In-Vitro Antibacterial Activity of Bioactive Plant Extracts against Methicillin-Resistant Strains of *Staphylococcus aureus*

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Abstract

Methanol extract of leaves of five different Nigerian plants were tested for activity against three drug resistant strains of Staphylococcus aureus by the agar well diffusion and broth microdilution techniques. The S. aureus strains were composed of two clinical isolates and one reference strain. Promising antimicrobial activity was displayed by three of the five plants tested. Minimum inhibitory concentration (MIC) of the extracts ranged from 25 mg/ml to 100 mg/ml. The clinical isolates had comparable level of sensitivity to the three most active extracts. Both were also more susceptible to the extracts than the reference strain. Phytochemical analyses showed that secondary plant constituents were present in varied concentrations in all the extracts.

Keywords: Antibacterial activity, Minimum inhibitory concentration, Phytochemcial analyses

Introduction

Man has used indigenous plants for treatment of infections and diseases since prehistoric time because they are known to contain components of therapeutic value (Nostro *et al.*, 2000). Folklore medicinal practitioners claimed to have learnt by observing that sick animals change their food preferences to nibble at herbs they normally would not eat; possibly for curative effects (Huffman, 2003).

The increasing incidence of microbial resistance to previously choice drugs presently constitutes a huge medical challenge to quality health care delivery. As a result, medics are faced with narrow treatment options which often require the use of expensive and sometimes toxic alternatives. Practitioners across the disciplines of medical science continue to search for new compounds or leads that might birth the development of new and more potent antimicrobials. Those searches have hitherto moved in one direction – the direction of natural medicinal products and ethnopharmacology (Maoz and Neeman, 1998; Hammer *et al*, 1999; Nostro *et al*., 2000). One very important product of such 'foraging' was the discovery, long ago, that raw honey dressings were effective in the prevention and treatment of methicillin resistant *Staphylococcus aureus* infections (Cooper *et al*., 2001; Al-Waili, 2004; French *et al*., 2005; Blaser *et al*., 2007).

S. aureus produces disease by invasion and subsequent tissue destruction, whether locally or through the effect of haemolysins (Cooper *et al*., 2002). It has the greatest tendency to develop resistance to antibiotics to which the strains were previously sensitive. The resistance of *S. aureus* to all penicillins, including methicillin and other narrow spectrum β-lactam antibiotics has been reported (Saderi *et al*., 2008; Hon *et al*., 2008; Anas *et al*., 2008; Agil *et al*., 2008). According to Guignard *et al*. (2005), *S. aureus* became methicilin resistant (MRSA) by acquiring a mec A gene usually carried on a larger piece of DNA called Staphylococcal cassette chromosome, SCC mec, of which expression of mec A yields a penicillin binding protein, PBP2a, with reduced affinity for β- lactam antibiotic binding. Presently, orthodox therapies against MRSA are beset with several challenges. Vancomycin and teicoplanin; both glycopeptide antibiotics currently used in the treatment of MRSA (Schentag *et al*.,1998), have very low rate of absorption and must be administered intravenously to control systemic infections (Janknegt, 1997). Equally, several new strains of MRSA have been found showing resistance to Vancomycin and teicoplanin (Schito, 2006). Patients with *S. aureus* have, on the average, three times the length of hospital stay, three times the total charges and five times the risk of in-hospital death than non-Staphylococcal inpatients (Noskin *et al*., 2005).

These shortcomings in the treatment of MRSA infections propelled us to join the search for natural products that would be better treatment options in the management of microbial infections especially those caused by MRSA. In this study, the antibacterial activity of methanol extracts of five Nigerian plants against different strains of methicillin resistant *S. aureus* was investigated.

Materials and Methods

Plant extract preparation: The plants used in this study were gifts from the Herbarium of the Department of Botany, University of Nigeria, Nsukka and were as follows: *Acalypha wilkesiana, Costus afer, Phyllanthus muellerianus, Pterocarpus santalinus and Vitex doniana.* The whole leaf samples were rinsed thoroughly in running tap water, air dried under ambient conditions (temp. 28 ± 2^0C) and ground into fine powder in a waring blender (Waring International, New Hartford, CT, USA) before extraction. Samples (30 g) of powdered plant materials were each macerated in 150 ml of analytical grade methanol for 72 h. The

macerate was first passed through double layered muslin cloth, then filtered with Whatman No.1 filter paper (Whatman Lab. Division, Springfield Mill, Maidstone, Kent, England) before the filtrates were evaporated to dryness by a steady air current for about 48 h in previously weighed Petri dishes. After evaporation, the Petri dishes were weighed with the residue and the weight of the dry extracts determined. The extracts were sterilized by exposure to ultraviolet radiation for several hours. Sterility was confirmed after 24 h if there were no growth on streaked Nutrient Agar plates (Nkere and Iroegbu, 2005). One gram portion of each extract was subjected to phytochemical screening according to the methods of Harbone (1973), while the rest were preserved aseptically in amber bottles at 5^0C until required for further use (Gupta *et al*., 1996).

Organisms and growth conditions: The microorganisms used in this study were different cultures of *Staphylococcus aureus* labeled as Test strains 1-3. The organisms were obtained from the culture collections of the Medical Microbiology Laboratory, Department of Microbiology, University of Nigeria, Nsukka, Nigeria. Test Strain 1 was a clinical isolate from urethral swab (US) of an adult male patient while strain 2 was isolated from high vaginal swab (HVS) of an adult woman. Strain 3 was a reference strain from the American Type Culture Collection ATCC 6538P. Cultures were maintained on Blood agar (BA) (Oxoid) slants at 4^0C. In BA plates, cultures appear golden yellow; have smooth colonies with entire edges and narrow zones of haemolysis. Inocula were prepared by diluting 16 h cultures in Mueller-Hinton Broth (MHB; Oxoid) with sterile physiological saline (PS; Oxoid) to approximately $1.5x10^8$ CFU/ml. The suspensions were further diluted with saline as required.

Agar well diffusion assay: For determination of the minimum inhibitory concentration (MIC) of the plant extracts, the agar well diffusion method of Okeke *et al.* (2001) was employed. Specifically, a series of 2-fold dilutions of the extracts were made in 1% (v/v) dimethylsulfoxide (DMSO; BDH, Milan, Italy) a dispersing solvent (Hili *et al*., 1997; Nostro *et al*., 2000), to achieve a decreasing concentration of 100-6.25 mg/ml. Inocula was standardized by diluting further in sterile saline so that the final concentration of each organism was $5x10^5$ CFU/ml. Each suspension was uniformly smeared on the surface of media plates into which sterile 20 ml each, of Mueller-Hinton Agar (MHA) had previously been poured, cooled and predried. Excess inoculum was removed by a sterile syringe before allowing drying for 20 mins at room temperature. Wells (6 mm diameter) were made aseptically on the surface of each agar plate. Subsequently 100 µl of each extract dilution was introduced into the wells in the already seeded MHA plates. Sterile saline and Vancomycin (Upjohn Company, Kalamazoo, Mich.) diluted over a range of 15-0.5 µg/ml were added as controls. Ampicillin (Beecham Laboratories, Bristol, Tenn.) of 1 mg - 0.5 µg/ml concentration was also included as control. After allowing 1h at room temperature for the extracts to diffuse across the surface, the plates were incubated at 35^0C for 24 h. The inhibition zone diameter was measured in millimeter (mm) and the assay was replicated three times for each extract. The MIC was defined as the least concentration of the extract that inhibited the growth of the test organism by showing a clear zone of inhibition.

Minimum bactericidal concentration (MBC) assay: One millilitre volume of two fold serial dilution of each extract was introduced into wells in a 96-well microtitre tray (Falcon, Becton Dickinson and Co., Lincoln Park, N.J) over the concentration range of 100-6.25 mg/ml. Overnight broth cultures of each test organism were prepared in MHB and diluted with sterile saline to a final concentration of $5x10^5$ CFU/ml. The viability of each suspension was confirmed by performing viable counts on BA before inoculating into each well already containing the extract dilutions. Growth controls were included in each test. Trays were incubated aerobically at 35^0C for 24 h before the MBCs were determined.

To determine MBCs 10 µl of broth was removed from each well and spot inoculated onto MHA. The number of surviving organisms was determined after overnight aerobic incubation at 35^0C. The MBC was defined as the concentration of extract dilution where less than 0.1% of the initial inoculum survived (Hammer *et al*., 1996). Tests were replicated three times and the mean found.

Results

Yield of extracts: The yields of the methanolic extracts of the plant leaves are shown in Table 1. Of the five plant materials, *Costus afer* and *Pterocarpus santalinus* gave the highest yield. The rest yielded approximately the same quantity of extracts. Methanol was probably a better solvent for the extraction of the two highest yielding plant species than the rest.

Table 1: Yield of the methanolic extracts of the plant leaves

Plant	Yield (g)	Yield (%)
Acalypha wilkesiana	2.8	9.33
Costus afer	2.5	11.67
Phyllanthus muellerianus	2.7	9.00
Pterocarpus santalinus	2.8	12.66
Vitex doniana	2.6	8.67

Phytochemical screening of plant materials: The results of the phytochemical analyses of the five plants show a preponderance of phytocompounds in the plant leaves (Table 2). Though no particular plant had all the phytoconstituents tested, *C. afer and P. muellerianus* showed absence of only reducing sugars and anthroquinone respectively. The other three plant extracts showed absence of anthroquinone and cyanogenic glycosides in addition to one or two other compounds. Alkaloids and terpene were absent in *Acalypha wilkesiana* and *P. santalinus.*

Minimum inhibitory concentration of extracts: The MIC values indicated by the IZD showed that most of the extracts had measurable activity against

Table 2: Phytochemical constituents of the plant extracts

Constituent	*A. wilkesiana*	*C. afer*	*P. muellerians*	*P. santalinus*	*V. doniana*
Alkaloid	-	+++	+	++	++
Flavonoid	+++	++	+	++	++
Terpene	++	+++	++	-	+
Saponin	-	++	+++	+	+++
Anthroquinone	_	+	-	-	-
Cyanogenic glycoside	-	++	++	-	-
Tannin	++	++	++	+	+
Reducing sugar	+	-	++	++	++
Carbohydrate	++	+	+++	+	+

Key*: - = Absent; + = Present in trace amounts; ++ = Present in moderate amounts; +++ = Present in large amounts*

Table 3: Minimum inhibitory concentration (MIC) of the extracts

Test strains	Concentration of Plant Extract (mg/ml)					Antibiotic control (µg/ml)	
	A. wilkesiana	*C. afer*	*P. muellerianus*	*P. santalinus*	*V. doniana*	Vancomycin	Ampicillin
1	50(13.7)	25(7.0)	25 (14.2)	25 (8.9)	25 (13.75)	1.95 (16.9)	-
2	50 (10.3)	50 (9.3)	25(13.4)	25 (10.7)	25 (12.15)	1.95 (17.7)	-
3	100 (12.5)	50 (8.7)	50 (12.1)	50 (10.3)	50 (9.10)	1.95 (19.2)	-

NB: *Inhibition zone diameter (IZD) corresponding to the MIC's are enclosed in brackets.*

Table 4: Minimum bactericidal concentration (MBC) of the extracts

Test strains	Concentration of Plant Extracts (mg/ml)					Antibiotic control (µg/ml)	
	A. wilkesiana	*C. afer*	*P. muellerianus*	*P. santalinus*	*V. doniana*	vancomycin	Ampicillin
1	25	>100	12.5	100	25	1.95	-
2	50	>100	25	100	12.5	1.95	-
3	>100	>100	50	100	>100	1.95	-

the clinical strains at concentration of 25 mg/ml (Table 3). Only *A. wilkesiana* was active at 50 mg/ml for both clinical isolates. The reference strain was sensitive to *A. wilkesiana* at 100 mg/ml concentration but for the other plant extracts, it was sensitive at 50 mg/ml. *A. wilkesiana, P. muellerianus and V. doniana* extracts had the widest inhibition zones against both clinical and reference strains implying that the extracts had higher activity against the test strains than *C. afer* and *P. santalinus* which elicited lower responses. The test strains were sensitive to similar concentration of vancomycin (1.95 µg/ml) but were resistant to all the concentrations of ampicillin tested.

Minimum bactericidal concentration: Apparently due to low antibacterial activity, the MBC of *P. santalinus* was 100 mg/ml, while that of *C. afer* fell outside the range tested (Table 4). Among the plants showing promising activity, it was noted that the MBC values were often lower or equal to the recorded MIC values. It is clear from the table that the reference strain was sensitive to higher extract concentrations than the clinical strains. *P. muellerianus* was more active than both *A. wilkesiana* and *V. doniana by* its low MBC values.

Discussion

The antimicrobial activity of plant extracts are known to be influenced by the presence of phytocompounds acting alone or in combinations (Nostro *et al*., 2000). Interactions explainable on the basis of antagonism or synergism have been reported (Hili *et al*., 2007). The results obtained for the MIC and MBC determinations of *A. wilkesiana, P. muellerianus* and *V. doniana* (Tables 3 and 4) might be considered sufficient for further investigation aimed at isolating and identifying the active phytocompound(s) and evaluating their possible synergism.

Agar diffusion technique is frequently used to screen plant extracts for antimicrobial activity (Smith-Palmer *et al*., 1998) but it is limited to the generation of preliminary quantitative data (Rios *et al*., 1988; Janssen *et al*., 1987). Anecdotal evidence and the traditional use of plants for medicinal purposes provide the basis for such surveys (Hammer *et al*., 1999). Often due to the hydrophobic nature of some extracts, uniform diffusion of compounds through the agar medium is prevented with the result that the MIC values measured by the IZD is affected (Janssen *et al*., 1987; Rios *et al*., 1988). Agar and broth dilution methods are more commonly used but results obtained by these methods are inconsistent because several factors such as differences in microbial growth, microbial responses to the extracts as well as solubility are important sources of variation (Hili *et al*., 1997; Hammer *et al*., 1999). The MIC results showed that the extracts were more active against the clinical isolates than the reference strain. The MIC of the test strains was between 25 and 50 mg/ml, while that of the reference strain fell within the range of 50-100 mg/ml.

The extracts of *A. wilkesiana, P. muellerianus* and *V. doniana* were more active against the ampicillin resistant strains than *C. afer* and *P. santalinus.* Several extracts showing activity against multidrug resistant *S. aureus* have been

documented (Anas *et al*., 2008; Agil *et al*., 2005). Unfortunately, it is difficult to compare data with literature because severe variables such as environmental and climatic conditions of plant as well as the choice of the extraction method and antimicrobial test technique influence results (Nostro *et al*., 2000). Equally, standard criteria for the evaluation of the plant activity are lacking, so, the results obtained by different authors are often widely different (Van den Berghe *et al*., 1991). However, it is important that plants hitherto administered traditionally as potential antimicrobial compounds be scientifically investigated to ensure their effectiveness and safety. Thus *in-vivo* studies may be required to confirm the validity of some of the results obtained.

Finally, this study shows that many plant extracts possess *in vitro* activity against drug resistant strains of *S. aureus.* However, issues of safety and toxicity must be addressed before their immense antibacterial potential can be fully exploited.

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References

Agil, F., Khan M.S., Owais M. and Ahmad, I (2005) Effects of certain bioactive plant extracts on clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus. J. Basic Microbiol. 45:* 106-114.

Al-Waili, N.S. (2004). Investigating the antimicrobial activity of natural honey and its effect on the pathogenic bacterial infections of surgical wounds and conjunctiva *J. Med. Food 7:* 210-222.

Anas, K., Jayasree, P.R., Vijayakumar, T and Manish-Kumar, P.R (2008). *In vitro* antibacterial activity of *Psidium guajava Linn.* leaf extracts on clinical isolates of multi-drug resistant *Staphylococcus aureus. Indian J. of Exp. Biol. 46:* 41-46.

Blaser, G., Santos, K, Bode, U., Vetter H., and Simon, A. (2007) Effects of medical honey on wounds colonized or infected by MRSA. *J. Wound Care 16:* 235-328.

Cooper, R.A., Molan, P.C., Krishnamoorthy, G and Harding, K. (2001). Manuka honey used to heal a recalcitrant wound. *Eur. J. Clinic. Microbiol & Infect. Diseases 20:* 758-759.

Cooper, R.A., Molan, P.C., and Harding, K.G. (2002) The sensitivity to honey of Gram positive cocci of clinical significance isolated from wounds. *J. Appl. Microbiol* 93: 857-863.

French, V.M., Cooper, R.A. and Molan, P.C. (2005). The antibacterial activity of honey against coagulase negative *Staphylococci. J. Antimicrb. Chemo. 56:* 228-231.

Guignard, B., Entenza, J.M. and Moreillon, P. (2005) Beta-lactams against methicillin-resistant *Staphylococcus aureus. Curr. Opin Pharmacol. 5:* 479-489.

Gupta, V.P., Govindaiah P. and Datta, R.K. (1996) Plant extracts: a non-chemical approach to control *Fusarium* diseases of mulberry. *Curr. Sci. 71:* 406-409.

Hammer, K.A., Caron, C.F. and Riley, T.V. (1996) Susceptibility of transient and commensal skin flora to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Am. J. of Infect. Cont. 24: 186-189.*

Hammer, K.A., Carson, C.F and Riley, T.V. (1999). Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol. 86:* 985-990.

Harbone, J.B (1973) Methods of plant analysis. In: *Phytochemical methods.* Chapman and Hall, London pp 1-32.

Hili, P., Evans C.S. and Veness. R.G. (1997) Antimicrobial action of essential oils: the effect of dimethylsulfoxide on the activity of cinnamon oil. *Lett. in Appl Microbiol. 24:* 269-275.

Hon, K.L., Leung, A.K., Kong A.Y., Leung, T.F and Ip, M. (2008) Atopic dermatitis complicated by methicillin-resistant *Staphylococcus aureus* infection. *J. Natl. Med. Association 100:* 797-800.

Huffman, M.A. (2003) Animal self-medication and ethnomedicine: exploration and exploitation of the medicinal properties of plants. *Proc. Nutr. Soc. 62:* 371-381.

Janknegt, R. (1997) The treatment of *Staphylococcal* infections with special reference to pharmacokinetic, pharmacodynamic and pharmacoeconomic considerations. *Pharm. World and Sci. 19:* 133-141.

Janssen, A.M., Scheffer, J.J.C. and Baerheim-Svendsen, A. (1987) Antimicrobial activity of essential oils: a 1976-86 literature review. Aspects of the test methods *Planta Medica 53:* 395-398.

Maoz, M. and Neeman, I. (1998) Antimicrobial effects of aqueous plant extracts on the fungi *Microsporon canis* and *Trichophyton rubrum* and on three bacterial species. *Lett. Appl. Microbiol. 26:* 61-63.

Nkere, C.K and Iroegbu, C.U. (2005). Antibacterial screening of the root, seed and stembark extracts of *Picralima nitida. Afr. J. Biotechnol. 4:* 522-526.

Noskin, G.A., Rubin, R.J., Schentag, J.J., Kluytmans, J.I., Hedblom, E.C., Smulders, M., Lapetina, E. and Gemmen, E. (2005). The burden of *Staphylococcus aureus* infections on hospitals in the United States: an analysis of the 2000 and 2001 nationwide in-patient sample database. *Arch Intern Med. 165:* 1756-1761.

Nostro, A., Germano, M.P., D' Aneglo, V., Marino, A and Cannatelli, M.A. (2000) Extraction method and bioautography for evaluation

of medicinal plant antimicrobial activity. *Lett. Appl. Microbiol. 30:* 379-384.

Okeke, M.I., Iroegbu, C.U., Eze, E.N., Okoli, A.S and Esimone C.O (2001) Evaluation of extracts of the root of *Landolphia owerrience for* antibacterial activity *J. Ethnopharm. 78:* 119-127.

Rios, J.L., Recio, M.C. and Villar, A. (1988) Screening methods for natural products with antimicrobial activity: a review of the literature. *J. Ethnopharm 23:* 127-149.

Saderi, H., Owlia, P. and Habibi, M. (2008) Mupirocin resistance among Iranian isolates of *Staphylococcus aureus. Med. Sci Monit 14:* 210-213.

Schentag, J.J., Hyatt J.M., Carr, J.R., Paladino, J.A., Birmingham, M.C., Zimmer, G.S and Cumbo, T.J (1998). Genesis of methicillin resistant *Staphylococcus aureus* (MRSA): how treatment of MRSA infections has selected for vancomycin-resıstant *Enterococcus faecium* and the importance of antibiotic management and infection control. *Clin Infec. Dis. 26:* 1204-1214.

Schito,G.C (2006).The importance of the development of antibiotic resistance in *Staphylococcus aureus*.*Clin .Microbiol. Infect.*1:3-8.

Smith-Palmer, A., Stewart, J. and Fyfe, L. (1998) Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett. Appl. Microbiol. 26:*118-122.

Vanden Berghe, D.A and Vlietinck, A.J. (1991) Screening methods for antibacterial and antiviral agents from higher plants. *In:* Methods in Plant Biochemistry (Dey, P.M and Harbone, J.B eds.) London Academic Press. pp 47-67.