ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

TLC Analysis and Bioactivity Screening of the Stem Bark Extract of *Anogeissus Leiocarpus* Against Multi-Resistant *Staphylococcus Aureus* and Quantification of Its Phytoconstituents.

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ABSTRACT

Research on natural antimicrobials has increased due to the emergence of microbial strains resistant to conventional antibiotics. Current strategies to overcome the global problem of antimicrobial resistance include research in finding new and innovative antimicrobials from plants. *Anogeissus leiocarpus* is a medicinal plant known in Nigerian ethnomedicine to treat microbial infections and rich in phytochemical constituents. This study was carried out to determine the anti-multi-resistant *Staphylococcus aureus* activity of the stem bark extract of *Anogeissus leiocarpus* against multi-resistant *Staphylococcus aureus* prevalence in the society. The crude aqueous methanol extract was obtained by maceration with 70% methanol for 72 h. The clinical isolate of the multi-resistant *Staphylococcus aureus* was used for the activity screening. The anti-multi-resistant *Staphylococcus aureus* test was performed using an Agar diffusion method at different concentrations range of 50-400 mg/ml. Minimum Inhibition Concentration was determined for the partitioned fractions that showed some efficacy against the tested microorganism, multi-resistant *Staphylococcus aureus*. Penicillin and cloxacillin (5 mg/ml) were used as standard controls. The range of anti- multi-resistant *Staphylococcus aureus* activity values (23-40 mm) exhibited by the partitioned fractions against the test organism are comparable to those of penicillin (25 mm) and cloxacillin (20 mm). Specifically, all derived fractions exhibited a significant anti-multi-resistant activity against multi-resistant *Staphylococcus aureus* while *n*-hexane lacked efficacy against MRSA. On the overall, the results of the sensitivity test showed that methanol partitioned fraction (100 mg/ml at $P< 0.05$) contained more potential antimicrobial agents against multi-resistant *Staphylococcus aureus* when compared with the remaining two fractions (chloroform and ethyl acetate; 200 mg/ml at $P< 0.05$). The results revealed that chloroform fraction is the most active fraction at all concentrations with zone of inhibition ranging from 35 to 40 mm except at 25 mg/ml. The minimum bactericidal concentration for methanol fraction was at 100 mg/ml while chloroform and ethyl acetate fraction was at 200 mg/ml. This *in vitro* anti-multi-resistant *Staphylococcus aureus* study corroborated the use of *Anogeissus leiocarpus* in ethnomedicine for treatment of bacterial infections. This plant could be potential source of new anti-multi-resistant *Staphylococcus aureus* agent.

Keywords: *Anogeissus leiocarpus*, anti-multi-resistant *Staphylococcus aureus*, Multi-resistant *Staphylococcus aureus*, MRSA, Phytochemical constituents

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ISSN: 0975-8585

INTRODUCTION

Infective diseases accounts for approximately one half of all the deaths in most developing countries of the world. Bacterial infections of human being are common phenomenon worldwide [1, 2]. *Staphylococcus aureus (S. aureus)* is considered a major pathogen associated human infections. It is an opportunistic pathogen affecting both immunocompetent and immunocompromised individuals frequently resulting in high morbidity and complications which constitute problems to health care institutions [3]. It is associated with various clinical infections, ranging from minor skin diseases to life threatening infections including septicaemia, pneumonia, wound sepsis, septic arthritis, post-surgical toxic shock syndrome as well as scalded skin syndrome in humans [4-7]. *S. aureus* is the commonest caused of infections in the hospitals and is most liable to infect newborn babies, old and malnourished persons, patients with diabetes, and other chronic diseases [8].

Resistance of microbes (bacteria, fungi and viruses) to available antimicrobial agents is a major global public health problem. Exposure and inappropriate use of the antibiotics is the major cause of Multi-Drug Resistant (MDR), both in developed and developing regions of the world. *S. aureus* has a record of developing resistance quickly and successfully to antibiotics and has overcome all the therapeutic agents that have been developed in the past 50 years [9]. It is very common in most important pathogens such as Multi-resistant *Staphylococcus aureus* (MRSA). MRSA was reported after one year of introduction of methicillin in 1961 and has emerged as one of the most important nosocomial pathogens especially in the last two decades [10]. Hospital acquired infections due to MRSA have been associated with an increase in length of hospital stays, mortality rates, and health care costs [11, 12]. MRSA colonizes healthy individuals and causes severe infection in hospitalized patients and is a serious therapeutic challenge [13]. It is an important nosocomial pathogen worldwide and has limited treatment options [14]. *Staphylococcus aureus* is a coagulase-positive multiple resistance by microorganisms to antibiotic such as penicillin, methicillin and oxacillin. MRSA is resistant to the β-lactam antibiotics. However, antibiotics inhibit bacterial growth by interfering with one or more cellular processes. β-lactams are a large group of cell wall active antibiotics used to treat a wide variety of infections. *S. aureus* cell wall synthesis is dependent on the proper functioning of a number of enzymes. The β-lactam antibiotics exert their effect by binding with one specific type of enzyme, transpeptidase, thus interfering with its ability to catalyze the final stage of peptidoglycan synthesis, resulting in defective cell wall formation. The β-lactams comprise four main groups of antibiotics; all have the β-lactam ring as their basic chemical structure: Penicillins (penicillin, oxacillin/methicillin, ampicillin and piperacillin), Cephalosporins Carbapenems and Monobactams. The spectrum of antimicrobial activity is dependent upon the particular structural modification of the β-lactam ring. Therefore, the most common strategy used by *S. aureus* to circumvent the action of the methicillin, oxacillin and penicillin is by the production of the enzyme β-lactamase, which hydrolyses the β-lactam ring, rendering the entire compounds inactive [1]. The use of methicillin and β-lactamase resistant penicillin, initially overcame the problem experienced with β-lactamase producing bacteria. Unfortunately certain groups of bacteria, including the staphylococci, have evolved new strategies that led to the emergence of methicillin-resistant strains. This has had the greatest impact in human medicine, where methicillin resistant *S. aureus* has emerged as a

major nosocomial pathogen. The term methicillin-resistant is historically used to describe resistance to any of this class of antimicrobials even though methicillin is no longer the drug of choice. The acronym MRSA persists and is used interchangeably with ORSA – oxacillin-resistant *Staphylococcus aureus*. Oxacillin/methicillin resistance implies resistance to all penicillins, cephalosporins, monobactams, carbepenems and β-lactam/β-lactamase inhibitor combinations. *S. aureus* intrinsically produces β-lactamase enzymes that breakdown β-lactam antibiotics (eg, penicillin). Resistance of *S. aureus* to methicillin, penicillin and oxacillin has a wide distribution [1]. Therefore, Penicillin Resistance *Staphylococcus aureus* (PRSA) and Oxacillin Resistance *Staphylococcus aureus* (ORSA) were used for screening of Methicillin-resistant *Staphylococcus aureus* (MRSA). Basic exploratory research, like the present study is of utmost importance to identify the antimicrobial lead compounds from natural sources.

Medicinal plants are rich sources of developed secondary metabolites, which are potential remedies for different ailments. Extreme interest in plants with microbial activity has revived due to resistance, associated with antibiotics presently in use. The main advantage of natural agents is that they do not enhance the antibiotic resistance, a phenomenon commonly encountered with the long-term use of systematic antibiotics. There is growing interest in correlating phytochemical constituents of the plant with its pharmacological activity. Many works have been done which aim at knowing the different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistant. This has led to investigation of several medicinal plants for anti-multi-resistant *Staphylococcus aureus* potentials [15, 16]. About1400 fully characterized natural and synthetic compounds were evaluated by high throughput screens against MRSA using various clinical isolates in comparative studies. Tree barks represent an interesting source of bioactive molecules for the discovery of new drugs. Many studies indicate that Combretaceae species are commonly used in Africa to treat various infectious diseases.

Anogeissus leiocarpus is a graceful tree of Africa, commonly known as "Axle wood tree or African birch" because of the silvery cast of the foliage like the temperate birch. It is a tall evergreen tree native to savannah of tropical Africa as shown in Figure 1. It extends from the Sahel to forest zones and Senegal to Sudan and Ethiopia with savanna regions as its habitats [17]. In Nigeria, *A. leiocarpus* is popularly known with these local names: Hausa: *marike, marke*; Nupe: *shici*; Fulfulde: *galaldi, kojoli,* Yoruba: *ayin, pako ayin, orin-odan*, Igbo: *atara*. Ethnobotanically, the decoction and maceration of the stem bark (trunk shown in Figure 1) are used against anorexia, constipation, malaria, jaundice, fatigue, itching, eczema, psoriasis, carbuncles, wounds, sores, boils, cysts and various forms of hepatitis and ulcers including diabetic ulcers; helminthosis, schistosomiasis, leprosy, diarrhea and amoebic dysentery, bacterial infections and used as chewing sticks [17, 18]; trypanosomiasis [19]; cough and tuberculosis [20, 21] and treatment of sexually transmitted infections in Mali [22].

A review by Mann *et al*. [23] indicates that *Anogeissus leiocarpus* has many pharmacological activities. For instance, pharmacological investigations indicated that its bark, fruit, and leaves possess antimicrobial activities [24-43]. It is one of the Nigerian

chewing sticks that possess antimicrobial activity against oral microbial flora such as *Staphylococcus aureus* [44-48]. Furthermore, it also found to exhibit numerous biological activities such as: antitrypanosomal activity [49, 50]; anticancer/antiproliferative activities [51]; anthelmintic effect [52-54] and antiplasmodial activity [55, 56]. Its aqueous leaf extract toxic effects in rats using changes in haematological and biochemical parameters were evaluated [57].

Figure 1: Matured tree and stem (trunk) of *Anogeissus leiocarpus*

The preliminary phytochemical screening of extracts from *A. leiocarpus* indicated presence of anthraquinone, carbohydrate, saponins, steroids, tannin, and terpenoids [25, 31, 32, 34, 35]. Moreover, the active compounds isolated from this plant have been shown to be mainly triterpenes and ellagic acid derivatives; flavonoids and phenolic compounds

like flavogallonic acid bislactone [50, 58]. For instance, anogelline and dakaline are obtained from its bark are used as cosmetics with anti-ageing properties.

The fact that this plant was active against both clinical and laboratory isolates is also an indication that it can be used against drug resistant microorganisms prevalent in hospital environment. Several studies have been conducted in the past that focus on the antimicrobial properties of herbs, spices and their derivatives such as extracts and decoctions against multi-resistant *Staphylococcus aureus* [59-62]. However, numerous bioactive compounds such as 2-(2', 4'-dibromophenoxy)-4, 6-dibromophenol that exhibited potent and broad spectrum anti-multi-resistant *Staphylococcus aureus* activity had been isolated natural sources [63]. Therefore, these reported high potential antimicrobial values have attracted our attention to determine the *in vitro* anti-multi-resistant *Staphylococcus aureus* activity potentials of stem bark extract of *Anogeissus leiocarpus* grown in Kataeregi, Niger State, Nigeria.

MATERIALS AND METHODS

Plant Collection

The stem bark of *Anogeissus leiocarpus* was collected from a forest at Kataeregi along Bida-Minna road, Niger State, Nigeria. The plant was taxonomically identified and authenticated by Umar S. Gallah, a taxonomist in the Department of the Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen (No.167) was deposited at the herbarium of the University.

Preparation of the Crude Aqueous Methanol extract and its Partitioning into soluble fractions

The crude aqueous methanol extract and its fractions were prepared according to the method of Mann [64]. The fresh stem bark of *A. leiocarpus* was air-dried at room temperature and pulverized into a dry coarse powder (500 g), and macerated with 70% methanol in water for 72 h with constant shaking [64]. The resultant mixture was filtered using whatman (No 1) filter paper. The extraction process was repeated three times to exhaustively extract the plant material. The filtrates were concentrated using rotary evaporator finally evaporated to dryness in water bath in a fume cupboard and the amount of extract obtained was quantified to give a yield of 7.7% (w/w). The extract was stored in a sample bottle and kept at room temperature. The partition of the crude aqueous methanol extract was carried out following the procedure of Mann [64] by dissolving extract (35 g) in 70% methanol. It was then partitioned successively with *n*-hexane (100 ml x 3), ethyl acetate (100 ml x 3), *n*-butanol (100 ml x 3) to give four fractions (*n*-hexane, ethyl acetate, *n*-butanol and methanol fractions). The four solvent soluble fractions were concentrated separately using a rotary evaporator and the concentrates evaporated to dryness at 28^{o}C and air dried to constant weight [64].

Thin Layer Chromatography (TLC) Analysis of the Crude Aqueous Methanol extract and its partitioned fractions

Each of the partitioned fractions was concentrated and was used for TLC analysis and anti-multi-*Staphylococcus aureus* activity. Standard Whatman TLC pre-coated plates (LK6D Silica Gel 60A) were then activated overnight at 120^{o}C in oven. Chemical constituents of the crude aqueous methanol and its soluble fractions were analyzed by TLC using glass-backed plates (LK6D Silica Gel 60A). Crude aqueous methanol and its soluble fractions (10 mg) each were dissolved in 1ml of 70% methanol and applied on a pre-coated silica gel (0.2-0.3 mm) and activated glass plates (20×20 cm) in a oven at 100°C for 30 min, 1cm from the bottom edge of the plates. These plates were developed using gradient mixtures of *n*-hexane, ethyl acetate, *n*-butanol and methanol, air dried and visualized under UV light leading to the observed spots which were further confirmed by spraying agents [64]. Development of the chromatograms was done in a closed tank in which the atmosphere had been saturated with the eluent vapour by lining the tank with filter paper wetted with the eluent. Visible bands were marked under daylight and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600) before spraying with freshly prepared *p*-anisaldehyde (1 ml *p*-anisaldehyde, 18 ml ethanol, 1 ml sulphuric acid) or vanillin (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) spray reagents [65]. The plates were carefully heated at 105ºC for optimal colour development. Therefore, Retardation factors (Rf. values) were then calculated using the formula [21]:

$$\text{Retardation factor} = \frac{\text{distance moved by the compound}}{\text{distance moved by the solvent}}$$

Preparative Thin Layer Chromatography

Preparative TLC of the fractions of *A. leiocarpus* was carried out on silica gel coated and activated (0.4-0.5mm thick) glass plates in the selected solvents. Spots of Rf values were marked in each plate and were collected and eluted with ethyl acetate. Elutes were pooled, completely dried and re-chromatographed to test their purity.

Phytochemical studies

The crude aqueous methanol extract and fractions of *A. leiocarpus* were subjected to phytochemical screening according to standard methods [66-70].

Quantitative determinations of the Crude Aqueous Methanol Extract

The detected phytochemical constituents were quantified as described:

Determination of Alkaloids

0.2 g of the plant bark extract was weighed into a 250 ml beaker and 8ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. It was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated NH_4OH was added drop wise to the extract until the precipitation was

completed and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. The percentage alkaloid was calculated using the formula of Kumar and Bhardwaj *et al*. [71].

$$\% \text{ alkaloid} = \frac{\text{Mass of alkaloid (residue)}}{\text{Mass of sample}} \text{ x } 100$$

Let W_1 = weight of sample
W_2 = Weight of filter paper
W_3 = Weight of filter paper + alkaloid

$$\% \text{ alkaloid} = \frac{W_3 - W_2}{W_1}$$

Determination of Tannin

0.1 g of the plant sample was weighed into a 50 ml plastic sample bottle. 10 ml of distilled water was added and shaken for 60 min in a mechanical shaker. This was filtered into 50 ml volumetric flask and made up to the mark. Then 5ml of the filtrate was pipette out into a test tube and mixed with 2ml of 0.1M $FeCl_3$ in 0.1MHCl and 0.008M potassium ferocyanide [$K_3(Fe(CN)_6$]. The absorbance was measured with spectrophotometer at 120 nm with 10 min [67]. The measurement was repeated and the average absorbance was taken.

UV- Ms spectrophotometer reading

1st reading	2nd reading
0.248	0.235

$$\text{Average value} = \frac{0.248 + 0.235}{2} = \frac{0.483}{2} = 0.2415\text{ppm}$$

The % tannins can be calculated as follows:

$$\% \text{ tannins} = \frac{A_n}{A_s} \text{ x } C \text{ x} \frac{100}{W} \text{ x} \frac{V_f}{V_a}$$

Where A_n: Absorbance of test sample
A_s: absorbance of standard solution
C = concentration of standard solution
V_f = total volume of sample used
V_a = Volume of extract used
W = weight of sample used

Determination of Flavonoids

0.5g of the plant sample was repeatedly extracted with 10ml of 8% aqueous methanol of room temperature. The mixture was then filtered using what man no 1 filter

paper. The filtrate was transfer into 250ml beaker and was put into a water bath and allowed to evaporate to dryness and weighed [72]. The percentage flavonoid was calculated using the formula.

$$\% \, flavonoids = \frac{Weight \, of \, flavonoids}{weight \, of \, plant \, sample} \, x \, 100$$

Let W_1= Weight of sample
W_2 = weight of empty beaker
W_3= weight of empty beaker + flavonoids

Determination of Saponins

2 g of plant sample was weighed into a 250 ml conical flask. 10 ml of 20% ethanol (C_2H_5OH) was added. The mixture was heated over a hot water bath for 4 hours with continuous stirring at about 55^{o}C. The mixture is then filtered and the residue re-extracted with another 20 ml of 20% ethanol (C_2H_5OH). The combine extract was concentrated to 16 ml over a water bath at about 90^{o}C. The concentrated extract was then transferred into 250 ml separating funnel and 20 ml of diethyl ether $(CH_3CH_2)_2O$ was added to the extract and shaken vigorously. The aqueous layer was recovered while the diethyl ether $(CH_3CH_2)_2O$ layer was discarded and the purification process was repeated. 60ml of n-Butanol (C_4H_9OH) was then added and the combined n-Butanol was washed with10ml 5%NaCl. The remaining solution was then heated on a water bath to evaporate to dryness and the residue was then weighed [72]. The % saponin was calculated as:

$$\% \text{ saponin} = \frac{\text{Weight of saponin (w3} - \text{w2)}}{\text{weight of plant sample(w1)}} \text{ x } 100$$

W1 = Weight of sample
W2= Weight of empty beaker
W3= Weight of beaker + saponin

STATISTICAL ANALYSIS

Data were expressed as mean ± S.D for each analysis and significant differences were determined by Analysis of Variance (ANOVA), followed by Scheffe's test. $p<0.05$ and $p<0.001$ values were considered significant. Computer statistical package SPSS (version 16) software (SPSS Inc., Chicago, IL, USA) was used for the analysis.

Source of microorganisms

Pure culture of multidrug resistant *Staphylococcus aureus* MRSA isolated from patient from a private hospital in Lapai, Niger State. The organism was subcultured and maintained on Nutrient agar at 37^{o}C for 6 h at the Department of Microbiology, Faculty of Natural and Applied Science, Federal University of Technology, Minna, Nigeria prior to antimicrobial testing.

Methicillin-Resistant Staphylococcus aureus (MRSA) Activity Determination

The sensitivity test of the partitioned fractions was carried out using modified Agar diffusion method [44, 73, 74] to determine the antimicrobial activity. Nutrient agar was prepared by arranging four glass petri dishes on the work bench, after which 19 ml of the nutrient agar was dispense into each of the petri dishes. The plates were prepared in duplicate using nutrient agar. The bacterial isolate (MRSA) was inoculated into each of the petri dish using surface inoculation method. The preparation was left to gel and dry under a hood. Spots where fractions were to be introduced into the plates were carefully marked using a flame-sterilized cork-borer (6 mm internal diameter) and the agar disc was carefully removed with sterile forceps and 1 ml of fraction of different concentrations were added. 1ml of different concentration of the fractions were introduced into the wells using different syringe for different fractions and kept to diffuse for 30 sec. The two antibiotics (penicillin and cloxacillin) were obtained in a local pharmacy store in Kano in ampoule vials as powder for injection. Standard penicillin and cloxacillin were employed as controls at 5 mg/ml in aqueous dilution, reflecting hospital practice in the use of antibiotic discs. Blot disc papers were soaked in each of the control antibiotics as penicillin and cloxacillin (as sodium salt BP) placed on each of the inoculated plates. The plates for both partitioned fractions and controls were kept at 4°C for 1h for diffusion of fractions and drugs respectively, thereafter were incubated at 37°C for 24 h. The zone of inhibition or depressed growth around the wells were observed, measured and recorded. The presence of zone of inhibitions shows the antibacterial activity of the partitioned fractions and no observable activity against MRSA for both penicillin and cloxacillin depicts that the bacterium is resistant the antibiotics. The zone of inhibitions were evaluated by calculating the difference between the clear zone around well and the diameter of the well in accordance with Clinical and Laboratory Standard Institute guidelines [73].

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined for partitioned fractions showing antimicrobial activity against test pathogen using the method of El-Mahmood *et al.* [75] and Vinoth Kumar *et al.* [76]. Under aseptic environment conditions, 0.2 g of the each of the fraction was weighed and dissolved in 5ml of the solvents to give the concentration of 400 mg/ml for each partitioned fractions. Serial dilutions of the partitioned fractions were prepared to give final concentration in the range of 25, 50, 100, 200, 400 mg/ml. 2 ml nutrient broth was dispensed into 20 test tubes each for five different concentrations of 400, 200, 100, 50 and 25 mg/ml for the five partitioned fractions and autoclave for 121^{o}C at 15 min. 1 ml of the different concentration of the fraction was introduced into each test tube as labeled appropriately. A loop fill of the cultured organism was taken and inoculated into these test tubes. The inoculated test tube was cork and incubated at 37^{o}C for 24 h. The test tubes were observed after 24 h for turbidity which interpreted as visible growth of microorganism. The lowest concentration with no turbidity or inhibited growth (i.e. clear test tube) indicates the minimum inhibitory concentration (MIC) of the fractions.

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration was determined using the procedure of Mann [77]. This was determined by selecting test tubes that did not show turbidity or visible growth during MIC determination. A loopful was taken from the test tubes and inoculated on sterile nutrient agar by surface streak plate method in duplicates. The plates were incubated at 37^{o}C for 24 h. The lowest concentration of the fractions that did not showed colony growth on the solid medium was taken as the minimum bactericidal concentration.

RESULTS AND DISCUSSION

In the present investigation, the qualitative phytochemical examination of the crude methanolic extract of *A. leiocarpus* stembark revealed the presence of tannins, saponins, alkaloids, flavonoids, steroids, carbohydrate and phlabotanins but anthraquinone was absent in all the fractions (Table 2); which is in conformity with the previous findings [33]. This study also estimate some of the phytochemical constituents quantitatively which has not been reported in the earlier reports on this plant. The finding indicates that the crude methanol extract of the stem bark of *A. leiocarpus* gave 98.52% recovery (Table 1) and table 3 contained high percentages of tannins (18%), alkaloids (20%), flavonoids (10%), and saponins (12.5%) Plants used in the treatment of disease are said to contain active principles which are phytochemicals with biological activity, some of which are responsible for the characteristic odours and colours of plants while others give a particular plant its medicinal properties [70]. From the present results of the qualitative and quantitative analyses, the derived fractions were found to contain tannins, alkaloids, flavonoids and saponins which are well-known to possess antimicrobial activities [78]. Flavonoids have been reported to be synthesized by plants in response to microbial infection and have been shown to have antibacterial activities [79]. Tannins were also reported have demonstrated activity against bacteria [79]. These kinds of compounds were found to be present in the extract of this plant (Table 2). The extract was found to demonstrate a good antibacterial activity against multi-resistant *Staphylococcus aureus* (Tables 5-7). Various natural products have been shown to possess anti-multi-resistant *Staphylococcus aureus* activities [59-62]. This was measured using zone of inhibition as measurement of greater than or equal to 10 mm indicating good antibacterial activity [77, 79]. Zone of inhibition shown by the partitioned fractions obtained from *Anogeissus leiocarpus* stem bark against multi-resistant *Staphylococcus aureus* (MRSA) are displayed in Figure 2. A concentration dependent activity was exhibited by the extract against this organism, an increase in concentration giving rise to an increase in the zone of inhibition. Out of the four fractions of *A. leiocarpus* stem bark tested the anti-multi-resistant *Staphylococcus aureus* activity of *A. leiocarpus,* methanol fraction was significantly higher (100 mg/ml at $P< 0.05$) than that exhibited by chloroform and ethyl acetate fractions; while *n*-hexane virtually had no activity against *S. aureus* (Tables 5-7). This implies that the methanol fraction has caused inhibition of growth of MRSA at concentration of 100 mg/ml; while chloroform fraction and ethyl acetate fractions caused inhibition at concentration of 200 mg/ml. On the other hand *n*-hexane fraction has not caused any inhibition to the growth of MRSA at any level of its concentration. This is in agreement with the findings of Akande and Hayashi [44]. MIC and MBC values as shown Tables 6 and 7 respectively were evaluated for the partitioned fractions which had shown activity in 'Disc Diffusion Assay'. In the present investigation lowest MIC values (100 mg/ml)

was recorded for MeOH fraction against MRSA, whereas MIC 200 mg/ml was observed for chloroform and ethyl acetate fractions indicating significant antimicrobial potential of partitioned fractions. MIC and MBC values were found to be equal for the two fractions of *A. leiocarpus* indicating cidal activity by their nature. Methanolic fraction at concentration of 100 mg/ml showed significant activity against MRSA (23 mm) better than the remaining two fractions (chloroform and ethyl acetate). The standard controls (penicillin and cloxacillin) produced zones of growth inhibition 25 mm and 20 mm respectively against MRSA. The present findings demonstrated that the stem bark partitioned fractions were sensitive to the test microorganism (MRSA), and thus showed that the partitioned fractions contained anti-multi-resistant *Staphylococcus aureus* agents. On the overall, the results of the sensitivity test showed that methanol partitioned fraction (100 mg/ml at $P< 0.05$) contained more potential antimicrobial agents against MRSA when compared with the remaining two fractions (chloroform and ethyl acetate; 200 mg/ml at $P< 0.05$). It is likely that presence of these metabolites tannins, saponins, alkaloids, flavonoids, steroids and carbohydrate as found in the present study, may be responsible for the anti-multi-resistant *Staphylococcus aureus* activities. Since this test organism, MRSA is associated with various forms of human infections and the present scenario is that existing antibiotics are gradually lost activity against pathogenic microorganisms, this kind of studies should highly be encouraged, so that new and alternative sources for future antibiotics may be explored.

Table 1: Weights and colours of Partition fractions of the Crude aqueous methanolic extract of *Anogeissus leiocarpus* stem bark

Solvent	Fraction	Colour	Weight (g)
Methanol	Crude	Dark brown	29.00
n-hexane	*n*-Hex	Light yellow	2.34
Chloroform	$CHCl_3$	Brown	5.71
Ethyl acetate	EtOAC	Brown	7.10
Methanol	MeOH	Dark brown	13.42

Key: *n*-Hexane = n-Hex, Chloroform = $CHCl_3$, Ethyl acetate = EtOAC, Methanol = MeOH

Table 2: Phytochemical constituents of the Crude aqueous methanolic extract of *Anogeissus leiocarpus* stem bark

S/n	Phytochemical constituent	Test	Observation	Inference
1.	Tannins	Ferric chloride test	Blue-black coloration	+
2.	Saponins	Frothing test	Persistent foam	+
3.	Alkaloids	i. Mayer's test ii. Weagner's test iii. Dragendoff's test	Creamy white ppt Reddish brown ppt Reddish brown ppt	+ + +
4.	Steroids	Salkowki's Test	Reddish brown coloration at interface	+
5.	Carbohydrates	i. Molisch's test ii. Fehling test	Purple ring at interface Reddish brown ppt	+ +
6.	Anthroquinones	Filtrate + HCl +$CHCl_3$ + NH_4OH	Rose pink coloration	-
7.	Phlabotannins	Filtrate + HCl	Red ppt	+
8.	Flavonoids	Filtrate + HCl + Mg	Dirty brown coloration	+

Key: + = present, - = absent

ISSN: 0975-8585

Table 3: Quantitative analyses of phytochemical constituents of the Crude aqueous methanolic extract of *Anogeissus leiocarpus* stem bark

S/n	Phytochemical	Constituent (%)
1.	Tannins	18
2.	Alkaloids	20
3.	Flavonoids	10
4.	Saponins	12.5

Table 4: Thin Layer Chromatography analyses of the Partitioned fractions of *Anogeissus leiocarpus* stem bark

Fractions	Solvent system	No of spot	Rf values	Colour		
				UV	Iodine crystal	Visible light
MeOH	*n*-Hexane: Methanol (4:1)	2	0.59 0.71	Light green	Brown	Colorless
	$CHCl_3$: Ethyl acetate (4:4)	3	0.62 0.78 0.96	Green	Brown	Brown
	$CHCl_3$:Methanol:H_2O (5:1:5:0.5)	1	0.63	Green	Purple	Faint brown
	n-Hexane:Ethyl acetate(4:1)	2	0.19 0.26	Light green	Purple	Brown
	Methanol: $CHCl_3$ (0.5:4.5)	3	0.13 0.33 0.95	Green	Purple	Yellow
n-Hexane	$CHCl_3$: Ethyl acetate (4:4)	3	0.76 0.90 0.94	Green	Yellow	Yellow
	n-Hexane: Ethyl acetate (4:1)	2	0.15 0.46	Yellow	Purple	Colorless
	Methanol: $CHCl_3$ (0.5:4.5)	3	0.15 0.33 0.56	Green	Purple	Yellow
	n-Hexane: Methanol (4:1)	2	0.70 0.82	Light brown	Brown	Yellow
	$CHCl_3$:Methanol:H_2O (5:1.5:0.5)	1	0.15	Light green	Colorless	Colorless
Chloroform	*n*-Hexane: Ethylacetate (4:1)	2	0.06 0.43	Green	Colourless	Colourless
	Methanol: $CHCl_3$: (0.5:4.5)	3	0.10 0.26 0.74	Light green	Purple	Yellow
	n-Hexane: Methanol (4:1)	4	0.27 0.32 0.39 0.49	Light green	Brown	Brown
	$CHCl_3$:Ethylacetate (4:4)	3	0.80 0.88 0.96	Green	Yellow	Yellow
	$CHCl_3$:Methanol:H_2O (5:1.5:0.5)	3	0.37 0.74 0.87	Green	Purple	Faint
Ethyl acetate	*n*-Hexane: Ethyl acetate (4:1)	3	0.04 0.09 0.17	Pink	Purple	Brown
	Methanol: $CHCl_3$ (0.5:4.5)	4	0.23 0.26 0.82 0.97	Green	Purple	Brown
	$CHCl_3$:Methanol:H_2O (5:1.5:0.5)	2	0.74 0.91	Light green	Purple	Faint brown
	n-Hexane: Methanol (4:1)	1	0.61	Green	Colorless	Faint brown

Rf: Retardation factor

ISSN: 0975-8585

Table 5: Sensitivity test of the partitioned fractions against MRSA

Fraction/Zone of inhibition (mm)	Concentration of fractions (mg/ml)				
	400	200	100	50	25
MeOH	S	S	S	S	R
Zone of inhibition (mm)	36	27	24	23	0
Chloroform	S	S	S	R	R
Zone of inhibition (mm)	40	37	35	0	0
n-Hexane	R	R	R	R	R
Zone of inhibition (mm)	0	0	0	0	0
Ethyl acetate	S	S	S	R	R
Zone of inhibition (mm)	37	32	28	0	0
Penicillin (mm at 5 mg/ml)				25	
Cloxacillin (mm at 5 mg/ml)				20	

Key: MRSA = Multi-resistant *Staphylococcus aureus* strain, 0 = no inhibition, R = Resistance, S = Sensitive

Figure 2: Zone of inhibition shown by the partitioned fractions obtained from *Anogeissus leiocarpus* stem bark against multi-resistant *Staphylococcus aureus* (MRSA)

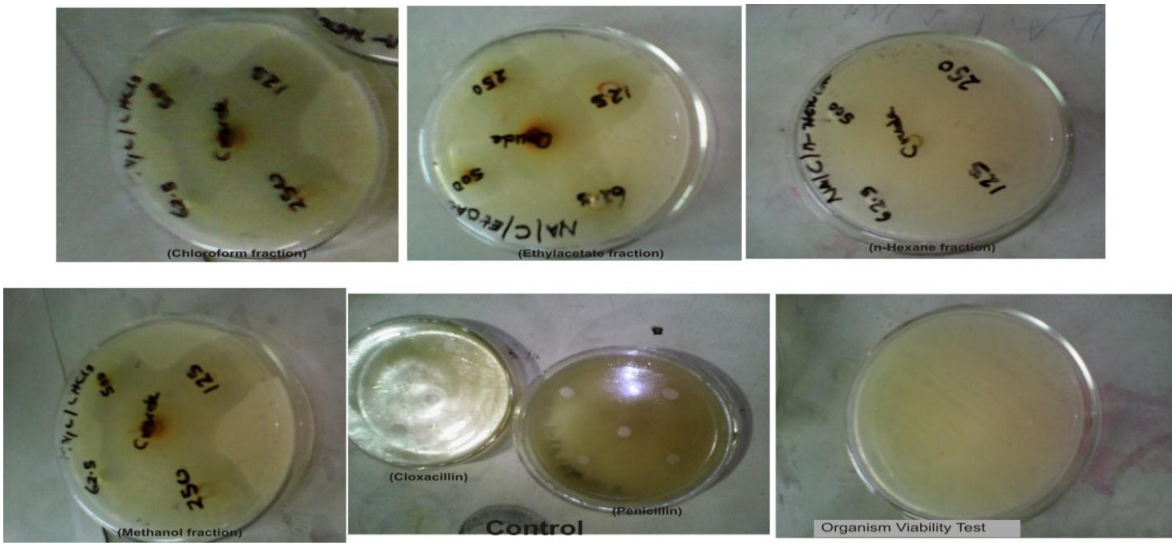

Table 6: MIC of methanol, chloroform and ethyl acetate fractions against MRSA

Fractions	Concentration of fractions (mg/ml)				
	400	200	100	50	
MeOH	-	-	*	+	
Chloroform	-	*	+		
Ethyl acetate	-	*	+		

Key: MRSA = Multi-resistant *Staphylococcus aureus* strain, + = Growth observed, - = No growth, * = MIC

Table 7: MBC of methanol, chloroform and ethyl acetate fractions against MRSA

Fractions	Concentration of fractions (mg/ml)			
	400	200	100	50
MeOH	-	-	*	+
Chloroform	-	*	+	
Ethyl acetate	-	*	+	

Key: MRSA = Multi-resistant *Staphylococcus aureus* strain, + = Growth observed, - = No growth, * = MBC

ISSN: 0975-8585

CONCLUSION

Phytochemical screening and anti-methicillin resistant *Staphylococcus aureus* activity of *A. leiocarpus* stem bark extract reveal that it is a valuable medicinal plant with curative and antibacterial properties. The methanolic extract of the stem bark of *A. leiocarpus* contained good numbers of bioactive chemical constituents which may be responsible for the anti-methicillin resistant *Staphylococcus aureus* activity. The findings of this study, could therefore justify the use of this plant in the management of bacterial infections in traditional medicine. It can be used as a good source for the isolation of safe and natural antistaphylococcal compounds. Further studies are needed to isolate, purify and characterize the active principles responsible for the antistaphylococcal potential of this medicinally important plant, *A. leiocarpus*. This may, therefore, explain the rationale behind the use of the aqueous extracts of *A. leiocarpus* stem barks in the management of infections in traditional medicine.

ACKNOWLEDGEMENTS

The authors are grateful to the Management and members of Staff of the Departments of Chemistry and Microbiology, Federal University of Technology, Minna, Niger State, Nigeria for providing their support and encouragement to carry out this study.

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