BIOASSAY-GUIDED FRACTIONAL ISOLATION AND IDENTIFICATION OF ANTI-MRSA COMPOUNDS BY GC-MS FROM TRIDAX PROCUMBENS L

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ABSTRACT

Introduction: The plant Tridax procumbens is well known for its pharmacological properties and ethano-botanical usages. Objective: The present study deals with the isolation and identification of anti-MRSA compounds as active secondary metabolites from the root part of Tridax procumbens L. Method: Antibacterial activity was assayed using agar well diffusion method. The metabolites profiling of bioactive extract was done by pre-coated silica gel TLC plates grade F254 & anti-MRSA compounds were located by TLC bio-autography, GC-MS analysis, NIST data bank. Conclusion: The study concludes the plant had great potential for the growth inhibition of methicillin-resistant S. aureus and serves as potential source of anti-MRSA agents.

KEYWORDS: Tridax procumbens L, TLC Bio-autography, GC-MS analysis, Solvent extracts, Secondary metabolites and anti-MRSA compounds.
INTRODUCTION
Nature has gifted with the wide variety of the natural products and serves as the elementary source of compounds with an abundance of reflective and promising medicinal qualities, since antiquity. The tremendous diversity found in species of plants, animals, marine organisms and microorganisms makes it possible for nature to serve as an appealing source of novel therapeutic candidature compounds.\textsuperscript{[1]} In recent year, microbial resistance to commonly used antibiotics has been increased to great extent & become a major solicitude to public health.\textsuperscript{[2]} The alleged consumption should be done according to the dosage prescribed by physicians as over prescription of antimicrobial drugs is an essential contributor to drug resistance phenomenon.\textsuperscript{[3, 4]}

\textit{Staphylococcus aureus} is an opportunistic pathogen often carried symptomatically on the human body. Methicillin-resistant \textit{S. aureus} (MRSA) includes those strains that have acquired a gene giving them resistance to methicillin and essentially all other beta-lactam antibiotics. MRSA was first reported in 1961, soon after methicillin was introduced into human medicine to treat penicillin-resistant \textit{Staphylococci}. This group of organisms has since emerged as a serious concern in human medicine.\textsuperscript{[5, 6, 7, 8, 9]} MRSA was first reported as a nosocomial pathogen in human hospitals. Researchers now engaged in developing new molecules with potent activity and greater stability for resolving the problem of antimicrobial-resistance phenomenon in the pathogens. Plant derived antimicrobials have been proposed their suitability for developing new active drugs.

\textit{Tridax procumbens} L. also dispensed as “Bhringraj”, which is well known Ayurvedic medicine for liver disorders, jaundice and antioxidant properties. The leaf juice possesses antiseptic, insecticidal and anti-parasitic properties. It is also used to check hemorrhage from cuts, bruises and wounds. Traditional method includes application of rubbed aerial parts of this plant on minor cuts and wounds. An aqueous extract of the plant produced reflex tachycardia and showed a transient hypotensive effect on the normal blood pressure of dogs; it had also a marked depressant action on the respiration. Reported literature showed the presence of various pharmacological properties of this plant.\textsuperscript{[10]} Therefore the study was designed for the management of MRSA by the use of the plant derived drug for the future therapeutics of the community.
MATERIAL AND METHODS

Plant extract preparation – Root of the *Tridax procumbens* L plant (*Asteraceae* family) were collected from different localities of Wardha, District of Maharashtra state (India), located between latitude 20°-21° North and longitude 78°-79° East. Collection was done during January 2012 to April 2012, from nearby areas of railway routes. The plant material was authenticated in the Department of Botany, Adarsha Mahavidyalaya Dhamangaon (Rly). The cleaned plant roots were allowed for the complete shade drying and then made to fine powder with a mechanical grinder. A powdered plant roots were extracted successfully with the polar solvent methanol by using Soxhlet assembly. The extraction was carried out for 24 – 48 hours at room temperature with mild shaking.

MRSA Isolation: All the isolates of *S. aureus* recovered from admitted patients in the private hospitals and Microbiological Laboratories from Nagpur District (Central Region). The isolation of *S. aureus* strains were done by phenotypic characterization test and were screened for methicillin resistance based on Kirby-Bauer disk diffusion method using oxacillin (OX1) & methicillin (MET 5) discs (Hi-Media Pvt. Ltd, Mumbai). The isolates were considered methicillin resistant if the zone of inhibition was 10 mm or less, according to the standard zone size interpretation chart as recommended by NCCLS, 2003 guidelines.[11]

Anti-MRSA Activity of root extract: The previously prepared root extract of the plant was tested by agar well diffusion technique for anti-MRSA activity against the clinical MRSA isolate. The root extract concentration of 300 mg/ml was used for the assay while the pure solvent methanol alone was also tested as a control.

TLC standardization: Thin Layer Chromatography was performed on a pre-coated silica gel TLC plates grade F254 (E-Merck, Darmstadt, Germany) to determine the number of compounds present in the methanol root extract. A total of 5 μl of sample was spotted at 1 cm from the bottom of silica gel plates using capillary tubes. Different solvents at various combinations and concentrations were used for metabolites profiling. Development of the chromatogram was done in closed tanks, in which the atmosphere has been saturated with eluant vapour by wetting a filter paper lining. The chromatogram was visualized under UV light (365 nm and 254 nm) and iodine vapour, and Rf values of the compounds were calculated.
**TLC bio-autography**: The TLC bio-autography was determined by agar-overlay method\cite{12}. The TLC plate was placed in a sterile Petri plate and a lukewarm 20 ml of molten Mueller Hinton Agar was poured into plate (Hi-media, Mumbai, India). The plates were allowed to solidify and left for two hours absorption. After which 18 h grown (OD adjusted 0.5 McFerland standards) 100 µl of MRSA culture was transferred onto plate and made culture lawn by using sterile L-rod spreader. The plates were incubated at 37°C in a 40 W flescent light source (~ 400 nm) for 24 h and antibacterial activity of the compound was visualized.

**Gas Chromatography**: An Agilent 6890 gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D., 0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35°C, hold for 2 minutes, then ramp at 20°C per minute to 260°C and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode).

**Mass Spectrometry**: A JEOL GCmate II benchtop double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000 software was used for all analysis. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

**Mass spectrometry library search**: Identification of the components of the purified compound was done by matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software.

**RESULTS & DISCUSSION**

**Anti-MRSA activity of plant extract**: A total of 134 MRSA isolates form different clinical specimens were tested against the methanol root extract (at concentration of 300 mg/ml). Methanolic root extract displayed a broad spectrum antibacterial activity for methicillin resistant *Staphylococcus aureus* isolates. The growth inhibition zone was ranging from 17 mm to 23 mm (Data depicted in Table1). The methanol root extract showed two potent compounds which could be the novel drug in the treatment of infection caused by MRSA.
Table 1: Zones of inhibition of MRSA against root extract

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Specimen</th>
<th>No. of S. aureus isolates obtained</th>
<th>No. of MRSA isolates screened</th>
<th>Root extract inhibition zone range (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pus</td>
<td>82</td>
<td>33 (40.24%)</td>
<td>17 -23</td>
</tr>
<tr>
<td>2</td>
<td>Blood</td>
<td>90</td>
<td>42 (46.66%)</td>
<td>17 -23</td>
</tr>
<tr>
<td>3</td>
<td>Urine</td>
<td>60</td>
<td>28 (46.66%)</td>
<td>17 -23</td>
</tr>
<tr>
<td>4</td>
<td>Sputum</td>
<td>27</td>
<td>11 (40.74%)</td>
<td>17 -23</td>
</tr>
<tr>
<td>5</td>
<td>Miscellaneous</td>
<td>50</td>
<td>20 (40.00%)</td>
<td>17 -23</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>309</td>
<td>134 (43.36 %)</td>
<td></td>
</tr>
</tbody>
</table>

Several reports of different researchers are available indicating the efficacy of plant extracts towards anti-MRSA potential. In regards to earlier reports, present study showed the higher inhibitory activity of methanol root extract of _Tridax procumbens_ plant against the methicillin resistant _Staphylococcus aureus_ isolates. Kabir _et al._, (2005) evaluated the anti-MRSA potential of water and ethanol extracts of six medicinal plants viz. _Terminalia avicennioides, Phyllanthus discoideus, Bridella ferruginea, Ageratum conyzoides, Ocimum gratissimum_ and _Acalypha wilkesiana_ and reported the much efficacy of ethanol extract than aqueous extract against MRSA.\(^{[13]}\) Similarly Yousef _et al._, (2013) screened the medicinal plants for the anti-MRSA activity and reported the potent antimicrobial activities of _Cistus salvifolius, Salvia officinalis, Arbutus pavarii, Pistacia anatrica_ and _Myrtus communis_ plant extracts, with zone of inhibition ranging from 6-18 mm against the MRSA.\(^{[14]}\) Also the Sucilathangam _et al._, (2012) evaluated the MRSA potential of _Quercus infectoria_ and reported significant antibacterial activities of ethanolic and aqueous extracts of _Quercus infectoria_ against all strains of methicillin-resistant _Staphylococcus aureus_ (MRSA). They also screened the ethanolic and aqueous extracts against the 103 clinical isolates of _Staphylococcus aureus_ and reported the zone of inhibition of 12 to 16 mm for aqueous extract & 11-17 mm for ethanolic extract.\(^{[15]}\) In the present study, the methanol extract from the root part of the _Tridax procumbens_ plant was evaluated and showed comparatively higher inhibitory activity against the MRSA than the earlier reported plants. The present findings that supports the anti-MRSA activity of _Tridax procumbens_ plant was reported by Dhanabalan _et al._, (2008). They reported the antibacterial activity of _Tridax procumbens_ aqueous and methanolic leaf extract against the methicillin and penicillin resistant _Staphylococcus aureus_ clinical isolates obtained from Bovine Mastitis disease of cattle by disc diffusion and agar well diffusion methods. The methanol extract reported to be the most active extract with significant antibacterial activity (7.8 – 8.2 mm inhibitory zones) against _Staphylococcus aureus_ isolated from four different breeds of cows suffering from mastitis.\(^{[16]}\) Our finding in the present study showed much
higher inhibitory activity of root methanol extract of *Tridax procumbens* against the MRSA isolates with inhibition zone ranging from 17-23 mm (Table1).

**TLC standardization:** TLC standardization of methanol root extract was done by using three different solvent systems. These solvent systems were Chloroform (10:0), Chloroform: Methanol (8:2) & Chloroform: Methanol (6:4). These solvent systems showed presence of different bands of phyto-compounds visualised under UV light (365 nm and 254 nm). After the standardization chloroform: methanol TLC plates were chosen for further studies.

**TLC bio-autography:** Two antimicrobial compounds showed MRSA inhibition in the (8:2) TLC plate by bio-autography assay. These two antimicrobial compounds (AM1 & AM2) showed Rf values of 0.92 and 0.21, respectively. Among the two anti-MRSA compounds, zone of inhibition of AM2 was higher than the AM1. The MIC value of 10 µl was determined for AM2 compound against MRSA. Both of the compounds were subjected to GC-MS analysis for structural determination, characterization & identification.

**GC-MS Studies:** GC-MS analysis revealed that, the compound AM1 was matched with NIST database spectrum with convincing MS fragment of Dasycarpidan-1-methanol, acetate (ester). The compound AM2 was matched with Milbemycin B, 6, 28-anhydro-15-chloro-25-isopropyl-13-dehydro-5-O-demethyl-4-methyl (Fig 1 & 2).

Fig1. Mass spectra of AM1 compound
It is a fact that antimicrobial potency of the plant derived drug is directed towards the active phytochemicals or the secondary metabolites present in the plant. The use of specific bioassay technique for the identification of bioactive compounds is the concern matter for the researchers. Chemist, biologist and microbiologists have continuously engaged in the activities concerning the easiest way for isolating the bio-molecules which have been proved earlier for the pharmacological properties.

Bharathi et al., (2012) evaluated the antibacterial potential of Tridax procumbens Linn using ethyl acetate and methanol extract and showed significant inhibitory activity of both extracts with inhibition zone range from 8 mm to 18 mm. This study also validates the involvement of bioactive secondary metabolites of Tridax procumbens in antibacterial activity. Many researchers attempted for searching the bioactive compound responsible for the anti-MRSA activity. 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 16 mm from alkaloids, 13 mm from glycosides, 14 mm from flavonoids and 24 mm from terpenoids against the Staphylococcus aureus. The terpenoids results in permeability of the cell membrane of the bacteria & showed the inhibitory effect. Sharma and Kumar (2009) extracted the free & bound flavonoids from the different parts such as leaf, stem, flower & root of Tridax procumbens. They evaluated the antibacterial potency of free & bound flavonoids against the Staphylococcus aureus and reported 0 – 16 mm inhibition zones for
free flavonoids and 0 – 22 mm for bound flavonoids. The bound flavonoids from flower part of *Tridax procumbens* was reported to be the most potent for the *Staphylococcus aureus* [10]. In the present study two anti-MRSA compounds AM1 & AM2 were detected in the methanolic extract of *Tridax procumbens*. Previously AM1 (Dasycarpidan-1-methanol, acetate (ester), molecular formula C_{20} H_{26} N_{2} O_{2}) compound was reported in methanolic extract of *Oldenlandia umbellate* plant as one of the phyto-component by GC-MS studies [19]. Another researcher Li Yu-mei, (2012) reported the presence of compound AM1 in the *Semen cassia* and *Semen seeds tea* of *Cassia tora* L. plant belonging to family *Leguminosae*. They extracted the volatile oils from the *Semen cassia* and *Semen seeds tea* and indentified the volatile components by GC-MS studies [20]. In present study, anti- MRSA activity of the AM1 compound was detected against different clinical isolates of MRSA. This confirmed the antimicrobial activity of AM1 compound in the methanolic extract of *Tridax procumbens*. The second compound AM2 detected in the root methanol extract was constituent member of milbemycin group of compounds, which is well known veterinary drugs used in cats and dogs for anti-parasitic, insecticide, anti-helmentic (wormicidal) activity [21, 22]. One of the member of the Milbemycin compounds, Milbemycin b, 13-chloro-5-demethoxy-28-deoxy-6, 28-epoxy-5- (hydroxyimino)-25-(1 methylethyl)- (6r,13r,25r)- molecular formula C_{33} H_{46} CLNO_{7} was isolated and identified from acetone extract of *Caralluma truncato-coronata* belonging to sub-family *Asclepiadoideae* by GC-MS studies [23]. This confirms the antimicrobial nature of the two compounds in regards to anti-MRSA activity. The study validates the use of the *Tridax procumbens* as the potential source of anti-MRSA agents. In present study, two antimicrobial compounds AM1 & AM2 with significant level of anti-MRSA activity were detected by GC-MS studies. This could be the first attempt to report different anti-MRSA compounds (AM1 & AM2) from the root part of *Tridax procumbens*.

**CONCLUSION**

The study concludes that plant *Tridax procumbens* had the anti-MRSA activity at the optimum level and the isolated bioactive compounds could be used as an anti-MRSA agent for future reference.

**REFERENCES**


