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RESEARCH ARTICLE

In vitro Assessment of Essential Oils against Methicillin Resistant *Staphylococcus aureus* (MRSA) from Chronic Wound

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ABSTRACT

Skin wounds are particularly prone to bacterial infections because the wounds provide an ideal medium for bacterial proliferation and a portal of entry into the bloodstream. The infection can be readily treated with a variety of antibiotics if the bacteria involved are susceptible. However, there are only extremely limited or no treatment options when antibiotic resistant strains are involved in the wound infection like Methicillin Resistant *Staphylococcus aureus* (MRSA). Essential oils are concentrated natural extracts derived from plants, which were proved to be good sources of bioactive compounds with antioxidative and antimicrobial properties. This study followed the effect of some commonly used essential oils like Eucalyptus oil (*Eucalyptus globules*), Clove oil (*Eugenia aromatic*), Mango Ginger oil (*Curcuma amada*), Tea tree oil (*Melaleuca alternifolia*) and Oregano oils (*Origanum vulgare*) were tested against MRSA from chronic wound sample. The antibacterial effect of the essential oils Mango Ginger oil, Tea tree oil and Oregano oils gave the highest antibacterial effect on all MRSA strains with inhibition ranged from 16-24 mm. While, Eucalyptus oil, Clove oil were moderate effect with inhibition ranged from 13-17 mm. The investigation suggests potentials of some essential oil as an alternative to antibiotics for the treatment of wound associated infection regardless of antibiotic susceptibility.

Key-words: Essential oil, Antimicrobial activity, *Staphylococcus aureus,* Methicillin, MRSA.

INTRODUCTION

Virulent strains of *Staphylococcus aureus* (*S. aureus*) attacks the human body causing infective endocarditis, skin and soft tissue infection, hospital-acquired pneumonia, vertebral osteomyelitis, associated epidural abscess and surgical wound infections (1). Methicillin resistant *Staphylococcus aureus* (MRSA) is a strain of genus *Staphylococcus*, initially resistant to methicillin as well as many beta-lactamase antibiotics. MRSA is mainly hospital-acquired or nosocomial

infection (2). Although, there are community associated - MRSA infections (CA-MRSA) which are MRSA infections found in healthy people in a community outside the hospital environment. Wounds or abscesses also occur within body become infected when microorganisms from the outside environment, or from within the person's body, enter the open wound and multiply. A wound that is red, painful, swollen and draining pus is probably infected (3).

The Centre for Disease Control (CDC) estimates that each year, nearly 2 million people in the United States acquire an infection while in hospital, resulting in 90,000 deaths. More than 70% of the bacteria that causes these infections are resistant to at least one of the antibiotics commonly used to treat (4). For MRSA, Infectious Diseases Society of America guidelines recommend treatment with vancomycin or daptomycin. However, each antimicrobial agent has limitations. Several issues restrict the utility of vancomycin, including slow bactericidal activity, low tissue penetration and increasing reports of resistance and failure. While daptomycin is effective against MRSA bacteremia, treatment-emergent non-susceptibility is concerning, and evidence suggests prior vancomycin treatment may encourage daptomycin resistance in *S. aureus (*5). It is clear that the currently available antibiotics are insufficient to control these superbugs and, hence, more research and novel antimicrobial sources are highly demanded.

There is a need to find alternative strategies to deal with infections resulting from drug-resistant bacteria especially MRSA. In this regard, plant essential oils and their major chemical constituents are potential for antibacterial agents. Essential oils (EO) were proved to be good sources of bioactive compounds, with antioxidative and antimicrobial properties. Many plant parts contain oils that can be extracted: leaves, seeds, bark, resin, berries, flowers, roots or fruits. The composition is complex and consists mostly of terpenes (mostly monoterpenes and sesquiterpenes), terpenoids (oxygenated compounds such as phenols, alcohols, aldehydes, ketones or ethers) and aromatic compounds (6). Essential oils were already successfully used in treatment of several conditions such as releasing of pain associated with chronical conditions or with medical procedures, reducing postoperative nausea or autonomic response to pain (7) for possible symptom relief in people with cancer and even to treat pediculosis in children (8).

MATERIALS AND METHODS

Screening of Methicillin Resistant *Staphylococcus aureus* (MRSA)

A total of 31 pus specimens were collected from chronic wound for *S.aureus* screening. The samples were obtained from various health care hospitals in Erode District, Tamil Nadu, India. Wound swabs were streaked on mannitol salt agar and incubated at 37^{o}C for 24 to 48 h. Growth and fermentation of mannitol on MSA was examined. *S. aureus* were identified using gram stain and biochemical tests based on Bergey's manual of systematic bacteriology (9). Detection of MRSA by using CHROM agar with Oxacillin 4 mg/l; Oxacillin Resistant Screening Agar Base (ORSAB) with oxacillin 2mg/l and Blood Agar (BA) with Oxacillin 2mg/l, Baird Parker Agar (BPA) with Ciprofloxacin 8mg/l, Mueller Hinton Agar with 4% NaCl and 6mg/l Oxacillin, Mannitol Salt Agar (MSA) with Oxacillin 4mg/l. The plates were incubated at 37°C for 24 to 48 h (10).

Detection of *mecA* gene

Detection of the *mec*A gene in the *S. aureus* isolates were performed by polymerase chain reaction. The PCR procedure was based on a modification method (11) and this were used as the gold standard for all isolates. Oligonuclotide used were *mec*A F primer 1282 (5'–AAA–ATC–GAT–GGT–AAA–GGT–TGG–C–3') and *mec*A R primer 1793 (5'– AGT-TCT-GCA-GTA-CCG-GAT-TTG-C-3'), which gives a PCR products of 533bp. PCR was performed on cooled thermocycler 5333, Eppendorf version 2.30.33-09, using a reaction mixture of 20 µl consisting of *Taq* polymerase buffer 2µl, 1 µl of each primer, DNA sample 1 µl, *Taq* polymerase enzyme 0.2 µl and distilled water 12.8 µl. 20 µl of PCR product was then analyzed by 1.2% agarose gel electrophoresis.

Antibiotic susceptibility test

The antibiotic resistant and sensitivity test for each isolates were carried out on Muller Hinton Agar by Kirby bauer disc diffusion method. Amikacin (30mcg), Cefazolin (30mcg), Ceftazidime (30mcg), Ceftizoxime (30mcg), Cephoxitin (30mcg), Chloramphenicol (30mcg), Ciprofloxacin (5mcg), Clindamycin (2mcg), Co-Trimoxazole (Trimethoprim / Sulphamethoxazole) (1.25/23.75mcg), Erythromycin (15mcg), Gentamicin (10mcg), Kanamycin (30mcg), Methicillin (5mcg), Nalidixic acid (30mcg), Netillin (30mcg), Norfloxacin (10mcg), Ofloxacin (5mcg), Oxacillin (1mcg), Penicillin G (10 units), Rifampicin (5mcg), Tetracycline (30mcg), Vancomycin (30mcg) and Moxalactam (30mcg) by the disc diffusion method The plates were incubated at 37◦C and the zone of inhibition was observed after 24 h (12).

Minimum Inhibition Concentration (MIC)

Minimum inhibition concentrations of Ciprofloxacin, Oxacillin and Vancomycin antibiotics were tested by the checkerboard assay method (13). Plate wells from each column in row 1 were marked and 100 µl of antibiotics were added. Later, 50 µl of sterile distilled water was added to row 2-11. Two fold serial dilutions were performed by transferring 50 µl of solution from row 1 to row 2. This was repeated down to row 12. 40 µl of double strength nutrient broth and 10 µl of bacterial solution were added to all the wells, so the final concentration on inoculum in all the wells is 100 µl. Plates were covered by plastic cover and incubated at 37◦C overnight. The bacterial growth was determined after addition of 40 µl of tetra-zolium red (0.2 mg/ml). The MIC of isolates was taken as the lowest concentration of the antibiotic of which the bacterial tested did not show visible growths.

Collection of Essential Oils

Five of the most commonly used essential oils in our geographic area were tested for their antibacterial properties. Eucalyptus oil (*Eucalyptus globules*), Clove oil (*Eugenia aromatic*), Mango Ginger oil (*Curcuma amada*), Tea tree oil (*Melaleuca alternifolia*), Oregano oils (*Origanum vulgare*) were acquired from specialty retailer shops. We followed to get good quality, natural oils, with no added synthetic compounds.

Disc diffusion method

The agar disc diffusion method was used to determine the antibacterial activity. Sterile discs (Hi-media, India) were loaded with 50 µl of the sample oil and were left to dry for 30 min at 37ºC. All discs were applied on the nutrient agar medium inoculated with 100 µl of bacteria suspension and plates were incubated for 37ºC for 24 h. Zone of inhibition around the disc was measured after incubation period and recorded.

RESULTS AND DISCUSSION

Isolation of *S. aureus*

Totally 31 clinical wound isolates of *S.aureus* were collected from four major medical centers distributed in Erode District, Tamil Nadu, India. All the wound samples were tested on Mannitol Salt Agar (MSA) for the isolation of *S. aureus.* On MSA *S. aureus* colonies were appeared yellow zone, which was containing mannitol to detect mannitol fermentation.

Antimicrobial Susceptibility Test

Twenty three antibiotic discs were used for antimicrobial susceptibility test against *S. aureus.* It showed 92.85%, 81.74%, 76.19% and 74.60% of the isolates resistant to Netillin, Co-Trimoxazole, Penicillin-G, and Tetracycline respectively. Low resistant was observed against Clindamycin (30.15%) and Cefazoline (30.95%). All MDR

Staphylococcus aureus were resistant to any one of the antibiotic like Ciprofloxacin, Methicillin and Oxacillin. The multiple antibiotic resistance (MAR) index were calculated by using the following formula.

$$\text{MAR index for isolates} = \frac{\text{Number of antibiotics to which the isolates is resistance}}{\text{Number of antibiotics tested}}$$

The MAR index of isolated bacteria was greater than 0.2, which implies that strains of such bacteria originate from an environment where several antibiotics were used (14). MAR index of isolates in present study indicate large portion of bacteria were exposed to the antibiotics showing MAR index more than 0.6 (Table 1). Most of wound isolates showed multiple antibiotic resistances in the study area. It may be due to large portion of the bacteria isolate being previously exposed to several antibiotics. This data gives idea about the common trend of increased antibiotics resistance of wound causing *S. aureus* in this region, which may be due to geographic variation on indiscriminate use of antibiotics.

Phenotypic Identification of MRSA

All the *S. aureus* isolates were cultured on CHROM agar with Oxacillin (Ox), ORSAB, Baird Parker agar with ciprofloxacin, Mannitol Salt agar with Ox, Blood agar with Ox, and Mueller Hinton agar media with Ox and incubated for 48 h at 37°C (Table 2). The plates were examined at 24 and 48 h, which were mauve color on CHROM agar, blue on ORSAB, yellow on MSA Ox, black on Baird Park agar, white on Blood agar and Ox-MH agar. 100% of the MRSA strains were recovered by the use of CHROM agar and ORSAB agar after 48 h of incubation. 100% positive MRSA strains were screened from CHROM and ORSAB agar media when it compared with genotypic confirmation.

Genotypic Identification of MRSA

The genotypic and phenotypic expression of pre-confirmed 31 clinical wounds *S. aureus* isolates were examined in this study. All the isolates were tested for the phenotypic confirmation for MRSA and hence were all genetically confirmed to be MRSA using PCR. Among 31 *S. aureus*, 6 isolates (SaW 2, 3, 25, 26, 29 and 31) were positive for the *mec*A gene in the molecular weight of 533bp (Table 2). The remaining *S. aureus* were negative for the *mec*A gene. Currently, multiple antibiotic resistant *S. aureus* strains constitute a major healthcare problem, since they are the etiologic agent of several nosocomical and skin infection. For that reason, accurate detection of resistant isolates constitutes a critical goal of clinical microbiology. Therefore PCR assay have become an essential tool in laboratory programmes. The utility of PCR for the accurate detection of the *mec*A gene and the possibility of simultaneous identification of *S. aureus.(*15)

Minimum Inhibition Concentration

Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of 6 MRSA isolates were tested against ciprofloxacin, oxacillin and vancomycin (Table 3). None of the ciprofloxacin susceptible in MRSA isolates, the MIC for these isolates were between 0.125 mg/ml and 0.25 mg/ml. All MRSA isolates were resistant to Ox (MIC > 0.002 mg/ml) and vancomycin (0.025mg/ml). Similarly the method (16) reported that the ciprofloxacin resistant MRSA isolates were isolated from the patients, none of the patients who yielded ciprofloxacin susceptible MRSA had used ciprofloxacin previously.

Anti MRSA Activity of Essential Oils

The selected five Essential Oils (EO) have been screened for their antibacterial activities against MRSA using the disc diffusion test. The results were represented as the diameter of inhibition zone (Table 4). MRSA strains were

susceptible to mango ginger, tea tree and oregano essential oil with a zone of inhibition (ZOI) ranging from 16 to 24 mm larger than those of methicillin and ciprofloxacin reference antibiotics, while eucalyptus and clove oil showed moderate ZOI from 13–17 mm. The positive controls (methicillin and ciprofloxacin) showed ZOI ranging from 11 to 15 mm, the negative control vehicle dimethylsulfoxide (DMSO) showed no inhibition against MRSA.

A rhizome paste has traditionally been used for healing of wounds, cuts and itching (17). The external use of the rhizome paste for sprains and skin diseases is also an old practice (18). Antibacterial activity of free and bound phenolics from mango ginger rhizomes has been reported (19). Tea tree oil presented better antibacterial activity toward anaerobic bacteria than aerobic bacteria (20, 21). They used mass spectrophotometry to separate two major components, terpinen-4-ol and 1, 8-cineole, were used to evaluate skin toxicity by a single topical application. The oregano oil showed a significant antibacterial activity over PBS controls against four *Acinetobacter baumannii* strains and two MRSA strains, with the MICs ranging from 0.08 to 0.16 mg/ml (22). Oregano oil also exhibited similar antibacterial activities against established biofilms (24-h-old) formed by the 13 bacterial strains within 1 h, with complete inactivation of the biofilms of *A. baumannii*, *P. aeruginosa* and MRSA at the concentrations of 0.3, 1.0, and 0.4 mg/ml, respectively.

Further Scope

As the antimicrobial efficacy of the tested essential oil have been established, further research is required keeping the limitations in mind. In this study, we have used commercial essential oils since preparing fresh oil wasn't feasible in our study. These results could be compared with freshly prepared essential oils. In our investigation we tested essential oils on selected MRSA strains those are responsible for chronic wound infection. These essential oils would be more acceptable for treatment if the tested oils were applied directly or synergistic treatment or hydrogel form on infection area with safe doses instead of individual bacteria.

CONCLUSION

In this study, it's been proven that some essential oils have noticeable antimicrobial activity against MRSA which are responsible for chronic wound infection. In the final analysis, the potential of mango ginger oil, oregano oil and tea tree oil to be used as natural antimicrobial agent is recommendable as antimicrobial action against methicillin resistant *Staphylococcus aureus*. However bacterial resistant to chemical antibiotics seem to be capable of overcoming the action of essential oil which required further improvement before the EO base product development.

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Table 1. Multiple antibiotic resistant index of MRSA isolates.

S. No.	Isolates	No. of Resistant isolates	No. of Sensitive isolates	MAR Index	% Frequency
1	SaW2	17	6	0.739	73.91
2	SaW3	16	7	0.695	69.56
3	SaW25	22	1	0.956	95.65
4	SaW26	14	9	0.608	60.86
5	SaW29	18	5	0.782	78.26
6	SaW31	14	9	0.608	60.86

SaW- *Satphylococcus aureus* Wound
MAR - multiple antibiotic resistant

Table 2. Phenotypic and Genotypic Identification of *Staphylococcus aureus* using Selective Media and PCR

S.No.	Isolates	Phenotypic Identification Selective Media for MRSA						Genotypic Identification	
		CHROM Agar[a]	ORSAB[b]	BPA[c]	MSA[d]	BA[e]	MHA[f]	*mec*A gene	Class
1	SaW 2	+	+	+	+	-	-	+	MRSA
2	SaW 3	+	+	+	+	+	+	+	MRSA
3	SaW 25	+	+	-	+	+	+	+	MRSA
4	SaW 26	+	+	+	-	-	+	+	MRSA
5	SaW 29	+	+	+	+	+	+	+	MRSA
6	SaW 31	+	+	-	-	+	+	+	MRSA

SaW- *Satphylococcus aureus* Wound, + : Positive, -: Negative, MRSA: Methicillin Resistant *Staphylococcus aureus*
[a]CHROM agar *Staphylococcus aureus* with oxacillin 4mg/l,
[b]ORSAB - Oxacillin Resistant Screening Agar Base with Oxacillin 2mg/l,
[c]BPA - Baird Parker Agar with Ciprofloxacin 8mg/l,
[d]MSA - Mannitol Salt Agar with Oxacillin 4mg/l,
[e] BA - Blood Agar with Oxacillin 2mg/l,
[f]MHA - Mueller Hinton Agar with 4% NaCl, 6 mg/l Oxacillin.

Table 3. Minimum Inhibition Concentration of Ciprofloxacin, Oxacillin and Vancomycin for MRSA Isolates

S.No.	Isolates	Ciprofloxacin (1mg/ml)		Oxacillin (0.2mg/ml)		Vancomycin (6mg/ml)	
		MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml
1	SaW2	0.25	0.25	0.05	0.05	0.43	0.43
2	SaW3	0.25	0.25	0.025	0.05	0.43	0.43
3	SaW25	0.25	0.25	0.05	0.05	0.43	0.43
4	SaW26	0.25	0.25	0.025	0.025	0.43	0.43
5	SaW29	0.25	0.25	0.025	0.05	0.43	0.43
6	SaW31	0.125	0.25	0.05	0.05	0.43	0.43

SaW- *Satphylococcus aureus* Wound
MIC - minimum inhibition concentration
MBC - minimum bactericidal concentration

Table 4. Anti MRSA Activity of Different Essential Oil

S.No.	Isolates	Zone of Inhibition (mm)					Positive Control Antibiotics (ZOI –mm)	
		Essential Oils						
		Eucalyptus oil	Clove oil	Mango ginger oil	Tea Tree oil	Oregano Oil	M	Cf
1	SaW2	16	13	21	21	22	12	14
2	SaW3	16	15	18	19	19	14	12
3	SaW25	13	14	18	16	16	11	13
4	SaW26	16	16	19	19	17	13	15
5	SaW29	15	17	21	14	19	14	15
6	SaW31	17	15	24	17	23	15	14

SaW- *Satphylococcus aureus* Wound
M- methicillin and Cf - ciprofloxacin