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Incidence and *in vitro* Susceptibility of Methicillin-Resistant *Staphylococcus aureus* Isolated from Ekiti State to Saponin Extract from *Phyllanthus niruri*

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ABSTRACT: The incidence of Methicillin-resistant *Staphylococcus aureus* (MRSA) in the hospitalized patients and the community for a 2-year period and the antibacterial activities of saponin extracted from *Phyllanthus niruri* on the bacterium were investigated. Of the 269 MRSA isolates, 45 isolates were from in-patients while 224 were from out-patients. The predominant isolates in the out-patients were from abscess, cellulites and impetigo at 38%, 29%, and 16% respectively. For the in-patients, the predominant isolates were from abscess and traumatic wound each at 7%. The proportion of *S. aureus* infections caused by MRSA remained stable over the 2 year period. Community-acquired *S. aureus* (CA-MRSA) strains increased from 4%-23% whereas the proportion caused by Hospital-acquired *S. aureus* (HA-MRSA) decreased from 25%-5%. The susceptibility of MRSA to crude saponin showed that of the 269 isolates,197 isolates showed inhibition zone of diameter ranging between 5.0mm and >9.0mm while 72 (27%) of the isolates showed zone of inhibition of diameter $\leq$ 4.0mm. This shows that only 27% of the MRSA isolates were resistant to saponin extract. The risk factor and the spread of MRSA were ascribed to numerous factors.

Keywords: Methicillin-resistant *Staphylococcus aureus, Phyllanthus niruri*, Incidence, Saponin, *in-vitro* susceptibility.

INTRODUCTION

Staphylococcus aureus is a well known pathogen of humans and animals. Methicillin-Resistant *S. aureus* (MRSA) was recognised as a nosocomial pathogen in the 1960s and now represents a substantial proportion of *S. aureus* infections in hospitalized (in-patients) and community (out-patient) settings (Klevens, *et al*.,2006). Risk factors of healthcare associated MRSA(HA -MRSA) are well defined and included hospitalization, surgery, dialysis, residence in a long- term care facility, and use of indwelling catheters or other percutaneous medical devices (Fridkin *et al*., 2005). Factors that appear to facilitate transmission of Community association MRSA (CA-MRSA) include frequent skin- to-skin contact, crowding, compromised skin integrity, sharing of potentially contaminated items and lack of personal hygiene (Dancer, 2008).Hospital-acquired (HA- MRSA) and Community-acquired (CA-MRSA) possess

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resistance to β-Lactam antimicrobial agents, conferred by the Staphylococcal cassette *mec* element (Hiramatsu *et al*., 2002). However, CA-MRSA strains are typically less resistant to non- β-lactam antimicrobial agents (Leonard and Markey, 2008).

Historically, plants have provided antimicrobials that produced successful results in the treatment of notable bacterial infections. Their potency has been ascribed to possession of bioactive agents (phytochemicals) which act either singly or synergistically. One of such plant is *Phyllanthus niruri,* a Euphorbiaceae, which has been known to be used in the treatment of jaundice, urinary discharge, malarial, hepatitis B- virus anuria, asthma and as a diuretic (Mukherjee and Wahile, 2006). Owing to its bitter taste, it is also believed to be beneficial to the digestive system as an appetite stimulant and a fortifying tonic (Rain, 2002). In India, it is used as a mild laxative for dysentery. It is also known to relieve intestinal pain. Fakirs use it as an intestinal anaesthetic (Ghendon, 1987). Topically, it seems to have a soothing effect against scalp, genital and anal itching (Rain, 2002). Saponins, one of the phytochemicals in *P. niruri* are naturally occurring surface – active glycosides that derived their name from their ability to form stable, soap-like foams with aqueous solution. They are known for their hypocholesterolic, anticarcinogenic, immune boostering and antibacterial properties (Ovesna *et al*., 2004). Their physiological, immunological and pharmacological properties have provoked considerable clinical interest. Saponins are divided into three major groups: triterpenoid, basic steroidal and steroid saponins.

This study investigated and compared the incidence of MRSA in hospitalized patient and the community setting between August 2006 and July 2008. It evaluated the antibacterial activity of saponin extract from *P. niruri* on the isolates.

MATERIALS AND METHODS

Collection of plant material

Phyllanthus niruri was collected from shrubs around the Federal Polytechnic compound, Ado- Ekiti**,** Nigeria between the months of July and September, 2008 and was identified at the Department of Plant Science, University of Ado-Ekiti, Ekiti- State, Nigeria. A voucher specimen (No 06/242) was deposited at the herbarium of the Department of Science Technology, Federal Polytechnic, and Ado-Ekiti. The samples used for the analysis were air-dried at room temperature of $\pm 27^0$C and pulverized

Extraction of Crude Saponin

The saponin was extracted according to the method described by Otsuka *et al.* (2005). The milled plant (170g) was defatted using 700mL of petroleum ether for 72h with the aid of Soxhlet for about 24h. Seven hundred (700mL) millilitre of methanol was used to extract saponin from defatted sample and the residue was left overnight under reflux at 70^oC. It was then filtered and the filtrate evaporated to dryness. The yield was dissolved in 300mL distilled water and mixed at ratio 1:1 with methanol in a separating funnel. The set up was allowed to stand for three days and two layers were formed. The bottom layer was discarded while the upper layer, which contained the crude saponin, was allowed to evaporate for 2 weeks.

Methicillin–Resistant *Staphylococcus aureus* (MRSA)

From year 2006 through 2007 specimen samples were collected from patients visiting General Hospitals in Ado-Ekiti, Ikere-Ekiti, Ifaki- Ekiti and Medical centre, Ido-Ekiti; in Ekiti State, Nigeria. The samples collected included wound/pus, sputum, urine, blood, medical devices, respiratory swabs, gynaecologic specimens, stool and synovial fluid. Samples for MRSA detection were put on plates. *S. aureus* was confirmed by bound coagulase test. Isolates found to be oxacillin resistant ($\leq$ 10mm) or had intermediate resistance to oxacillin (11-12mm) on a Kirby-Bauer disk diffusion assay were further tested by E- test on Mueller-Hinton agar with 2% Sodium chloride incubated for 24h, at 37^oC. Those with a MIC $\geq$ 4µmol/mL were considered to be MRSA (Louiselle *et al.*,

2006). MRSA identification was performed by using standard method (Harbarth *et al*., 2006) according to the guidelines of the clinical and laboratory standard institute (CLSI) recommendations (CLSI, 2007).
An infection was considered to be healthcare onset if MRSA culture was obtained within 48h after a patient had no evidence of the infection at the time of admission. A MRSA culture obtained within 48h of hospital admission or evidence of infection on admission was considered as an indication of community – onset infection.

Antimicrobial assay
The disk diffusion method described by Brady and Katz (1990) was employed. Various concentrations of the extract were prepared on test tubes (0.625mg/mL-10mg/mL). Disks obtained from Whatman No 5MM filter paper were sterilized in an oven at 60^{o}C for 30minutes and soaked in the extract for 2hrs. A loopful of the final dilution (10^3) of the test bacterial suspension was spread on dried nutrient agar (Oxoid). The disks of different concentrations of the extracts were placed equidistance on the agar and incubated at 37^{o}C for 24hrs. Zones of inhibition were measured in millilitres with a metre rule. Experiments were in triplicates.

Statistical analysis
Trends in MRSA incidence were evaluated by using Poisson regression with time in years as the predictor variable, case number as the dependent variable and population size as an additional exposure variable (Agresti, 2002). A sub analysis, comparing in-patients with out-patients, was also performed. χ^2 or Fisher exact test as appropriate were a 2-sided significance level of 0.0001 was used. Data analyses were performed by using statistical package version 9**.**

RASULTS AND DISCUSSION

Of the 269 *S. aureus* hospital isolates, 45 from inpatients and 224 were from outpatients. 42% were cultured in the hospital setting, 14% in the Emergency Department, 42% in the Ambulatory Care and 2% from long-term-care units. For the outpatients, the highest number of isolates was recovered from abscess, cellulites and impetigo at 38%, 29% and 16% respectively. For the inpatients, the isolates were cultured from abscess and trauma wound at 7% each among inpatients. Table 1 shows the proportion of *S. aureus* infections caused by MRSA remained stable over the two year period.
CA-MRSA strains increased from 4%-23% of all *S. aureus* infections (χ2 for trend ($p <$ 0.0001) over the two year study period, whereas the proportion caused by HA-MRSA decreased from 25% - 5% (χ^2 for trend $p <$ 0.0001) (Fig 1). The yearly increases in the CA-MRSA strains were relatively modest, but the cumulative effect was a major and previously unrecognized shift in the epidemiology of MRSA in the study area. This trend may be due to altered virulence or transmissibility of MRSA in general or particular strains (Tristan *et al*., 2007). Changes in the host that affect vulnerability (e.g. increasing levels of obesity and diabetes or intravenous drug use or transmission dynamics (e.g. increasing use of day care settings) (Smith and Cook, 2005) or changes in the environment such as widespread use of antimicrobial agents or changes in hygiene behaviour. However, these explanations are speculatory and ignorance of these factors during such major change is worrisome, particularly in view of the international concerns about emergency of CA-MRSA.

Table 1: Infection type for hospitalized patients with CA-MRSA infection

Infection type	Total No(%) infections n= 269	In-Patient n=45	Out-patient n=224	p-value
Skin (any)	169(62.8)	10 (44.4)	159 (71)	<0.0001
Impetigo	29(10.8)	2(11.1)	27	<0.001
Foliculitis	3 (1.1)	0	3	<0.0001
Abscess	65(24.2)	3	6	<0.001
Furunculosis	9(3.3)	0	9	<0.0001
Hidradenitis	1(0.4)	0	1	<0.001
Cellulitis	49(18.2)	2	47	<0.0001
Abrasion	4(1.4)	0	4	<0.001
Pressure wound	4(1.4)	0	4	<0.001
Trauma wound	3(1.1)	3	0	<0.001
Necrotic lesion	2(0.6)	0	2	<0.001

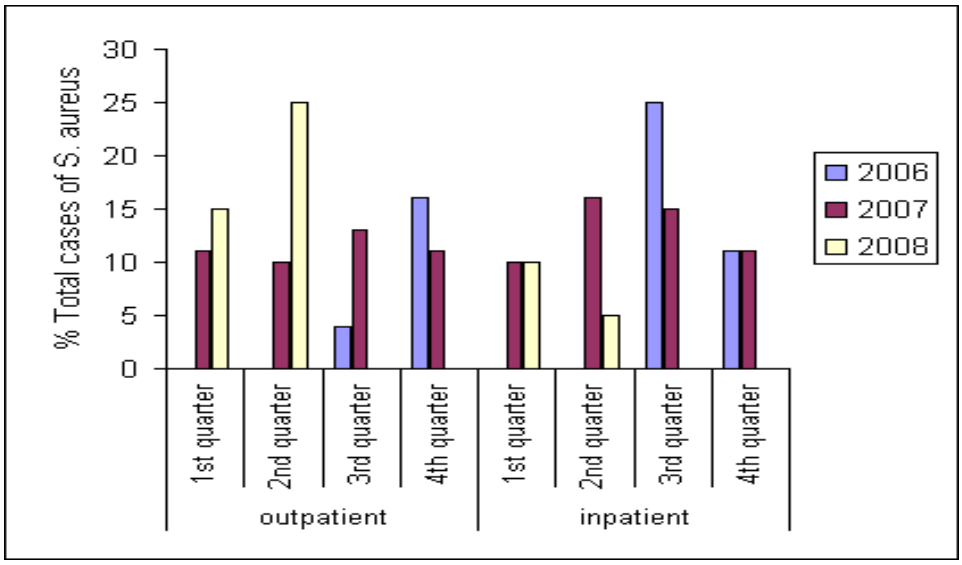

Fig 1: Proportion of *S. aureus* due to CA-MRSA infection and HA-MRSA infections by quarter and year Aug. 2006- July 2008

Patrice *et al*. (2008) reported that resistance to fusidic acid or susceptibility to gentamicin should not be used as phenotypic criteria for CA-MRSA since the isolation of MRSA in substantial population of the culture was common. The increasing incidence of PVL-producing type IV CA-MRSA isolates was reported by Patrice *et al.* (2008) to be worrisome and indicated emergence of new MRSA lineages with a particular fitness for community transmission.

Dancer (2008) observed that an increase in CA-MRSA has been ongoing for at least 15years, preceding the major (and largely separate) nosocomial increase in MRSA. These findings correlate with this study. Although there is marked increase in admissions, community-onset staphylococcal infections which are severe enough to merit admission; remain comparatively rare.

The use of medicinal plants constitutes an important part in traditional or folkloric medicine in Africa. Modern orthodox medicine has improved the health of many people all over the world. It is noteworthy that in many cultures, modern medicine complements traditional practices by what is obtained in China (Odugbemi, 2007). With the above findings, it is noticed that the saponin extract exhibited potency on many of the test bacteria.

In recent years, outbreaks of MRSA infection have been reported in different settings, out-patient settings visiting hospitals (Fenfang e*t al.*, 2005), hospitalized or surgery patients (Iyer and Jones, 2004), as well as nursing homes and conventional facilities (Louiselle *et al*., 2006). The problems of MRSA are increasing worldwide. MRSA is no longer restricted to hospital settings but found in homes, places of work and kindergartens (Wulf *et al.*, 2008). A number of risk factors for MRSA infection have been identified in these studies to include antimicrobial drug use, close contact with persons colonized with MRSA and barriers to medical care. Antibiotic drug self-medication is a matter of concern because it has contributed to the spread of antimicrobial resistance. Self treatment with a drug that is ineffective against a disease causative organism or with an inappropriate dosage may increase the risk of selection of resistant organism that may be difficult to eradicate. These resistant organisms may then be transferred into the community. Unrecognized CA-MRSA colonization during hospitalization could become an additional method of its dissemination in the community. Increased prevalence of CA-MRSA has been reported in Chicago, Los Angeles, Texas and Minnesota (Fergie and Purcell, 2001). Susceptibility to saponin was observed in 73% of the MRSA isolates. This result shows that saponin extracted from *P. niruri* could be a therapy in this circumstance.

The susceptibility of MRSA to the crude saponin is shown in Table 2. Of the 269 isolates, 197 (79%) isolates showed zone of inhibition of diameter ranging between 5.0mm to $\geq$ 9.0mm while 72 (27%) of the isolates had zone of inhibition $\leq$ 4.0mm in diameter.

Table 2: Susceptibility of Methicillin–Resistant *S. aureus* to Crude saponin (1.5 mg/ml) extract

Diameter of zone of inhibition (mm)	No (%)
$\geq$ 9.0	23 (9)
8.0	31 (12)
7.0	31 (12)
6.0	56 (20)
5.0	56 (20)
$\leq$ 4.0	72(27)*

*Resistant

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