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Anti-Staphylococcal potential of *Callistemon rigidus*

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Abstract: The last decade witnessed the emergence of *Staphylococcus aureus*- a versatile human pathogen, as a deadly superbug. The enormous genetic plasticity of the organism assists it to endlessly evolve resistance mechanisms against existing anti-infectives thus necessitating the need to control the spread of resistant staphylococcal isolates in hospitals and health care settings. This in turn demands the incessant exploration of newer biological matrices in search of diverse chemical entities with novel drug targets. Since time immemorial higher plants continue to be the best source of newer compounds with high therapeutic potential. Lead fractions from same or different plants can be developed into effective antibacterial polyherbal formulations. A lead fraction from methanolic extract of leaves of *Callistemon rigidus* exhibited a dose dependent antistaphylococcal activity during *in vitro* agar well assay against a panel of twenty seven clinical multidrug resistant *S. aureus* isolates. Further, minimal inhibitory concentration (MIC) evaluation by *in vitro* 96-well microplate based assay established a MIC range of 1.25 - 80 μg/ml as compared to 5 - 320 μg/ml of positive control, Cefuroxime sodium. The MIC_{50} and MIC_{90} of the methanolic lead fraction were 5 μg/ml and 40 μg/ml respectively. The present study thus signifies the vast potential of the lead fraction from *Callistemon rigidus* for future development into a herbal drug/ formulation and to impede global health crisis due to multidrug resistant *Staphylococcus aureus*.

Keywords: Antibacterial activity, multidrug resistance, Staphylococcus aureus, Callistemon rigidus, *minimal inhibitory concentration*

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1 Introduction

Staphylococcus aureus has long been recognized as the major human pathogen responsible for skin and soft tissue diseases [1], respiratory diseases like cough pseudo-membranous tracheobronchitis and bronchopulmonary infection [2, 3], blood stream infections, breast, ocular infections, osteomyelitis, endocarditis and meningitis [4]. Methicillin resistant *Staphylococcus aureus* (MRSA) is a global pathogen responsible for a variety of infections. It is not only endemic in hospitals but also in community [5, 6]. MRSA colonizes in the anterior nares of healthy individuals and easily spreads through nasal carriage [7, 8]. The infections caused by MRSA and its variants due to acquisition of resistance towards current antimicrobials pose a serious challenge for clinicians making the therapy problematic. Glycopeptides such as vancomycin are the only antibiotics of choice for the treatment of MRSA infections. The emergence and spread of vancomycin intermediate *S. aureus* and vancomycin resistant *S. aureus* strains across Japan, USA and India [9–11] demands a change in the empirical therapy for *Staphylococcus aureus* infections. Development of newer anti-infective and therapeutic regimes that revert or overcome drug resistance is need of the hour. One such approach emphasizes the search of biologically active pharmacophores with novel mode of action from natural resources [12, 13]. Phytomedicines derived from plants have been promising agents against multidrug resistant infections.

Callistemon rigidus R.Br. (Bottle Brush), family Myrtaceae is an upright shrub widely distributed across Asia and Australia. Essential oils from the plant find use in folklore medicine for the treatment of cough, bronchitis and respiratory tract infections[14]. However, experimental studies confirming its folklore use in treatment of infections were initiated by Saxena and Gomber [15]. The present study was designed to validate the *in vitro* antibacterial potential of a lead methanolic fraction from leaves of *C. rigidus* against a panel of twenty seven *Staphylococcal* clinical isolates classified as vancomycin resistant *S. aureus* (VRSA), vancomycin intermediate *S. aureus* (VISA), methicillin resistant *S. aureus* (MRSA) and multi antibiotic resistant *S. aureus* (MARSA).

2 Statistical methods and Experimental Procedures

2.1 Materials: Plant sample and Extraction

Leaves of *Callistemon rigidus* were collected from Thapar Technology Campus in October 2004 and the voucher specimen was identified (Voucher specimen #7San03). Healthiness of the leaves was confirmed by culturing of the cut sections on nutrient agar and potato dextrose agar plates. Fresh and healthy leaves were thoroughly washed under running water, dried at 35°C and ground to a fine powder. The pulverized plant material was extracted exhaustively by soxhlet using methanol as the solvent. The solvent was filtered and the filtrate evaporated. The brown residue so obtained was partitioned between chloroform and methanol (1:1). Separate fractions were obtained which were evaporated

and the residues so obtained were stored at -20°C until use.

2.2 Test Microorganisms

The test panel included drug resistant clinical isolates of *S. aureus* and *S. aureus* NCTC 6571 used as a reference isolate (Table 1). The antibiotic resistance pattern of all the isolates was predetermined [16, 17] based on which the isolates were grouped as VRSA, VISA, MRSA and MARSA (Table 1). Trypticase Soy Agar (TSA) slants were the maintenance medium and the cultures were activated in cation adjusted Mueller Hinton (MH) broth 18- 24 hours prior to the test.

2.3 Antimicrobial Susceptibility testing methods

2.3.1 Kirby Bauer Disk assay

Antibiogram is the resistance pattern of a microorganism against a battery of antimicrobial agents. The Kirby Bauer disk assay was used for testing 38 antibacterial drugs against the test microorganisms for profiling their resistance pattern according to Clinical Laboratory Standards Institute (CLSI, USA-formerly National Committee for Clinical Laboratory Standards -NCCLS) [16] guidelines. All the experiments were repeated three times. Based on the diameter of inhibition zone the cultures were classified as sensitive, intermediate or resistant.

2.3.2 Antibacterial Assay

Agar Well Diffusion Assay [18] was used to evaluate the antimicrobial potential of the chloroform as well as methanolic fraction residues. 5 mm wells were cut in MH agar plates. The fraction residues were evaluated at six different concentrations (16.65 μg, 33.3 μg, 66.6 μg, 99.9 μg, 199.8 μg and 399.9 μg). 30 μl of the test extract in Dimethylsulfoxide (DMSO) was dspensed in test wells. Solvent blank was included as control. The wells were sealed with molten MH agar. After 15 min. the plate was swabbed with 18-24 hours old 0.5 McFarland adjusted culture of the test isolate. Antibacterial activity was interpreted by determining the diameter of Inhibition Zone (DIZ) around the test wells. The assay was performed in triplicates.

2.3.3 Determination of Minimum Inhibitory Concentration (MIC)

MICs are considered as the 'gold standard' for determining the susceptibility of the organisms to antimicrobials. MICs are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC of antibiotics was evaluated using standard microbroth dilution method [19]. Based on the results of MIC determination the cultures were classified as resistant, intermediate and susceptible [20].

Table 1 Culture collection.

Species	Culture Id.	Repository	Resistance
PUS CULTURES			
Staphylococcus aureus	Sau (A)1	*AIIMS*	—
Staphylococcus aureus	Sau (A)2	AIIMS	—
Staphylococcus aureus	Sau(G)1	GMCP	VISA (MIC= 16μg/ ml)
Staphylococcus aureus	Sau(G)2	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau(G)3	GMCP	MRSA, VRSA (MIC= 32 μg/ml)
Staphylococcus aureus	Sau(G)10	GMCP	MRSA, MARSA
URINE CULTURES			
Staphylococcus aureus	Sau(A)3	AIIMS	MARSA
Staphylococcus aureus	Sau(G)4	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau(G)5	GMCP	MARSA
Staphylococcus aureus	Sau(G)11	GMCP	MRSA, MARSA
BLOOD CULTURES			
Staphylococcus aureus	Sau (G)6	GMCP	—
Staphylococcus aureus	Sau (G)12	GMCP	MRSA, MARSA
VAGINAL SWABS			
Staphylococcus aureus	Sau(G)7	GMCP	VISA (MIC= 16μg/ ml), MARSA
WOUND SUBCULTURES			
Staphylococcus aureus	Sau(A)4	AIIMS	—
Staphylococcus aureus	Sau(G)14	GMCP	MARSA
BURN SWABS			
Staphylococcus aureus	Sau(G)15	GMCP	MARSA
Staphylococcus aureus	Sau(G)9	GMCP	MRSA
Staphylococcus aureus	Sau(G)17	GMCP	VISA (MIC= 16μg/ml)
Staphylococcus aureus	Sau(G)18	GMCP	MRSA, MARSA
Staphylococcus aureus	Sau(G)19	GMCP	MRSA, MARSA
EAR DISCHARGE SWABS			
Staphylococcus aureus	Sau(G)8	GMCP	MARSA
CATHETER TIP ISOLATES			
Staphylococcus aureus	Sau (G)20	GMCP	MRSA
Staphylococcus aureus, coagulase negative	Sau (G)21	GMCP	MARSA
EYE ISOLATES			
Staphylococcus aureus	Sau (G)22	GMCP	VRSA (MIC= 64μg/ml), MARSA
OTHER CULTURES			
Staphylococcus aureus	Sau(A)5	AIIMS	—
Staphylococcus aureus	Sau (MTB)	Karolinska Institute, Sweden	MARSA, MRSA
Staphylococcus aureus	Sau(LHMC)	LHMC	Antibiotic Sensitivity Testing Strain

AIIMS: All India Institute of Medical Sciences, New Delhi, INDIA; GMCP: Government Medical College, Patiala, INDIA: NDRI; National Dairy Research Institute, Karnal, INDIA; LHMC: Lady Harding Medical College, New Delhi, INDIA. VISA (Vancomycin Intermediate *S. aureus*); VRSA (Vancomycin resistant *S. aureus*), MRSA (Methicillin resistant *S. aureus*); MARSA (Multi- antibiotic resistant *S. aureus*)

2.3.4 *In vitro* determination of Minimal Inhibitory Concentration [19]

Microbroth dilution method using 96 well microtitre plate and 3-(4, 5-Dimethyl-2-thiazolyl - 5-Diphenyl-2H)-tetrazolium bromide (MTT) assay was performed to evaluate the Minimal inhibitory Concentration (MIC) of the fraction residues. The range of antimicrobial dilutions evaluated was 320 - 0.15625 μg/mL. 50 μl of the bacterial suspension in saline was added to 125 μl of MH broth to achieve a final bacterial cell concentration of 10^5 cells in the well. Plates were then incubated at 37°C for 2.5 hours after which 25 μl of the test extract at different concentrations was added. After 24 hours, 20 μl of 0.02% MTT was added to each well. The assay was performed in triplicates. The same procedure was used to evaluate the MIC of the positive control, Cefuroxime sodium.

2.4 Statistical Methods

Pearson Correlation coefficient was calculated using Graph Pad Prism version 4.0. This statistical value indicates the correlation between dose of the bioactive fraction and antimicrobial activity. Symbolized by 'r' it predicts a linear relationship between two variables. It has a value between +1 and -1 indicating a perfect positive or perfect negative relationship respectively. When the value of r= 0 then there is no relationship between the variables. Pearson's correlation was calculated to study the dose response of the extract on the diameter of inhibition zone.

3 Results

Our study reveals potential dose dependent antistaphylococcal activity of methanolic fraction residue from the leaves of *C. rigidus* by *in vitro* agar well diffusion assay (Table 2). The chloroform fraction residues did not exhibit any antimicrobial activity. The methanolic fraction residue exhibited bactericidal activity against 93% of the test isolates with MIC ranging between 1.25 - 80 μg/ml (Table 2), which is fairly low as compared to positive control Cefuroxime Sodium which inhibited only 64% of test panel Staphylococci and had a MIC range of 5 - 320 μg/ml. The fraction however was not active against VRSA strains, viz. Sau G3 and Sau G22. Further statistical analysis using Pearson's correlation demonstrates a significant correlation (r^2) between dose of the extract and diameter of inhibition zone against most of the isolates (Table 2, Fig. 1)

4 Discussion

Antibacterial potential of crude extracts from numerous plants has been evaluated by agar well assay [21, 22] and MIC determined based on which anti-infective lead molecules are isolated for use as a therapeutic agent [23, 24]. Antibacterial activity of the alcohol extract of stem bark of *Picralima nitida* was tested against *Staphylococcus aureus* ATCC 12600 [25], where crude extract had a MIC range of 25 - 50 mg/ml. The extract inhib-

Table 2 Comparative *in vitro* antimicrobial activity of methanolic fraction residue from leaves of *Callistemon rigidus* and Cefuroxime sodium by Agar well diffusion and Microbroth dilution assays against multidrug resistant *Staphylococci*.

Culture Id	DIZ (mm) at 199.8 μg	MIC of methanolic fraction residue (μg /ml)	MIC of cefuroxime sodium (μg /ml)
Sau G1	29	1.25	80
Sau G2	24	10	80
Sau G3	0	NA	80
Sau G4	20	5	80
Sau G5	17	5	80
Sau G6	18	2.5	40
Sau G7	24	56	80
Sau G8	0	5	80
Sau G9	19	10	80
Sau G10	18	1.25	NA
Sau G11	18	2.5	NA
Sau G12	21	1.25	80
Sau G14	17	10	80
Sau G15	22	2.5	320
Sau G17	13	2.5	NA
Sau G18	17	20	NA
Sau G19	22	5	NA
Sau G20	24	80	NA
Sau G21	0	80	NA
Sau G22	0	NA	NA
Sau A1	25	10	40
Sau A2	24	40	40
Sau A3	21	20	20
Sau A4	17	20	10
Sau A5	14	80	80
Sau MTB	21	40	10
Sau LHMC	24	20	5

NA – No Activity.

ited only 50% of the isolates. Gentamycin was used as the positive control in the above study and further fractionation of the crude extract by column chromatography gave six partially pure alkaloidal fractions with a MIC range of 1.563 - 50 mg/ml. Methanolic extract of peels of grapefruit (*Citrus paradica*) inhibited *Staphylococcus aureus* at 3 mg/ml. Based on these results further studies were initiated and pure antibacterial compound naringin exhibiting a MIC of 1.5mg/ml was isolated [26]. Bioassay guided fractionation of the biologically active crude extract of the berries of *Piper nigrum* yielded four anti-

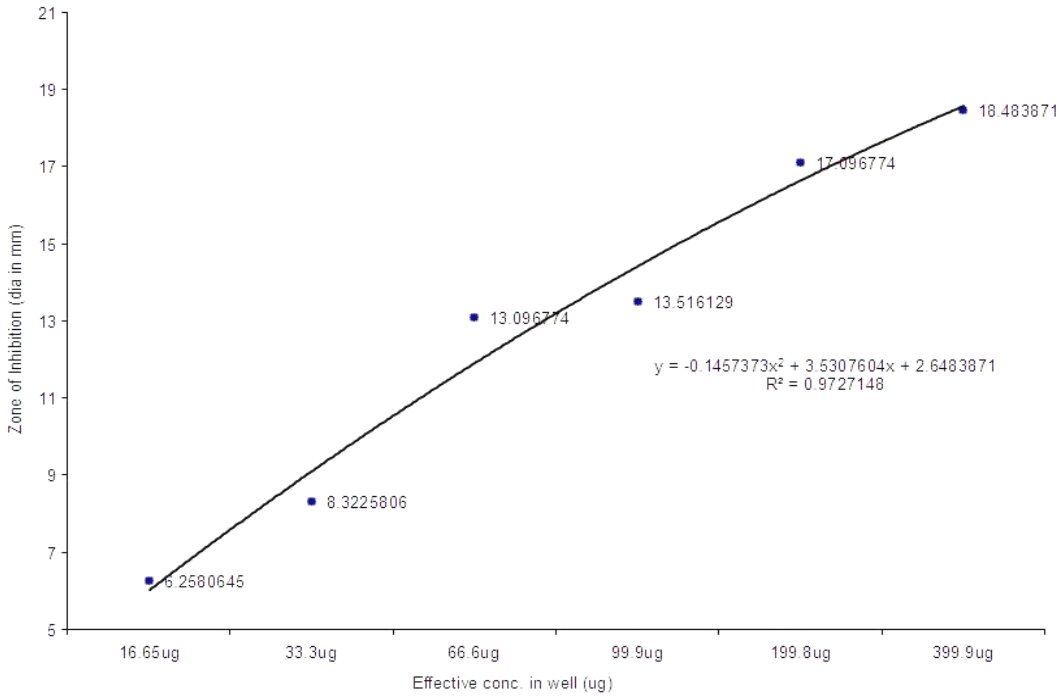

Fig. 1 Agar Well Diffusion Assay of test extract against *S.aureus*.

infective leads viz. pellitorine, trachyone, perguimidine and isopiperolein [27]. A study by Ajali and Chukwurah,[28] demonstrated potential activity of ethanol extract of roots of *Securidaca longipedunculata* against *Staphylococcus aureus* as compared to the positive controls chloramphenicol, penicillin- G and nystatin evaluated in the study. However, literature reveals that no prior work has been done on evaluating antistaphylococcal potential of *C. rigidus*. Methanolic extract of *Myrtus communis* also a Myrtaceae family member exhibits a MIC of 1mg/ml which is very high compared to our extract [29]. Similarly evaluation of antistaphylococcal activity of the aqueous and acetone extracts of the bark of *Syzygium jambos* (Myrtaceae) gave a MIC range of 500 - 1000 mg/L as compared to 0.25 - 64 mg/L for the positive controls ampicillin and erythromycin [30]. The antibacterial activity of the crude extracts was attributed to the high content of tannins in the extract, thus supporting the use of these extracts in traditional medicines to treat infectious diseases.

Our study confirms the bioactive potential of methanolic fraction residue from the leaves of *C. rigidus* and its possible use in the present armamentarium of antimicrobial therapy to combat the superbug *S. aureus* by isolation of the bioactive compound. Further studies for the characterization of the bioactive fraction residue are underway.

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