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Anti-MRSA studies on active extracts from *Tridax procumbens L.*

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ABSTRACT

The study was intended for the therapeutic management of methicillin resistant Staphylococcus aureus by the use of medicinal plant Tridax procumbens. A total of 472 clinical isolates of Staphylococcus aureus were included for the study. Anti-MRSA screening was done for methicillin resistance based on Kirby-Bauer disk diffusion method using oxacillin (1µg) and methicillin (5µg) discs. Antibacterial activity of MRSA was assayed using 20 different extracts obtained from polar and non-polar solvents. The significant inhibition zone range of 17-23 mm was obtained from methanol root extracts. The antibiotic susceptibility testing of S. aureus isolates revealed high degree of resistance (94.82%) to penicillin and ampicillin, while 5.17% isolates were sensitive to both antibiotics. The study concludes that, the screening of anti-MRSA potential of medicinal plants would help to rediscover the new therapeutic plant drugs for the management of emerging strains of the common pathogen.

KEYWORDS : Tridax procumbens, emerging strains, methicillin resistant Staphylococcus aureus and Anti-MRSA potential

INTRODUCTION

The emergence of resistant microorganisms in hospitals and in the community is causing problem for both the treatment of patient and infection control. The organism of particular concern includes methicillin resistant *Staphylococcus aureus*. The genus *Staphylococcus* includes pathogenic organisms in which *Staphylococcus aureus* is most important. It has overcome most of the therapeutic agents that have been developed in the recent years and hence the antimicrobial chemotherapy for this species has always been empirical (Jun *et al.,* 2004). The most notable example of this phenomenon was the emergence of methicillin resistant *Staphylococcus aureus* (MRSA), which was reported just one year after the launch of methicillin (Qureshi *et al.,* 2004). Many of these MRSA isolates are becoming multidrug resistant and are susceptible only to glycopeptide antibiotics such as vancomycin (Mehta *et al.,* 1998). Low level resistance even to vancomycin is emerging at present (Assadullah *et al.,* 2003). The prolonged hospital stay, indiscriminate use of antibiotics, lack of awareness, receipt of antibiotics before coming to the hospital etc. are the possible predisposing factors of MRSA emergence (Anupurba *et al.,* 2003). The plant *Tridax procumbens* L. belongs to the family of *Asteraceae*. This plant is used from long time in Indian traditional medicine as anticoagulant, antifungal and insect repellent; in bronchial catarrh, diarrhoea and in dysentery. Moreover, it possesses wound healing activity for minor cuts, burn, small injuries and promotes hair growth (Saraf *et al.,* 1991). Therefore the present study was intended for the therapeutic management of methicillin resistant *Staphylococcus aureus* by the active extract of *Tridax procumbens* L.

MATERIALS & METHODS

Plant material collection and extract preparation- Different parts of *Tridax procumbens* L. (root, stem, leaf, and flowers) were collected from different localities of Wardha, District of Maharashtra state. Collection was done during January 2012 to April 2012, from nearby areas like railway routes, gardens, and farms & washed thoroughly with distilled water. The plant was authenticated in the Department of Botany, Adarsha Mahavidhyalaya, Dhamangaon (Rly). The cleaned plant parts then allowed for the complete shade drying and then made to fine powder with a mechanical grinder and stored in an airtight container. A powdered plant parts were extracted successfully with the organic solvents with increasing of polarity by using Soxhlet Assembly. The extraction was carried out for 24 – 48 hours at room temperature with mild shaking. The shade dried plant material was subjected to Soxhlet extraction with ethanol, methanol, acetone, chloroform, and ethyl acetate. The plant material were finely ground and dried powder (25 g) of each part were extracted sequentially using Soxhlet extractor with 250 ml of pure organic solvent separately in order to extract non-polar and polar compounds. The crude extracts were then filtered through Whatman No.1 filter paper and concentrated at 40°C using a drier. The concentrated extracts were subsequently dried aseptically at room temperature.

Isolation and identification of *Staphylococcus aureus* -

A total of 472 clinical specimens and carrier screening samples such as urine, pus, sputum, vaginal swab, inguinal swab /fluid, throat swab, blood, pleural and synovial fluid, stool, catheter tip, implant specimen, tissue exudates, swabs from conjunctiva, ear, trachea swabs, nasal secretion and oral thrush were collected for *Staphylococcus aureus* screening. These clinical samples were obtained from various private hospitals, Pathological and Microbiological Laboratories. All the samples were aseptically handled and processed. The morphotypes were done for all the samples based on the Gram staining method to determine the likely organism present. Subsequently, the clinical specimens and carrier screening samples were inoculated on to blood agar (aerobic with 5% CO_2), Mac-Conkey agar and Baird Parker agar (Hi-media) for selective isolation and incubated at 37°C for 24 hours. The smear findings showed colonies of Gram-positive cocci present in clusters. All strains were further tested for the production of free coagulase enzyme using tube coagulase test based on standard methods. *Staphylococcus aureus* MTCC 96 of known coagulase production was included as control strain. The other biochemical characterization & sugar fermentation tests were also done for identification of *Staphylococcus aureus.*

Antibiotic susceptibility testing:

The antibiotic susceptibility profile was determined by the disc diffusion technique (Bauer *et al.,* 1966) using different antimicrobial agents. Antimicrobial discs were used in the present study were Erythromycin (E10), Ofloxacin (OF 5), Cefpodoxime (CPD 10), Cephalexin (CP 30), Ceftazidime/ Clavunic acid (CAC 30/10), Kanamycin (K 5), Ciprofloxacin (CIP 10), Tetracycline (TE 10), Chloramphenicol (C10), Trimethoprim (TR 10), Oxacillin (OX1), Ampicillin (A10), Penicillin (P10), Vancomycin (VA30) and Methicillin (MET 5). These discs were obtained from Hi Media Laboratories Pvt. Ltd, Mumbai. For susceptibility testing, Mueller Hinton agar medium was used. The solidified plates of Mueller Hinton agar was seeded with test bacterial suspension earlier matched with the 0.5 McFarland standards; a sterile cotton swab was dipped into the standardized inoculum and rotated firmly against the upper inside wall of the test tube to remove excess inoculum from the swab. Entire sterile and dried Mueller Hinton agar surface of the plate was streaked with the swab three times, by turning the plate 60^0 between each streaking. Excess surface moisture was allowed to dry for not more than 15 minutes. Commercial anti-

biotic disc were placed onto the agar surface at appropriate distance. The plates were incubated at 37^0 C for 24 hours. Zone size was measured and interpreted according to the standard zone size interpretative chart recommended by CLSI (CLSI, 2003).

MRSA screening of clinical isolates:
All previously confirmed *S. aureus* clinical isolates were subsequently tested for methicillin resistance based on Kirby-Bauer disk diffusion method using oxacillin (OX1) and methicillin (MET 5) discs obtained from Hi-Media Laboratories Pvt. Ltd. The isolates were considered methicillin resistant if the zone of inhibition was 10 mm or less, according to the standard zone size interpretation chart.

Anti-MRSA screening of plant extract-
The previously prepared extracts of all plant parts were tested by agar well diffusion technique for determining anti-MRSA activity against the clinical MRSA isolates.

The well-diffusion assay was used to determine the antibacterial assay (Nair *et al.,* 2005; Laouer *et al.,* 2009). The solidified plates of Mueller Hinton agar (3 to 4 mm depth) was seeded with test bacterial suspension earlier matched with the 0.5 McFarland standards. Entire sterile and dried Mueller Hinton agar surface of the plate was streaked with the swab dipped in standardized inoculums. Excess surface moisture was allowed to dry for 15 minutes.

A sterile borer was used to prepare two cups in the agar media. Stock solutions of different extracts were prepared with their respective solvents with a concentration of 300 mg/mL. To each plate, one bore was filled with 100 µL pure solvent and marked accordingly. To the other bore, 100 µL of the stock solution extract under study was added. Petri dishes were then incubated at 37^{0}C for 24 hrs and the zone of inhibition was measured using a zone reader and the results were noted by subtracting the zone of pure solvent.

RESULTS & DISCUSSION
Clinical Isolation & MRSA screening:
A total of 309 (65.46%) clinical samples showed presence of *Staphylococcus aureus* out of 472 tested clinical samples. These isolates were recovered from the pus 82 (26.53%), blood 90 (29.12%), sputum 27 (8.73%), urine 60 (19.41%) and miscellaneous samples 50 (16.18%) (Data shown in Table 1). Out of 309 isolates, 134 (43.36 %) exhibited the methicillin resistance phenomenon and isolates of *S. aureus* showed the drugs resistance to different antimicrobial agents. Amongst all tested isolates, the antibiotic penicillin and ampicillin showed high degree of resistance, 293(94.82%) were resistant to penicillin and ampicillin, while only 16 (5.17%) were sensitive. Next to this, the ceftazidime/ clavunic acid (CAC 30/10) exhibited resistance to 275 (88.99%) clinical isolates, while 34 (11.0%) isolates were sensitive. The same phenomenon was observed with the cefpodoxime (CPD 10), only 23 (7.44%) isolates demonstrated sensitivity to cefpodoxime, 12 (3.88%) isolates were intermediately sensitive and 274 (88.67%) were resistant isolates. This degree of resistivity directly reflects the inappropriate use of the antimicrobial agents in the treatment (Moon, 2011). The anti-biograms of the different antibiotic are shown in Table 2.

Anti-MRSA activity of plant extracts:
In the present study, 20 different extracts were prepared from four different plant parts viz. leaf, stem, flower and roots in different solvent systems such as ethanol, methanol, acetone, ethyl acetate and chloroform. All extract were screened for the Anti-MRSA activity against the clinical isolates. The methanol root & acetone flower extract was found to have significant activity against the MRSA strains. The methanol root extract was most potent amongst all the tested extracts, exhibiting the inhibition zone range 17 - 23 mm. Dhanabalan *et al.,* (2008) reported the antibacterial activity of aqueous extract and methanolic leaf extract of *Tridax procumbens* against the methicillin and penicillin resistant *Staphylococcus aureus* isolated from Mastitis disease of cattle by disc diffusion & agar well diffusion methods. The methanol extract reported to be the most active extract with significant antibacterial activity against *Staphylococcus aureus* isolated from four different breeds of cows suffering from mastitis. They reported 7.8 – 8.2 mm inhibitory zones against *Staphylococcus aureus*. Our finding in present study reports the significant inhibitory activity of methanol roots extract with higher zones of inhibition against the MRSA isolated from different human clinical samples. The present investigation agrees with Dhanabalan *et al.,* (2008) with reference to antibacterial activity of *Tridax procumbens* against *Staphylococcus aureus.* The emerging strains of *Staphylococcus aureus* that possess resistance for methicillin antibiotic (MRSA) have been recognised as the most common cause of infections among hospital environment, and many of the severe forms includes endocarditis, pneumonia and *Staphylococcal* toxic shock syndrome (STSS). In contrast with methicillin sensitive strains of *S. aureus* (MSSA), MRSA strains tends to be multidrug resistant (MDR), showing resistance not only to β-lactam group of antibiotics but also for different classes of antibiotic, such as fluoroquinolones, tetracyclines, macrolides, lincosamides and aminoglycosides. Earlier reports showed intermediate sensitivity or full resistance to vancomycin against the strains of *S. aureus* (Pantosti et al., 2012). Therefore, the discovery of novel anti-MRSA agents from the plant *Tridax procumbens* would be helpful for controlling the MRSA infection.

Bharathi *et al.,* (2012) evaluated the antibacterial potential of *Tridax procumbens linn.* They used ethyl acetate and methanol extract for determining the antibacterial activity and reported significant zone of inhibition of 15 mm against *Staphylococcus aureus* by disc diffusion method by both extracts, whereas by agar well diffusion ethyl acetate extract showed the 18 mm and methanol extract showed 8 mm inhibition zone. Their study also suggests the plant has the bioactive secondary metabolites which represents the antibacterial activity (Bharathi *et al.,* 2012).

Sathya Bama *et al.,* (2012) extracted the different bioactive compounds such as alkaloids, glycosides, flavonoids and terpenoids from *Tridax procumbens* and evaluated the antibacterial activity of each compound. They reported the zones of inhibition as 16mm for alkaloids, 13mm for glycosides, 14mm for flavonoids and 24mm for terpenoids against the *Staphylococcus aureus.* They further suggested that the inhibitory activity of terpenoids results in alteration in the permeability of the cell membrane of the bacteria (Sathya Bama *et al.,* 2012). In present study, we screened the anti-*Staphylococcus aureus* activity of 309 clinical MDR isolates, including the 134 MRSA isolates against the root methanol extract of *Tridax procumbens.* From the above study it was concluded that the methanol roots extract contains the bioactive compounds responsible for the anti-MRSA activity. The study validates the use of plant *Tridax procumbens* as source of anti-MRSA agent.

Table1. Isolation of *Staphylococcus aureus* form clinical origin.

S. No.	Clinical Specimen	No. of Isolates	Percentage
1	Pus	82	26.53 %
2	Blood	90	29.12 %
3	Sputum	27	08.73 %
4	Urine	60	19. 41 %
5	Others	50	16.18 %

Table 2. Antibiotic profiling & Methicillin resistance screening

S. No.	Antibiotics used	Resistance	Intermediate	Sensitive
1	Erythromycin E10	118(38.18 %)	132(42.71%)	59(19.09 %)
2	Ofloxacin OF 5	115 (37.21%)	59 (19.09%)	135(43.68%)
3	Cefpodoxime CPD 10	274 (88.67%)	12 (3.88%)	23(7.44%)
4	Cephalexin CP 30	205 (66.34%)	49(15.85%)	55(17.79%)
5	Ceftazidime/Clavunic acid CAC 30/10	275(88.99%)	0	34 (11.00 %)
6	Kanamycin K 5	222(71.84 %)	63(20.38 %)	24 (7.76 %)
7	Ciprofloxacin CIP 10	75 (24.27 %)	151(48.86 %)	83(26.86 %)
8	Tetracycline TE 10	88 (28.47 %)	88 (28.47 %)	133(43.04 %)
9	Chloramphenicol C10	73 (23.62 %)	95 (30.74 %)	141(45.63 %)
10	Trimethoprim TR 10	91(29.44 %)	114(36.89 %)	104(33.65 %)
11	Oxacillin OX1	135(43.68 %)	88(28.47 %)	86(28.47 %)
12	Ampicillin A10	293(94.82 %)	0	16(5.17 %)
13	Penicillin P10	293(94.82 %)	0	16(5.17 %)
14	Vancomycin VA 30	126(40.77 %)	40(12.94 %)	143(46.27 %)
15	Methicillin MET 5	134(43.36 %)	149(48.22 %)	26(8.41 %)

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