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Distribution of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Apparently Healthy Population and its Susceptibility to Saponins from *Dialium guineense*

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Authors' contributions

This work was carried out in collaboration between all authors. Author UN designed and provided the background for the study. Author WOO wrote the first draft of the manuscript. Authors CON and FCK carried out the bench work and managed the analyses of the study while author MOA anchored the literature searches for the study and manuscript preparation. All authors read and approved the final manuscript for submission to BJPR. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The ethanolic extracts of stem bark and fruit pulp as well as saponins from *Dialium guineense* were assayed for antibacterial activity against Gram positive and

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negative strains and clinical strains of methicilin resistant *Staphylococcus aureus* (MRSA) isolated from different locations on human body aged 20-30 years within the University of Nigeria community.
Methodology: Agar diffusion technique was adopted.
Results: The results showed that MRSA is predominant in apparently healthy population of the University community with 100% in males and 92.3% females showing positive case in nasal swab, 87.5% and 96.6 % positive from ear swabs of male and female volunteers respectively; and 77.7% positive from the high vaginal swabs of females. MRSA and other clinical isolates showed higher susceptibility to saponins compared to crude extracts; however, *Bacillus cereus* (NRRL 14724 and 14725) were not susceptible to the saponins from *D. guineense*. The MICs of the saponins were 31.25 mg/mL (*B. subtilis* ATCC 6051, *P. aeruginosa, S. typhi, S. knitambo, P. mirabilis* and *S. aureus*), 62.50 mg/mL (*E. coli*) and 125 mg/mL (*P. aeruginosa* ATCC 10145). Comparable MICs of higher values were obtained with the crude ethanolic extracts of stem bark and fruit pulp against MRSA and clinical isolates.
Conclusion: The present findings revealed wide distribution of MRSA in an apparently healthy population in Nigeria and the susceptibility patterns showed the presence of a broad spectrum antibacterial agent in *D. guineense*.

Keywords: Staphylococcus aureus; saponins; methicillin-resistant; inhibition zone diameter; Dialium guineense.

1. INTRODUCTION

The World Health Organization (WHO) estimates showed that about 80 % of world's population depended on medicinal plants for treatment of diseases [1]. One of such plants is *Dialium guineense* Wild (*Fabaceae*) generally referred to as velvet tarimand or black velvet. The shrub is well distributed in West Africa [2,3] and is locally used in improvement of lactation and control of genital infection [2], treatment of haemorrhoid, bronchitis, malaria fever, and severe cough [4], enteric and respiratory infections [5]. Various morphological parts of *D. guineense* also possess antimolluscicidal, antiulcer, antidiarrhoea and antioxidant properties [6]. Despite threats of extinction facing velvet tarimand in Africa as a result of deforestation, encroachment upon logging and human settlement, the pulp, leaves, stem, bark and roots of the plant still find usefulness in herbal medicines and folkloric management of stubborn infectious diseases. MRSA also called oxacillin-resistant *Staphylococcus aureus* (ORSA) is a strain of *Staphylococcus aureus* that has developed resistance to beta-lactam antibiotics and to the cephalosporins which are responsible for difficult-to-treat human infections such as necrotizing pneumonia, sepsis and toxic shock syndrome. Such resistance is mediated by acquisition of extra-chromosonal genetic elements containing resistant genes such as plasmids, transposable genetic elements and genomic islands [7-9]. MRSA contains genomic island called staphylococcal cascatte chromosome (*SCCmec*) which encodes *mecA* gene responsible for antibiotic resistant [9-11] and based on its virulence and prevalence, has been classified into hospital acquired- (HA-MRSA) and community acquired- (CA-MRSA). Infections by CA-MRSA, such as skin infection, bone and joint infection and abscess formation [12], are completely asymptomatic; infections due to HA-MRSA are more virulent but less prevalent in developed world. Documented preventive measures for MRSA included personal hygiene [13] such as hand washing with antiseptics and disinfectants [14,15], copper alloys [16-17], surface sanitizers and restricted use of glycopeptides, cephalosporins, and fluoroquinolones [18,19]. The standard treatment

guidelines for MRSA showed that CA-MRSA has greater spectrum of antimicrobial susceptibility to sulfa drugs (sulphamethoxazole/trimethoprim), tetracyclines, clindamycin, vancomycin and teicoplanin [20,21] while HA-MRSA is susceptible only to vancomycin, daptomycin and linezoid [21-25]. Despite promising susceptibility patterns to different antibiotics and effective preventive measures, MRSA has persisted in the community and hospital environment causing devastating health challenges. The current challenges posed by MRSA has been attributed to the global increase in the HIV/AIDS and other related risk factors [26], antibiotics abuse and emergence of the new MRSA bacterium called vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) [27,28]. While the presence of MRSA is hardly noticeable, available remedies are very costly [29] and inaccessible to many people resident in regions worst hit by the infections. In view of these, we investigated the distribution of MRSA in an apparently healthy population, selected from community and hospital environment, and explored alternative efficacious and safe remedy for the treatment of infections caused by MRSA using crude extracts and saponins from *Dialium guineense.*

2. MATERIALS AND METHODS

2.1 Materials

The materials used in the experiment were based solely on the design of extraction from the plant materials and the antimicrobial test. These include saline tablet, nutrient agar, nutrient broth, methanol, petroleum ether, acetone, *n*-butanol and diethyl ether, all were of analytical grades procured from Sigma-Aldrich, Germany.

2.2 Methods

2.2.1 Collection and preparation of plant materials

The plant, black velvet (*Dialium guineense*) stem bark and fruit pulp were collected from Enugu-Ezike, Nigeria. The plant materials were identified and authenticated by Mr. A. Ozioko of the International Centre of Ethnobotanical and Ethnomedicinal Development (INTERCEED) and a voucher specimen was deposited in the herbarium unit of the Department of Botany, University of Nigeria, Nsukka. The stem bark of *D. guineense* was properly washed with distilled water, chopped into tiny chunks, dried under shade at 25ºC and pulverized. The fruits were dehusked and the fruit pulp was kept to dry at room temperature and then, milled into fine bits. The pulverized samples were stored at room temperature until used.

2.2.2 Extraction of *Dialium guineense*

About 100 g of the pulverized stem bark was macerated in 400 mL ethanol, while the same quantity of fruit pulp were macerated in 400 mL of both cold and hot water, each for 8 h and filtered. The filtrates were concentrated to dryness in a steady air current at room temperature for 24 h. The concentrates were stored in a sterile glass container at refrigeration temperature of 4ºC until used.

2.2.3 Extraction of saponins from *Dialium guineense*

The pulverized stem bark of *Dialium guineense* was defatted with petroleum ether. The marc obtained was dried and extracted with aqueous methanol (80:20). The aqueous residue was further partitioned into *n*-butanol (200 mL) and filtered. The mixture was allowed to stand for 12 h in a separating funnel. After the phase separation, the n-butanol soluble which contains the saponins were re-extracted with 50 mL methanol and then precipitated with 50 mL acetone.

2.2.4 Phytochemical analysis of extracts from *Dialium guineense*

The phytochemical constituents of the extracts were determined by standard methods [30,31].

2.2.5 Isolation of methicillin resistant *Staphylococcus aureus* (MRSA)

Sterile swab sticks were used to collect swab samples from high vaginal region in females, nasal and ear swabs from both males and females with full consent of the volunteers. The swab samples were cultured in manitol salt agar and incubated at temperature of 30ºC after which identification was carried out. Identification of the isolates was carried out microscopically. The MRSA isolates appeared in tiny clusters at magnification of x500. The isolated *Staphylococcus aureus* were subjected to methicillin antibiotic disc and the strains resistant were kept for further studies while susceptible strains were discarded. The sample populations were university undergraduates and University of Nigeria Medical Centre (UNMC) personnel with age ranges between 20-30 years.

2.2.6 Collection of test bacteria strains

The test bacteria strains were cultures of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi* and *Proteus mirabilis* all of clinical strains were collected from the Virology unit, Department of Microbiology, University of Nigeria Nsukka. Type cultures of *S. aureus* ATCC 12600*, Bacillus subtilis* ATCC 6051, *Salmonella kitambo* SSR 113, *E. coli* ATCC 1175, and *P. aeruginosa* ATCC 10145 were collected from the International Centre for Ethnobotanical and Ethnomedicine Development (INTERCEED) Nsukka while type cultures of *Bacillus cereus* NRRL 14724 and 14725 as well as clinical strains of MRSA were variously isolated from high vaginal swabs (HVS), nasal swabs (NS) and ear swabs (ES) respectively.

2.2.7 Preparation and standardization of inocula

The 12 test bacteria isolates, aside the MRSA were sub-cultured from nutrient agar slants into sterile nutrient broth and incubated at 37ºC for 18 h. Each of the test organisms was standardized to 0.5 McFarland's standard which is equivalent to 1.5 x 10^8 cfu/mL using sterile normal saline for seeding on the Meuller-Hilton agar (MHA) plates.

2.2.8 Susceptibility of MRSA to crude extracts of *Dialium guineense*

Agar diffusion technique was employed for the determination of the susceptibility of MRSA to crude extracts of *D. guineense* [32-34]. Briefly, 500 mg of dried ethanolic extract of *Dialium guineense* was weighed into a test tube containing 2 mL distilled water and solubilized on votex mixer. Sterile dishes containing 25 mL solidified Meuller Hinton agar medium were

labeled with the test microorganism and seeded with the corresponding standardized test microorganisms. A 6 mm cork borer was used to bore five holes per dish and 100 µL pipette was used to introduce two drops of different concentrations (250, 125, 62.5, 31.25 and 15.625 mg/mL) of the extract to the respective holes. The plates were incubated at 37ºC for 24 h after a diffusion time of 20 min was allowed. The inhibition zones of test strains susceptible to the extracts were measured.

2.2.9 Susceptibility of the bacteria to saponins extracted from *Dialium guineense*

A 500 mg/mL concentration of saponins was prepared with sterile distilled water and further diluted serially two-fold to obtain five different concentrations (250-15.625 mg/mL) of the extract. Agar diffusion technique was also adopted in the study [33].

2.2.10 Minimum inhibitory concentrations (MICs) of the ethanolic extracts and saponins

The MICs of the ethanolic extracts and saponins from *Dialium guineense* determined using agar diffusion method [34] were evaluated by plotting the IZD^2 (mm^2) against the logarithm of concentration of the extracts/saponins. The point of intersection on the log concentration-axis was used to calculate the MICs.

3. RESULTS AND DISCUSSION

Table 1 shows the phytochemical composition of the crude ethanol extracts (stem bark and fruit pulp) of *Dialium guineense*. The phytochemical constituents showed the presence of alkaloids, saponins, reducing sugar and carbohydrate. Their presence, however, was in varied quantity with saponins being the most abundant in both the stem bark and fruit pulp extracts. Hence further extraction of the saponins was undertaken.

Table 1. The phytochemical composition of *Dialium guineense*

Constituents	Relative abundance	
	Stem bark	Fruit pulp
Alkaloid	++	++
Flavonoid	++	+
Glycosides	+	+
Saponins	+++	+++
Tannins	+	+
Terpenes	+	+
Reducing sugar	+	+
Carbohydrate	++	++

+=low concentration, ++=medium concentration, +++=high concentration, - =absence

Furthermore, the phytochemical analysis of the ethanolic extract of the stem bark of *Dialium guineense* showed the presence of alkaloids, tannins, glycosides, saponins and terpenes which are known to possess antibacterial/antimicrobial properties [2].

The distribution of MRSA in ear swab (ES) from male and female, nasal swabs (NS) from male and female, and high vaginal swabs (HVS) from female sample population is shown in Table 2. The results indicate that 100 and 87.5 % of the male NS and ES respectively were

positive to MRSA while 92.3, 96.6 and 77.7 % were positive in female NS, ES and HVS respectively.

Table 2. Distribution of MRSA in different swab samples

Swabs samples	Sex	Number of samples	Positive cases (%)	Negative cases (%)
Nasal	Male	14	14 (100 %)	0 (0.0 %)
	Female	26	24 (92.31 %)	2 (7.69 %)
Ear	Male	16	14 (87.5 %)	2 (12.5 %)
	Female	29	28 (96.56 %)	1 (3.44 %)
High vaginal	Male	0	0 (0.0 %)	0 (0.0 %)
	Female	9	7 (77.7 %)	2 (22.3 %)

MRSA were found to be widely distributed in the apparently healthy population that was sampled. From the nasal, ear and high vaginal swab samples taken, significant number of the population yielded positive strains of MRSA. The male sample population yielded more of the MRSA from their nasal swabs, yet apparently they remained healthy. This could be attributed to the asymptomatic characteristics of MRSA among the healthy populace who might be carriers of this microorganism. However, insight into the differences in the distribution of MRSA among carriers of different sexes is beyond the scope of this study.

The result of the susceptibility pattern of 39 strains (22 strains ES, 13 strains NS and 4 strains HVS) of MRSA to saponins and crude extracts of *Dialium guineense* stem bark and fruit pulp is shown in Table 3. Among the clinical strains and type cultures tested, it was observed that *B. subtilis* and *P. mirabilis* were the most susceptible to the ethanolic extract of stem bark and fruit pulp respectively while *S. typhi* and *P. aeruginosa* were the least susceptible to stem bark and fruit pulp extracts respectively.

Table 3. Susceptibility of strains of MRSA to crude extracts of *Dialium guineense*

Swabs/Strains	Number of swabs/strains	Inhibition zone diameters (mm) (Mean ± SD)		
		Fruit pulp	Stem bark	Saponins
Ear swab	22	18.0±0.5	22.0±0.7	25.0±0.9
Nasal swab	13	20.0±0.1	18.0±0.6	32.0±0.3
High vaginal swab	4	11.0±0.4	15.0±0.6	28.0±0.5
B. subtilis (ATCC 6051)	1	15.0±0.3	16.0±0.4	18.0±0.4
P. aeruginosa (ATCC 10145)	1	5.0±0.7	11.0±0.3	21.0±0.4
P. aeruginosa (clinical isolate)	1	8.0±0.8	9.0±0.9	20.0±0.3
S. typhi	1	12.0±0.3	8.0±0.8	13.0±0.5
S. kitambo (SSRL 113)	1	10.0±0.3	9.0±0.9	14.0±0.2
B. cereus (NRRL 14724)	1	13.0±0.3	6.0±0.7	0.0±0.0
B. cereus (NRRL 14725)	1	8.0±0.8	9.0±0.9	0.0±0.0
P. mirabilis	1	16.0±0.4	10.0±0.3	16.0±0.1
S. aureus (clinical isolate)	1	12.0±0.3	10.0±0.3	16.0±0.4
E. coli (clinical isolate)	1	14.0±0.3	13.0±0.4	21.0±0.4
E. coli (ATCC 11775)	1	8.0±0.8	12.0±0.3	17.0±0.8

It was observed that the ethanolic extract of the stem bark was more potent in inhibiting the growth of the strains of the tested microorganisms than the ethanolic extract from the pulp as could be seen from the IZD values. Of the 39 strains of MRSA subjected to the activities of the crude ethanolic extract and saponins from *D. guineense*, 82.05 % of the strains were susceptible while 17.95 % were resistant. This may be hinged on the fact that secondary metabolites yielded activity against these organisms while the resistant strains may have extra chromosomal substances quite different from others that may have conferred on it the resistance. The extracts also showed higher activity against Gram positive organisms, taking its activity against MRSA as an instance; however, both Gram positive and Gram negative strains responded by showing 100 % susceptibility to the test extracts, thus confirming the presence of a broad spectrum antibacterial agent in the plant extract used, which might be attributed to the phytoconstituents. The Gram negative test strains showed a 100 % susceptibility to the saponins fraction while the Gram positive showed 60 % susceptibility [35,36]. The reduction in the activity against the Gram positive test bacterial strains may be an indication of other substances acting in combination (synergism) with saponins in the crude fraction.

The results of the minimum inhibitory concentration (MIC) of the crude extracts and saponin from *D. giuneense* are shown in Table 4. The results further explained the non sensitivity of *B. cereus* NRRL type 14724 and 14725 strains to the saponins. It was observed that the saponins at concentration >> 500 mg/mL could not inhibit the growth of the strains of *B. cereus* as shown in the susceptibility and MIC results (Tables 3 and 4).

Table 4. MIC of ethanolic extracts and saponins from *D. guineense* against some clinical isolates and bacterial strains

Bacterial strains	Minimum inhibitory concentration (mg/mL)		
	Stem bark	Fruit pulp	Saponins
B. subtilis (ATCC 6051)	250.0	125.0	31.25
P. aeruginosa (ATCC 10145)	31.25	250.0	125.0
P. aeruginosa (clinical isolate)	31.25	250.0	31.25
S. typhi	62.5	125.0	31.25
S. kitambo (SSRL 113)	31.25	125.0	31.25
B. cereus (NRRL 14724)	250.0	125.0	>500
B. cereus (NRRL 14725)	250.0	250.0	>500
P. mirabilis	62.5	125.0	31.25
S. aureus (clinical isolate)	125.0	125.0	31.25
E. coli (clinical isolate)	31.25	125.0	62.50
E. coli (ATCC 11775)	31.25	250.0	62.50

4. CONCLUSION

MRSA which constitutes a major nuisance in the Western world but hardly talked about in the Third world has been shown to be widely distributed in the apparently healthy population of Nigerian society. However, *Dialium guineense* extracts proved to be active against these strains; so also, was activity against type cultures and other clinical strains high. Most importantly, the susceptibility patterns of the test strains showed the presence of a broad spectrum acting agent in the plant. Noting the fact that this plant, mostly, is used as food in the South-eastern part of Nigeria, and not common in herbal practices; however, this research has shown that even when it is consumed as food, it has antibacterial properties.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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