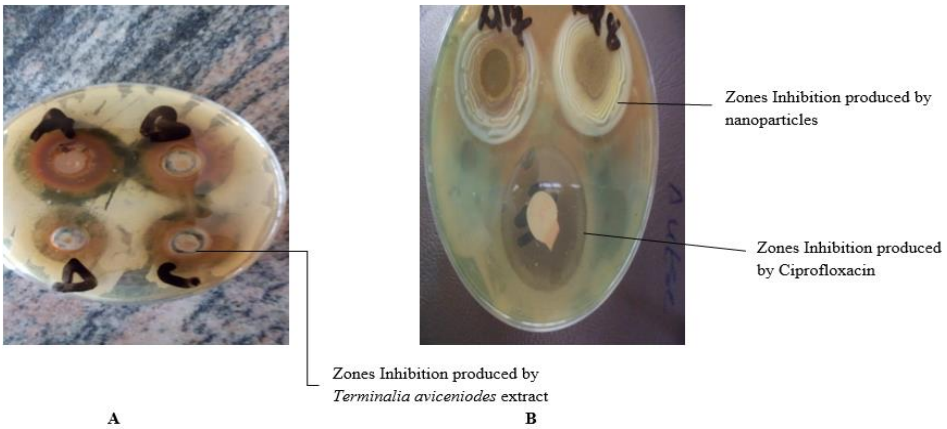
Table 5: Antibacterial Activity of Biologically Synthesised *Terminalia avicenoides* Extracts Derived Silver Nanoparticles Against Multidrug Resistant *Staphylococcus aureus* Strains

Organism	Variable	Mean ± SD Zone of growth inhibition (mm)	P-value at α = 0.05	Interpretation
Staphylococcus aureus Strains (DR_3, DR_5, DR_{11}, DR_{12}, DR_{19}, DDR_{21}, FSW_1, FSW_6, MSW_2, MSW_3, MSW_4)	Leave, Stem and Root Bark Extracts NPs Activity			There was a significant difference between silver nanoparticles and standard antibiotic (Ciprofloxacin) antimicrobial activity, with ciprofloxacin having larger zone of growth inhibition compare to the AgNPs. However, the zone growth inhibitions between the AgNPs showed no significant difference.
	NP_1			
	NP_2	29.200 ± 2.66^b	0.001	
	NP_3	30.65 ± 2.21^b	($P < 0.05$)	
		29.05 ± 2.10^b		
	NP_4			
	NP_5	30.10 ± 2.84^b		
	NP_6	28.50 ± 2.61^b		
		$30.25 \pm 1.90_b$		
	NP_7			
	NP_8	30.60 ± 2.68^b		
	NP_9	30.45 ± 2.57^b		
	Standard (Ciprofloxacin)	28.25 ± 1.90^b		
		40.55 ± 1.01^a		
	Plant Parts Extracts NPs and Ciprofloxacin Activity			There was a significant difference between silver nanoparticles and standard antibiotic (Ciprofloxacin) antibacterial activity, with ciprofloxacin having larger zone of growth inhibition compare to the AgNPs. However, the zone growth inhibitions between the AgNPs showed no
	NP_1, NP_2 and NP_3		0.001	
	NP_4, NP_5 and NP_6	29.63 ± 2.37^b	($P < 0.05$)	
	NP_7, NP_8 and NP_9	29.62 ± 2.53^b		
	Standard (Ciprofloxacin)	29.77 ± 3.31^b		
		40.55 ± 1.01^a		

			significant difference
Leave Extracts Nanoparticles			
NP_1	29.20 ± 2.66[a]	0.2567	Acetone, ethanol and aqueous Leave extracts AgNPs showed no significant different.
NP_2	30.65 ± 2.21[a]	(P > 0.05)	
NP_3	29.05 ± 2.10[a]		
Stem Bark Extracts Nanoparticles			
NP_4	30.10 ± 2.84[a]		
NP_5	28.50 ± 2.61[a]	0.2357	
NP_6	30.25 ± 1.90[a]	(P > 0.05)	Acetone, ethanol and aqueous stem bark extracts AgNPs showed no significant different
Root Bark Extracts Nanoparticles			
NP_7	30.60 ± 2.68[a]		
NP_8	30.45 ± 2.54[a]	0.2114	
NP_9	28.25 ± 4.22[a]	(P > 0.05)	
			Acetone, ethanol and aqueous root bark extracts AgNPs showed no significant difference.

Key: NP1 to NP9 = Synthesised Nanoparticles 1 to 9, DR = dressing room wound isolates, FSW=female surgical wound isolates, and MSW= male surgical wound isolates

Plate 2: A = Plate showing zone of growth inhibition of Terminalia *avicenoides* extract



B = Plate showing zone of growth inhibition of Biologically synthesized *Terminalia avicenoides* extract silver nanoparticles and standard antibiotic (Ciproxacin)

Table 6: Comparative Antibacterial Activity of *Terminalia avicenoides* Extracts and their Biologically Synthesised Silver Nanoparticles (AgNPs) Tested Against *Staphylococcus Aureus* Strains

Organism	**Variable**		**Mean ± SD Zone of growth inhibition (mm)**	**Df**	**t_{cal}**	**P-value at α = 0.05**	**Interpretation**
***Staphylococcus aureus* Strains** (DR3, DR5, DR11, DR12, DR19, DDR21, FSW1, FSW6, MSW2, MSW3, MSW4)	Extracts		19.88±13.09	178	-6.89	0.0001 (P<0.005)	The antibacterial activity between the extracts and their derived AgNPs showed significant difference. AgNPs showed larger zone of growth inhibition compared to extracts zone of growth inhibition
	Nanoparticles		29.60±2.83			0.0001 (P<0.005)	
	Leave	Extracts	17.40 ± 14.49	58	-4.51	0.0001	
		Nanoparticles	29.50 ±2.49			(P<0.005)	
	Stem bark	Extracts	19.63 ±12.45	58	--4.51	0.0001	
		Nanoparticles	29.60 ±2.50			(p<0.005)	
	Root bark	Extracts	22.60 ±12.09	58	-4.51	0.0001	
		Nanoparticles	29.70 ±3.49			(p<0.005)	
		Ciprofloxacin	40.50 ± 0.59		13.41	0.0001 (p<0.005)	The antibacterial activity between the standard antibiotic (ciprofloxacin) and extracts derived AgNPs showed significant difference. Ciprofloxacin showed larger zone of growth inhibition compared to AgNPs zone of growth inhibition
		Nanoparticles	28.399 ±2.9752		34.76	0.0001 (p<0.005)	

Key: DR = dressing room wound isolates, FSW=female surgical wound isolates, and MSW= male surgical wound isolates

DISCUSSION

This study isolated and identified *Staphylococcus aureus* strains from wound infected patients using both phenotypic and genotypic approaches. Cultural morphology of Phenotypic identification revealed the colonies of *Staphylococcus aureus* on mannitol salt Agar (MSA) as yellow, with flat and moderate shape. The production of yellow colonies on MSA has been reported by Fitzgerald to be as a result of fermentation of mannitol salt with consequent production of acid [20]. On Baird Parker medium, *Staphylococcus aureus* showed grey-black shining colonies with opaque halo surrounded by zone of clearing[20]. Silva *et al*. reported similar characteristics of *Staphylococcus aureus* on Baird parker medium, where it was reported that the formation of grey black shining colonies is due to reduction of potassium tellurite and the proteolytic activity through breaking down of egg yolk by Lecithinase causing clear zone around respective colonies, while the opaque halo surrounding zone of clearing is as a result of Lipase activity [21]. The gram stain cell revealed a characteristic of Gram-positive cocci which appeared in grape-like (cluster) under microscopic examination using x100 objective lens. Tong *et al*. reported similar cellular appearance of *Staphylococcus aureus* [22]. The biochemical characteristic showed that this organism is catalase and coagulase positive with characteristic production of beta-hemolysis on blood agar–a unique characteristic for phenotypic identification of pathogenic *Staphylococcus aureus* strains. Studies have reported that *Staphylococcus aureus* isolated from human have bound and free form of coagulase [11]. with characteristic formation of beta-hemolysis on blood agar. The presence of the enzyme coagulase is phynotypically employed to differentiate between the strain of virulent and less virulent *Staphylococcus aureus*.

The phenotypic identification approach in this study generally revealed cultural and biochemical characteristics related to *Staphylococcus aureus* isolates. However, due to the need for ethnobotanical studies to be conducted on pathogen-specific wound infection in this study with the selection of the organisms related directly to the reported traditional used of the plant *Terminalia avicenoides*, it became imperative to characterized the *Staphylococcus aureus* using molecular identification methods. This is for reproducibility of studies according to VanVuuren [23]. The molecular identification was employed to compare the genetic similarities of the *Staphylococcus aureus* isolated from wound infections with GenBank database according to Prescott *et al*. [24]. The results of the molecular analysis in this study showed the gel electrophoresis of amplified PCR 16SrRNA genes bands of Staphylococcus aureus isolates at 800bp of the 100 bp plus DNA marker. The sequences BLAST results of the presumptive *Staphylococcus aureus* isolates, S_1, S_2 and S_3 16SrRNA genes revealed the percentage identity and similarity of these isolates to those from the GenBank database as 76.87%, 91.64% and 86.94% respectively, confirming the identity of these isolates as *Staphylococcus aureus* strains.

Generally, the percentage identity and similarity revealed by the sequences BLAST results for all the *Staphylococcus aureus* strains ranged from 76.87%, -997.67% and Prescott *et al*. reported that since 1970s, it has been widely accepted that Prokaryotes whose genomes are at least 70% homologous belongs to the same species [24]. This supports the confirmation of identity of these isolates as *Staphylococcus aureus* in this study.

The findings in this study revealed all the *Staphylococcus aureus* as multi drug resistant isolates. According to the results (figure 1 and 2), imipenem was revealed to be the most potent antibiotic against the *Staphylococcus aureus* isolates followed by Gentamycin, because all the isolates were sensitive to these antibiotics. This means that imipenem must be carefully prescribed by clinicians to avoid development of resistance by the organism Also, sensitivity result should always be used as the basis for the prescription of the drugs to patients. There is also need to educate clinicians on this finding and the public health importance. Findings from this study are similar to that of susceptibility profile of Staphylococcus aureus in a study by Rashedul *et al*. who reported imipenem as the most potent antibiotic with 90% sensitivity, and with 75% isolates also showing resistance to oxacillin, methicillin, ciprofloxacin and tetracycline [25]. Kitara *et al*., and Brown and Ngeno reported that *Staphylococcus aureus* is capable of producing many antibiotic resistant strains and that this organism has the ability to acquire resistance to many antibiotics [26,27]. Brown and Ngeno also stated that *Staphylococcus aureus* resistance to antibiotics is a worldwide problem.

This study reported *Staphylococcus aureus* isolates to be resistant to chloramphenicol similar to findings by Rashedul *et al*. who reported *Staphylococcus aureus* isolates resistant to chloramphenicol [25]. Also, this study reported multi drug resistance exhibited by Staphylococcus aureus to ceftazidime similar to report by Aisha *et al*. [28]. Moreso, as Rashedul et al. studied reported that only 4 (36.63%) Staphylococcus aureus showed sensitivity to vancomycin and that it is considered as a serious threat to clinical setting [25]. also, this current study also reported vancomycin resistance to Staphylococcus aureus. The vancomycin resistance by *Staphylococcus aureus* isolates in this study indicated that the strains of this bacteria pathogens may presently be a serious problem to successful treatment of wound infections and may be an additional problem to the health system especially at the community level. According to Khan *et al*. and Julyan *et al*., vancomycin resistant *Staphylococcus aureus* (VRSA) is currently one of the great threats mankind faces because the antibiotic, vancomycin is the last resort for treating Staphylococcal infections [29,30].

Benjamin and Christopher recommended tetracycline, chloramphenicol and Gentamycin for treatment of wound infection cause by *Staphylococcus aureus* [31]. Similarly, Bowler *et al*. recommended Gentamycin, Vancomycin, Cefoxitin and imipenem for effective treatment of wound infection [32]. Findings from this study showed Imipenem, Gentamycin and Ciprofloxacin to be the most potent antibiotics indicating that they are still effective as recommended. Other recommended antibiotics were not potent to the tested bacterial isolates contradictory to the previous researches findings that recommended them. Moreso, Aisha *et al*. reported multidrug resistant *Staphylococcus aureus* against Gentamycin, Imipenem and Ciprofloxacin antibiotics, while in this study the Staphylococcus aureus strains were sensitive to these antibiotics [28]. The inconsistency might be due to some factors such as; bacterial acquisition of resistance genes, mutations, environmental changes factors, efflux pump mechanism, biofilm formation, possession of beta-lactamase, among others. This indicated the need for an alternative drug for effective therapy of bacterial wound infections, due to failure of the existing antibiotics.

In this study, extracts from *Terminalia avicenoides* were obtained from dried processed powdered of stem bark, root bark and leaves using three extracting solvents ethanol, acetone and water. The qualitative phytochemical analysis of *Terminalia avicenoides* extracts showed the presence of tannins, alkaloids, flavonoids, cardiac glycosides, phenolic compounds, terpenoids and saponins. Anthraquinones was not detected from all the category of the plant extracts. Odebumin *et al*. and Alaje *et al*. reported similar findings [33,34]. Also, in previous studies of biochemical compounds of medicinal plant, Irshad et al. reported that most chemical constituents of plant contain many bioactive compounds including alkaloids, tannins, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones [35]. Radhika *et al*. also stated that the most important of these bioactive compounds are the alkaloids, tannins, saponins, flavonoids and phenolic compounds [36]. According to Cragg and Newman, the presence of important phytochemical constituents is the bioactive bases for plant medicinal properties as these secondary metabolites are the chemical substances used by the plants for defense system and serve as bioactive principles for various drugs and modern therapy [37].

The important phytochemical constituents like steroids, tannins and

saponins have been detected in *Terminalia avicenoides* plant parts [38]. and the presence of these compound is known to confer antibacterial activity against bacteria pathogens [39]. To confer antibacterial activity of plant, flavonoids has been reported to be singly responsible for antibacterial activity associated with some ethnomedicinal plant [40]. It has also been reported that plants that are rich in tannins or phenolics compounds are inhibitory to wide range of bacteria, thus capable of conferring protection against some microbial infections [41]. The presence of the various phytochemical compounds is an indication that *Terminalia avicenoides* have potent antiseptic, bactericidal and other medicinal properties [42]. This may be due to the fact that each of the compounds identified has one or more therapeutic usage and may be acting singly or in consortium to bring about cidal or static effect on the organism. Thus, the presence of the phytochemical compound recorded in this study could be responsible for the *in vitro* antibacterial activity.

The *in vitro* antibacterial activity of the various *Terminalia avicenoides* extracts against multi drug resistant *Staphylococcus aureus* showed zone of growth inhibition. Antibacterial activity was characterized by a cleared zone between the wells (containing the samples) and certain distance. Formation of this inhibitory zones around the wells shows bacterial sensitivity to the extracts. The zone of growth inhibition ranged from 16.28±10.45–23.81±6.69 mm and showed significant difference ($P<0.05$), Udgire and Pathade suggested that plant extracts exhibiting inhibitory zones diameter greater than or equal to 10 mm and above against selected microbial pathogens should be considered to possess antimicrobial activity, whereas, those showing inhibitory zones greater than 20 mm against selected microbial pathogens should be considered noteworthy [43].

Several studies have attributed the antibacterial and therapeutic activities of *Terminalia avicenoides* extracts to the presence of flavonoids and a mixture of phenolic compounds and tannins [44]. The phenolic compounds are said to act as protoplasmic poison which penetrate and disrupt bacterial cell wall in addition to precipitation of cell proteins. The present study revealed that the Terminalia avicenoides extracts showed potent antibacterial activity against the bacterial strains. This implies that the *in vitro* antimicrobial activity of the *Terminalia avicenoides* extracts recorded in this study was due to availability of the plant secondary metabolites required for antibacterial activity. The ability of the extracts of *Terminalia avicenoides* to inhibit the growth of the multi drug resistant *Staphylococcus aureus* explains why it is being effectively used in folk medicine for treatment of wound infection. It can therefore, be deduced from the result obtained in this study that *Terminalia avicenoides* is a source of bioactive compounds with potential therapeutic benefit, because it portrays a good inhibitory effect against the multi drug resistant *Staphylococcus aureus.*

This study showed brown - like color of the biologically synthesised silver nanoparticles using both the leave, stem bark, and root bark extracts of the *Terminalia avicenoides*. The color of the biologically synthesis AgNPs solution; NP_1, NP_2 and NP_3 were light-brown, greenish-brown and light brown respectively. NP4, NP5 and NP6 were radish- brown, coffee brown and radish-brown respectively. NP_7, NP_8 and NP_9 were dark brown, coffee-brown and dark-brown respectively. Bal Shanmugam and Kalaichelvan, Henry *et al*., Suresh *et al*. and Qwidwai *et al*. reported similar findings [17-19], [45]. Henry *et al*. reported final greyish-brown and yellowish-brown color from biogenic synthesis of (AgNPs) using *Lantana camara* and Impatiens balsa mina extracts respectively [18]. Bal Shanmugam and Kalaichelvan similarly reported final dark- brown (AgNPs) from *Dodona viscose* Linn extracts [17]. Suresh *et al*. reported brown color with *Coccinea indica* leave extract [19]. and Qwidwai *et al*. reported colloidal - brown with leave extracts of *Phoenix sylvestris* [45]. The success of the biogenic synthesis of the *Terminalia avicenoides* extracts derived silver nanoparticles in this study was characterized by the appearance of visual brown color during synthesis, and also the increase in antibacterial activities of the AgNPs from that of the extract's activities. According to Suresh *et al.* and Henry *et al*. the initial brown- like color formation after addition of silver nitrates to plant extracts with application of mild heat at 60℃ for 1hour is because of some Ag^+ ions beginning to reduced due to heat effect and produces Ag^+ ions complex, which is responsible for changing color to finally brown during the synthesis [18,19]. More so, the final brown color observed from the biogenic synthesis of the AgNPs from plant extracts indicated formation of AgNPs. Formation of AgNPs accompanying by brown color has been attributed to the exposure of silver nitrate to plant extract which reduces Ag^+ to Ag^o. Khwaja *et al*. reported that plants and their parts contain carbohydrates, fats, proteins, nucleic acids, pigments and several types of secondary metabolites which act as reducing agents to produce nanoparticles from metal salts without producing any toxic by-product [46]. The presence of several polyphenolic components including flavonoids and terpenoids facillited the reduction of Ag^+ ions, and also stabilized the surface of the resultant AgNPs.

This study showed antibacterial activity of biologically synthesized *Terminalia avicenoides* extracts derived silver nanoparticles (AgNPs) against multidrug resistant *Staphylococcus aureus* isolates. The zone of growth inhibition produced by the biologically synthesised silver nanoparticles tested against *Staphylococcus aureus* isolates ranged from (28.25±1.90–30.65±2.21) mm and showed significant difference ($P<0.05$). Suresh *et al*. reported similar findings using biosynthesized silver nanoparticles derived from ethanolic extracts of *Coccinea indica* leaves against *Staphylococcus aureus* isolates [47]. Several studies including studies conducted by Skandalis *et al*. and Henry *et al*. also reported similar findings on the activity of silver nanoparticles derived from plant extracts against multidrug resistant *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Escherichia coli* [18,47]. The mechanisms of action of silver nanoparticles against bacterial pathogens has been reported by different researchers. Skandalis *et al.* reported that silver nanoparticles are independent of cell wall structure; and that the silver nanoparticles do disrupt the integrity of the cell membrane and cell wall of Gram negative and Gram-positive bacteria [47]. Henry *et al*., also reported that the small size nature of silver nanoparticles makes it easier to penetrate the outer cell wall of bacteria, enter the respiratory chain and thus inhibit cell respiration and bacterial death [18]. In the same manner, plant extracts derived biologically synthesised silver nanoparticles has been reported by Skandalis *et al*. to exert damage to bacterial cell membrane through membrane disruption affecting the bacterial cell shape, hence, leading to shrinkage of bacterial membrane and induction of holes observed with membrane damage [47].

The findings from the comparative antibacterial activity of *Terminalia avicenoides* extracts and their biologically synthesised silver nanoparticles (AgNPs) tested against multidrug resistant *Staphylococcus aureus* strains generally revealed zones of growth inhibition. Generally, the zone of growth inhibition between the extracts and their derived AgNPs showed significant difference ($P<0.05$), with the antimicrobial activity of the extracts having lower zone of growth inhibition (19.88±13.09 mm) compared to the lager zone of growth inhibition (29.60±2.832mm) produced by the (AgNPs). This study revealed that the biological synthesised silver nanoparticles possess higher antibacterial activity against multidrug resistant *Staphylococcus aureus* strains with larger zones of growth inhibition compared to *Terminalia avicenoides* extracts antimicrobial activity. High antibacterial activity of biologically synthesised plant derived silver nanoparticles has been reported in series of studies [48]. and this high antimicrobial activity may be attributed to the silver nanoparticle's ability to penetrate through flexible cell walls of bacteria made up of peptidoglycan linked by amino acids and cross-linked by tetra peptides [49]. Interestingly, the inhibition zones produced by the antibacterial activity of the biologically synthesised silver nanoparticles in this study can be categorized into strong inhibitory activity according to Davis and Stout [50]. This established the broad-spectrum antibacterial activity of the plant extracts derived silver nanoparticles against the studied bacterial isolates, hence, the *Terminalia avicenoides* extracts can be recommended as an effective biomaterial for biogenic synthesis of silver nanoparticles for

therapeutic applications. By and large, this study provided scientific evident on the potent antimicrobial activity of *Terminalia avicenoides* extracts derived silver nanoparticles against multidrug resistant *Staphylococcus aureus* isolates from wound infection that may further be explore for therapeutic purposes.

CONCLUSION

Staphylococcus aureus strains were isolated from wounds of out - and in - patients attending Barau Dikko Teaching Hospital Kaduna, Nigeria. All the *Staphylococcus aureus* were multidrug resistant strains. Out of all the antibacterial agents used, imipenem, ciprofloxacin, kanamycin and gentamycin were the most effective antibiotics against these wound pathogens. The *Terminalia avicenoides* extracts contain significant phytochemical compounds requires to exert bacteriostatic and bactericidal effects, and hence, exhibit noteworthy antibacterial activity against the multidrug resistant *Staphylococcus aureus* strains. However, the *Terminalia avicenoides* extracts derived silver nanoparticles exhibited higher antibacterial activity against the Multidrug Resistant *Staphylococcus aureus* strains in comparison to the plant extracts activity. The efficacy of the *Terminalia avicenoides* extracts and their derived silver nanoparticles against the multidrug resistant *Staphylococcus aureus* strains indicated that this plant extracts can be used to produce nanomaterial for effective therapy of drug resistant bacterial wound infections. However, exhaustive studies involving isolation and concentration of the specific bioactive or inhibitory compounds active against the multiding resistant *Staphylococcus aureus* strains is needed.

Conflict of Interest

None declared.

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