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Anti-infective and anti-biofilm activity of *Geodorum densiflorum* (Lam.) Schltr. against Methicillin resistant and sensitive *Staphylococcus aureus*

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ABSTRACT

Methicillin Resistant Staphylococcus aureus (MRSA) is one of the common pathogens in clinical which cause hospital acquired infections in surgical wounds and delayed the healing mechanism of our immune system. Thus MRSA infections became a worldwide serious health problem. The study was to comparatively evaluate the anti-infective and anti-biofilm activity of Geodorum densiflorum (Lam.) Schltr. ethanol extract against MRSA and MSSA. The disc diffusion method, MIC and Biofilm inhibition assay were performed with clinical isolates of MRSA and Staphylococcus aureus (MTCC 3160) an MSSA organism. MSRA clinical isolates shows sensitivity to G. densiflorum extract at 75mg/ml (7.5 mg/disc) concentration while MIC range 2mg/ml and anti-biofilm activity of plant extract shows sensitivity at the concentration of 100mg/ml and 25mg/ml for MRSA and MSSA respectively. The study clearly revealed that ethanolic extract of G. densiflorum having effective inhibitory activity on both MRSA and MSSA strains.

Key words: Biofilm formation, Anti-MRSA activity, Minimal Inhibitory Concentration, Multidrug Resistant *Staphylococcus aureus* and Biofilm suppression assay.

INTRODUCTION

During the past four decades Methicillin Resistant *Staphylococcus aureus* (MRSA) has spread throughout the world and has become highly endemic in many geographic areas (Mark *et al.*, 2002). In hospitals the infections caused by Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolates have increased greatly during the last decades (Giulia *et al.*, 2012) and the community (Yamaguchi *et al.*, 2012). MRSA infections are resistance to common antibiotics viz macrolides, tetracyclines, aminoglycosides, vancomycin etc, (Tenover *et al.*, 2001). The spread of infection in patients was controlled by topical argent Mupirocin a fermentation product from *Pseudomonas fluoresences* (NCIB 10586) increased use of the drug caused resistivity in MRSA strains (Cutler *et al*., 2004). Nowadays there was a developing attention in studying the microbial biofilms in order to overcome various human diseases. Biofilms are aggregates of microbes which develop multilayer bacterial load, facultative anaerobic bacterium colonizes, adaption with environment of partial or total oxygen depletion and development of resistance towards many antibiotics (Pompilio *et al.*, 2013), cause biofilm – associated diseases and infections (Tote *et al*., 2009). The continuing spread of *S.aureus* infections and diseases and its resistivity will highlights the need for development of alternative drug from natural source.

Geodorum densiflorum (Lam.) Schltr. is a glaborous orchid plant belongs to the Orchidaceae family, having many traditionally valuable therapeutic properties. The plant possess antimicrobial and antidibetic (Salecha *et al*., 2010), cytotoxic (Hossain *et al.*, 2012), thrombolytic (Hossen *et al.*, 2014), antioxidant (Habib *et al*., 2011), sedative, analgesic and anxiolytic activities (Khatun *et al*., 2013). The study will reveal the anti-infective and anti-biofilm activity of *G. densiflorum* against MRSA and MSSA.

MATERIALS AND METHODS

Isolation of MRSA strain from pathological sample

The clinical isolates of MRSA were obtained from patients having different impaired wounds. Sterile cotton swabs and needle aspiration method were used for collecting samples from the pathological sites. The obtained clinical samples were plated on selective media blood agar and were incubated at 37ºC in an incubator. Identification was done on the basis of morphology, cultural characteristics, biochemical reactions and resistant to *oxacillin* discs (1µg) using Mueller Hinton agar. The isolates were then tested for antibiotic sensitivity pattern *with cloxacillin, amoxicilin, streptomycin, erythromycin, tetracycline, cefotaxime* and *vancomycin*. The zone of inhibition was measured and results were interpreted.

Leaf Extracts

Geodorum densiflorum (Lam.) Schltr. whole plants were collected from Periyakombai hill area, Namakkal district, Tamil Nadu. The plants were washed with distilled water and were cut into small pieces and kept for shade drying. Dried samples were grounded into powder using the mixer and stored. Powder weighing 50 g was extracted with 500 ml of ethanol for 72 hrs. The solvent was recovered using rotary vacuum evaporator. The semisolid mass obtained was concentrated under reduced pressure and stored in air tight container at refrigerator for further use.

Microorganisms Used

The MTCC cultures of *S. aureus* (3160) which is MSSA and isolated MRSA colony culture were obtained and was sub cultured on Muller Hinton Agar Medium. The suspension culture was prepared in Mueller Hinton (MH) broth.

Growth Inhibition Assays

Antibacterial activity of the extracts was tested by disc diffusion method. Ethanol was used as a negative control for all the assays. The extracts at a varying concentrations (25, 50, 75 and 100 mg/ml) were tested for its influence in the growth and zone of inhibition of test bacteria.

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) was determined by Micro dilution method using serially diluted plant extracts. The extracts were diluted into different concentrations of 0.125 - 8mg/ml respectively with DMSO. Then each tubes was filled with 1ml of sterile nutrient broth and inoculated with 0.1ml of broth culture of the test organism (inoculums contains $1\text{-}2\times10^{-7}$ CFU/ml). The tubes were incubated aerobically at 37ºC for 18-24 hrs. The control tubes were maintained for each test tube. Inhibition of growth observed in those test tubes (No turbidity) which has lowest or minimum concentration of extract. This lowest or minimum concentration was considered as Minimum Inhibitory Concentration (MIC) (Hassan *et al.*, 2009).

Biofilm Suppression Assay

Anti-biofilm activity of plant extract was assessed using a static microplate biofilm formation assay. Inoculum was prepared at a density adjusted to 0.5 McFarland turbidity standards [(10^8 colony forming units (CFU/ml)] and diluted 1:10. The plant extract concentration ranged from 12.5 to 200 mg/ml. The final volume in each well of microplate was 210 µl (containing 150 µl of fresh nutrient broth, 30 µl of inoculums and 30 µl of plant extract). *Erythromycin* and DMSO were used as a positive and negative control respectively. Following 24 hours incubation at 37℃, the media containing test organisms and *G. densiflorum* extracts was discarded while surface-attached biofilm cells were stained with crystal violet for five minutes. The excess stain was rinsed off with tap water and optical biofilm density was determined using microplate reader at a wavelength of 570 nm.

RESULTS AND DISCUSSION

The Methicillin Resistant *Staphylococcus aureus* (MRSA) were isolated from different pathological sample collected. The MRSA isolates were tested for antibiotic sensitivity pattern; the present study reveals that the MRSA isolates have also developed resistance to other antibiotics tested. The clinical isolates showed resistance to *Cloxacillin* and *Streptomycin* and sensitive to *Tetracycline* and *Erythromycin* (Table - 1). The MSSA organisms did not developed resistivity to other antibiotics. The sensitivity pattern showed that they are susceptible to all antibiotics tested. These results will clearly prove the MRSA are entirely changed its biochemical machinery which empower them to quickly colonize on host tissue and guard themselves from host defence mechanism and cause infections worldwide and this phenomenon was reported elsewhere (Tahnkiwale *et al.*, 2002 and Poonam *et al.*, 2012).

Table 1: Antibiotic resistance of MRSA and MSSA

Antibiotics	Inhibitory activity of antibiotics	
	MRSA	MSSA
Amoxicillin (10 mg)	I	S
Streptomycin (25 mg)	R	S
Erythromycin (15 mg)	S	S
Cloxacillin (30 mg)	R	S
Cefotaxime (30 mg)	I	S
Tetracycline (30 mg)	S	S
Vancomycin (30 mg)	I	S

(R) – Resistant; (S) – Sensitive and (I) – Intermediate resistant

The antibacterial activity of *Geodorum densiflorum* (Lam.) Schltr. was assayed against both MRSA and MSSA, *in vitro* conditions by disc diffusion method. The inhibition of bacterial growth by ethanol extracts of *G. densiflorum* was summarized in Table 2. The results showed that the ethanolic extracts presented the highest activities about inhibition diameters of 14 – 29 mm and 10 – 24 mm for MSSA and MRSA respectively.

Table 2: Antibacterial activity of ethanol extract of *G. densiflorum* (Lam.) Schltr. by disc diffusion method

Microorganisms	Zone of inhibition in diameter (mm)			
	25 mg/ml	50 mg/ml	75 mg/ml	100 mg/ml
MRSA	7.4 ± 0.21	9.5 ± 0.7	14 ± 0.01	24 ± 0.24
MSSA	14 ± 0.30	17 ± 0.04	22 ± 0.23	29 ± 0.12

The data obtained, through the determination of MIC are shown in Table 3. The results showed variability in the inhibitory concentrations of ethanol extracts on both MRSA and MSSA. The MIC values for MRSA and MSSA were in the range of concentrations 0.125 mg/ml and 2 mg/ml respectively. There is little or no scientific information concerning the antibacterial activity of *G. densiflorum* against MRSA. The resistivity of MRSA was studied and reported scientifically. The results of the study were consistent with the antibacterial activity of *Acacia aroma* (Mattana *et al*., 2010), *Curcuma longa* (Yong *et al*., 2004) and *Boerhavia diffusa* (Ramesh *et al*., 2014) against MRSA strains. Data comparison was very difficult because the results were influenced by several factors, such as the geographic and climate conditions of the plant, and the extraction method and antibacterial test. Moreover, there were no standards for the estimation of the plant activity; therefore, the results reported by researchers could be different (Rios *et al*., 1988).

Table 3: Minimal Inhibitory Concentration of ethanol extract of *G. densiflorum* (Lam.) Schltr

Microorganisms	Minimal Inhibitory Concentration (mg/ml)						
	0.125	0.25	0.5	1	2	4	8
MRSA	-	-	-	-	-	B	α
MSSA	β	α	α	α	α	A	α

(-) – No inhibition; (β) – MIC; (α) – Inhibition present

Nowadays, microbial biofilm cells have been a great interest in development of antimicrobial agent due to its higher antibiotics resistance as compared to their planktonic counterparts. In the present study, inhibitory effect of *G. densiflorm* ethanol extract against formation of *S. aureus* biofilm was determined using static microplate assay system. Table 4 illustrates optical biofilm density at wavelength of 570 nm. It was observed that the inhibition of *S. aureus* biofilm formation both MRSA (optical biofilm density ranging from 0.069±0.005 to 0.097±0.007) and MSSA (optical biofilm density ranging from 0.052±0.001 to 0.094±0.017) environments at all test concentrations. At test concentration of 50 mg/ml, 100 mg/ml and 200 mg/ml, the anti-biofilm activity of *G. densiflorum* ethanolic extract showed significant activity on both MRSA and MSSA.

Table 4: Anti-biofilm activity of ethanolic extract of *G.densiflorum* (Lam.) Schltr

Concentration of sample (mg/ml)	OD of biofilm density at 570nm	
	MRSA	MSSA
200	0.072±0.012	0.057±0.009
100	0.069±0.005	0.054±0.014
25	0.086±0.004	0.052±0.001
12.5	0.097±0.007	0.094±0.017
+ve control	0.076±0.008	0.059±0.017
-ve control	0.120±0.028	0.114±0.01

Recently, Chusry *et al*. 2012 demonstrated that *Quercus infectoria G. olivier* extract and tannic acid increased *staphylococcal* cell surface hydrophobicity which upturn anti-biofilm activity very highly. The bioactive compound from natural source which disturbs the cell-surface interaction can control the biofilm formation. For understanding the mechanism of anti-biofilm formation the aspect of cell-surface interaction has been concentrated very much as reported (Jiang *et al.*, 2011). In that study, it was revealed that a bacterial exopolysaccharide (A101) inhibited the cell-surface interaction and multicellular aggregates formation happened on *S. aureus* and *P. aeruginosa* which was detected by phase-contrast microscopy with 600X magnifications. Considering the fact that a particular anti-biofilm agent can inhibit biofilm either by causing formation of multicellular clumps or interfering with the microbial cell-surface interaction, it was possible that the *Z. officinale* ethanolic extract exhibited the same anti-biofilm mechanism.

CONCLUSION

The present study demonstrated that ethanolic extract of *G. densiflorum* containing active compounds showing high activity against *Staphylococcus aureus* and MRSA strains. This is the first report on evaluation of plant extracts for this anti-biofilm activity against pathogens isolated from wounds. The study also revealed the antibacterial activity, Minimal Inhibitory Concentration and anti-biofilm activity of the plant extract against the MRSA strains an clinical isolates having Multidrug resistance and *Staphylococcus aureus* comparatively.

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