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Evaluation of Lemon Grass Essential Oil as an Antimicrobial Agent Against clinical isolates of MRSA, VRSA and VRE

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Abstract

Hospital acquired infections and wound infections are a major cause of morbidity and mortality worldwide. A 2015 study finds that the rates of hospital-acquired infections and antimicrobial resistance were clearly higher in India than those reported by the CDC in the U.S. The problem is complicated due to the appearance of newer antibiotic resistant strains. Herbal medicines, offer promising alternative. The essential oil of Lemon grass (*Cymbopogon citratus*) is assessed for its antibacterial activity against clinical isolates, MRSA (Methicilin Resistant *Staphylococcus aureus*), VRSA (Vancomycin Resistant *Staphylococcus aureus*) and VRE (Vancomycin Resistant Enterococci). Lemon grass essential oil (LGEO) was extracted and tested against multidrug resistant(MDR) bacterial wound pathogens isolated from pus samples. The nine selected strains, MRSA (based on presence of Mec A gene), VRSA and VRE (based on MIC of Vancomycin) were used for testing antimicrobial activity and MIC determination. Antibiotic resistance of the isolates was assessed by standard disc diffusion assay. LGEO showed antibacterial activity against all nine pus isolates of MRSA, VRSA and VRE. LGEO contains multiple constituents which show potent antibacterial activity against multidrug resistant clinical isolates of MRSA, VRSA and VRE. *In vitro* studies suggest that LGEO based formulation can be used for topical application to control pus forming MDR organisms.

Key words: Lemongrass essential oil, Citral, Antimicrobial activity, MRSA, VRSA, VRE, Wound pathogen, clinical isolates, MIC

1. INTRODUCTION

Wound and skin infections caused by *S. aureus* are becoming increasingly difficult to treat by conventional antibiotics in view of the emergence of antibiotic resistant strains. Antibiotics are used globally as a first line of treatment for any type of infection. Infections caused by Methicilin Resistant *S. aureus* (MRSA) have been associated with high morbidity and mortality rates. *S. aureus* is a major cause of hospital acquired infections involving skin and soft tissues. Approximately 2 – 10% of the U.S. population is now colonized with MRSA. MRSA can cause serious infections of surgical site wounds, blood stream infections, pneumonia and many more. MRSA has become the most frequent cause of skin and soft tissue infections and MRSA related mortality surpasses AIDS annually (Centre for Disease Control and Prevention USA, 2002).

MRSA is one of the common causes of hospital-acquired infections even in India. Methicilin resistance *S. aureus* has been reported from different hospitals in the range of 30-80% isolates. The prevalence rate of MRSA among *S. aureus* infections from Varanasi, Indore and New Delhi is more than 50 %. In a study from Hyderabad in 2011, 79.6% clinical isolates of *S. aureus* were identified as Methicilin Resistant *S. aureus* (MRSA) *(1).*

The health risks associated with MRSA infections, including its potential to produce invasive infections, particularly in vulnerable patients, and its resistance to multiple antibiotics, permit the implementation of monitoring programs to control its dissemination. Recommendations by clinical guidelines suggest Vancomycin to be the drug of choice to treat MRSA infections, but in reality the emergence of the Vancomycin-resistant *S. aureus* (VRSA) and Vancomycin-intermediate *S. aureus* (VISA) has made antibacterial therapy difficult. The existence of Vancomycin resistant *S. aureus* (VRSA) and multi drug resistant *S. aureus* has generated worldwide concern. VRSA infected patients are left with a choice of very few drugs or antibiotics like Linezoid, Trimethoprime and Daptomycin (1).

VRE first appeared in the late 1970s (2) (3) and has now spread worldwide (4) (5). In Germany, vancomycin resistance is almost exclusively restricted to strains of *E. faecium* (>99% of all VRE are *E. faecium*) (6) (7). Vancomycin-resistant *E. faecalis* are very rare (<1% of all *E. faecalis*) (6). Hence, despite differences in their pathogenicity, the two species are grouped together as VRE. There has been a trend towards an increase in vancomycin resistance in isolated *E. faecium* in recent years; it currently lies between 8% and 11% (8) (9) (10).

It has thus, become evident that there is an urgent need for novel antibacterial agent with broader spectrum, lesser side effects and without cross-resistance to antibiotics in use. Therefore, new chemotherapeutic compounds to treat and control infections by these microorganisms have been broadly studied and developed (11).

Medicinal plants have been used throughout the world as a source for safe drugs for thousands of years. They are therefore, receiving considerable attention due to their pharmacological effects such as antimicrobial, anticarcinogenic, and antioxidant properties (Balunas & Kinghorn, 2005). Natural products have been used as a major source of innovative and effective therapeutic agents throughout human history, offering a diverse range of structurally distinctive bioactive molecules (Jeon et al., 2011). Essential oils represent a cheap and effective antiseptic topical treatment option even for antibiotic-resistant strains as MRSA.

Cymbopogon citratus, commonly known as lemon grass has been cultivated over many years for medicinal purposes in south Asian countries. Researcher workers have found that lemon grass has antidepressant, antioxidant, antiseptic, astringent, bactericidal, fungicidal, nervine and sedative properties (12)

Present work was carried out to assess the effectiveness of Lemon grass essential oil (LGEO) against MRSA, VRSA and VRE isolated from clinical samples obtained from Pune, Maharashtra, India.

2. MATERIALS AND METHODS

2.1 Extraction of LGEO

The Leaves of Lemon grass (*Cymbopogon citratus*) were collected from Pune, India. Authentication and identification was done at Botanical Survey of India (BSI), Pune with voucher specimen (BSI-V. No. SOACYC1).

Leaves of lemon grass that were cut into small pieces (1 cm) were subjected to hydro-distillation for 2 h, using Clevenger apparatus. A pale yellow essential oil was obtained from lemongrass plant. Essential oil collected was stored in air tight container.

2.2 GC–MS analysis of LGEO

The LGEO was subjected to Gas chromatography–mass spectrometry (GC–MS) (Model: Accu TOF Gcv) analysis in order to identify the constituents.

GC conditions used were as follows: Column type: capillary column; class: semi-standard Non-polar; column length: 60m; column diameter: 0.25mm; start temp: 70^0C; end temp: 250^0C; phase thickness: 0.25 μm; Heat rate: 2.5 K/ min; start time: 4min; end time 20min; Injection temp: 250^0C-interface temp: 260^0C; ion source in MS is 200 and m/z is 35-6000range.

2.3 Isolation and characterization of clinical isolates

Bacterial wound pathogens isolated from pus sample were used for this study. The pus samples were obtained from pathology laboratory. They were characterized through microscopic examination, Gram staining, growth on mannitol salt agar and coagulase test for clinical isolates of *S. aureus*. Growth on Enterococcus agar was checked for clinical isolates of enterococci. Antibiotic resistance profile of each culture was determined by standard disc diffusion assay as per CLSI guidelines (13).

2.4 MRSA, VRSA and VRE Confirmation

Three strains each of MRSA, VRSA and VRE were selected based on following tests.

- Identification of MRSA by disc diffusion test. The isolates of pus sample were screened for MRSA by using standard discs of Oxacillin (1 μg/disc) and Cefoxitin (30 μg/disc) (HiMedia).
- Methicillin resistance arises by acquisition of a staphylococcal cassette chromosome SCC mec, and is conferred by the mec A gene, which encodes the low-affinity penicillin-binding protein PBP 2A (14). Therefore confirmation of MRSA by detecting presence of mec A gene by PCR amplification. (Model –DNA analyser 3730x1 – applied Biosystems, USA)
- PCR conditions were as follows –initial denaturation at 94^0C for 3 min was followed by 35 cycles of amplification (94^0C for 30 sec, 54^0C for 30 second 72^0C for 45 sec) and a final extension step was done at72^0C for 7 min. The primers used were mec A, F & R (Sigma) (1) (15).

  Following primers were used

- mec A F TCCAGGAATGCAGAAAGACC
- mec A R TCACCTGTTTGAGGGTGGAT

- Determination of MIC of Vancomycin by strip assay.

  Estimation of MIC of Vancomycin was done by Vancomycin Ezy MIC strip VAN (0.016 to 256 mcg/ml) (Hi Media) (16) (17).

2.5 Determination of antibacterial activity and MIC of LGEO against nine isolates

Antibacterial activity of LGEO against each culture was determined by standard disc diffusion assay as per CLSI guidelines (13). Test organisms (hospital isolates-

three MRSA, three VRSA and three VRE) were inoculated on Nutrient agar at 37^0C for 24 h. Saline suspension of 24 hrs old culture was prepared; (Standard 0.5 McFarland). 750 µl saline suspension was mixed with 20 ml of pre-sterilized, cooled Mueller Hinton agar butt and poured in sterile Petri plate. The plates were allowed to solidify at room temperature. Sterile Whatman filter paper discs were soaked (10µl) in respective concentration prepared and placed on agar surface. Organisms were exposed to 7 dilutions of LGEO, pure LGEO (100%), 50%, 25%, 12.5%, 6.25%, 3.12% and 1.56 % (v/v) dilutions. All dilutions were carried out using Dimethyl Sulfoxide. DMSO did not show inhibitory effect on organisms. Plates were kept at 4^0C for 30 min for pre-diffusion and incubated at 37^0C for 24 hrs. All exposures were carried out in triplicates. Diameter of zone of inhibition was measured and recorded. Minimum Inhibitory Concentration (MIC) was also determined.

3 RESULTS:

3.1 GC-MS Analysis of LGEO

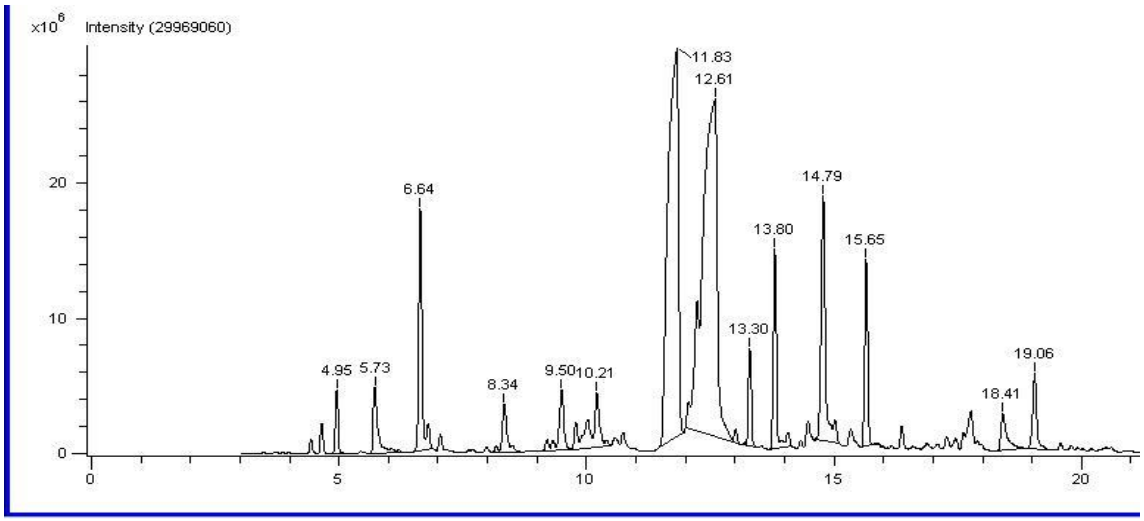

Fig 1. GC-MS Chromatogram of LGEO

3.1.1 Components of LGEO as identified by Gas chromatography–mass spectrometry

A pale yellow essential oil was obtained from fresh lemongrass plant. GC–MS analysis revealed the presence of many compounds representing the lemon grass essential oil.

Table 1: Components of LGEO

Peak No	Component	Retention time	Area %
1	5 –Hepten-2-one, 6-methyl	5.73	2
2	Limonene	6.64	6
3	1,6-Octadien3 ol, 3,7 dimethyl (linalool)	8.34	2
4	6-Octenal, 3,7-dimethyl (citronellal)	9.5	2
5	7-Oxabicyclo(4.1.0)heptanes, 1-methyl-4-(1-methylethenyl)- (Limonene oxide)	10.21	4
6	2-6,Octadienal, 3.7-dimethyl, (Z)β- Citral	11.83	27
7	2-6,Octadienal, 3.7-dimethyl, (E)α- Citral	12.61	35
8	3,7-Nonadien-2-one, 4,8 dimethyl-	13.31	2
9	4,8-Dimehyl-nona-3,8-dien-2-one	13.81	5
10	2-6,Octadien-l-ol, 3.7-dimethyl, acetate(citral)	14.79	8
11	Bicyclol(7 2 0) undec- 4-ene, 4,11,11-trimethyl 8 methylene	15.65	4
12	Napthalene, 1,2,3,5,6,8 a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-(1S cis)	18.41	1
13	Caryophyllene oxide	19.06	2
14	camphene	4.95	1

GC–MS analysis revealed the presence of many compounds representing the lemon grass essential oil. One of the main constituents of the many different species of lemongrass (genus *Cymbopogon*) is citral (3,7-dimethyl-2,6-octadien-1-al) (19). In present study the major components identified were α- Citral (35%) and β- Citral (27%), Limonene (6%) citronellal (2%) and limonene oxide (4%) were minor but significant components (Table 1).

3.2 Characterization of clinical isolates

To test the efficacy of lemongrass essential oil, nine cultures were selected as test organisms. Six hospital isolates of MRSA/VRSA were grown on Mannitol salt agar and checked for their ability to coagulate plasma by tube coagulase test. The cultures showed typical colony on Mannitol salt agar (ability to grow due to salt tolerance and change the colour of the medium around the colony to yellow due to mannitol fermentation) and coagulase test was positive. All six isolates showed typical colony

on Mannitol salt agar and coagulase test was positive (Table 2). Growth of three VRE isolates was checked on m-Enterococcus Agar with 6 µg/ml Vancomycin (Table 3).

Table 2: Characteristics of pathogens MRSA/VRSA isolated from pus sample

S. No.	Culture No	Growth on Mannitol Salt Agar	Coagulase test	*Mec A PCR	MIC Vancomycin µg/ml	# Sensitive/ Intermediate/Resistant
1	MRSA304	+	+	+	0.75	VSSA
2	MRSA299	+	+	+	0.5	VSSA
3	MRSA93	+	+	+	1	VSSA
4	VRSA 302	+	+	+	>16	VRSA
5	VRSA 298	+	+	+	>16	VRSA
6	VRSA 212	+	+	+	>16	VRSA

#Legend: (0.5 and 2 µg/ml(VSSA), 4-8 µg/ml (VISA) and ≥16 µg/mL are classified as vancomycin-resistant (**VRSA**) (CLSI guidelines).

Vancomycin MIC determined by Vancomycin Ezy MIC strip (Hi Media) showed a wide range of MIC from 0.5 to >16 µg/ml.

*Legend: The isolates of *S. aureus* showed 675bp amplicon of amplified mec A gene on 1% agarose gel, confirming to be MRSA.

Table 3: Characteristics of pathogens VRE isolated from pus sample

Sr. No.	Culture No	Growth on Enterococcus Agar+6 µg/ml Vancomycin	MIC of Vancomycin by Ezy strip (Hi Media) µg/m	# Sensitive/ Intermediate/Resistant
7	VRE1	+	>16	VRE
8	VRE2	+	>16	VRE
9	VRE3	+	>16	VRE

The isolates were subjected to determination of antibiotic sensitivity test using standard enzyme strips. (HiMedia) Antibiotic sensitivity profile of selected MRSA showed highest resistance to Penicillin (100% isolates), Cefoxitin (100%isolates), Amikacin, Tazobactum, Levofloxacin and Linezold (60% isolates for every antibiotic mentioned above), Cefotaxime (40% isolates) and carbenicillin (40% isolates) followed by Ciprofloxacin (20% isolates), Gentamycin (20%isolates), Azithromycin

(20% isolates). MRSA showed sensitivity against Erythromycin, Clindamaycin, Cotrimoxazole, Tetracyclin, Vancomycin, Teicoplanin, Cefazolin, Teicoplanin, Rifampicin (Table3).

Among three MRSA93, MRSA299 and MRSA 304 was resistant to five and four antibiotics respectively among 14 antibiotics tested. VRSA showed highest resistance against Penicillin (100% isolates), and Vancomycin (100% isolates) followed by Cotrimazole, Ciprofloxacin, tetracycline, Cefotaxime and Gentamycin (40% isolates for all antibiotics mentioned above) and 20% isolates showed resistance against Erythromycin, Clindamycin, teicoplanin and Carbepenicillin. VRSA showed sensitivity against Cefazolin. All the selected VRE are resistant to Amakacin (100%isolates), Azithromycin (100%isolates) and Levofloxacin(100%isolates) whereas 60% shows resistance against Cefotaxime. These multidrug resistant (MDR), MRSA, VRE and VRSA strains isolated from pus were selected and exposed to lemon grass essential oil to examine the antimicrobial activity of lemon grass essential oil.

3.3 Antibiotic Sensitivity profile of selected isolates

Table 4: Antibiotic sensitivity profile of nine isolates from pus

S No	Culture No	Resistant against	Sensitive against
1	MRSA304	Pen, Ery, Clind, Cipro, Ctx	Gen, Cotri, Tetra, Van, Linz, Teico, Nx, Nit, Tcy, Lz
2	MRSA299	Pen, Ery, Clind, Ctx	Cipro, Gen, Cotri, Tetra, Van, Linz, Teico, Nx, Nit, Tcy, Lz
3	MRSA93	Pen, Ery, Clind, Cotri, Ctx	Gen, Tetra, Cipro, Van, Linz, Teico
4	VRSA 302	Pen, Ery, Clind, Gen, Cotri, Cipro, Ctx, Tey	Tetra, Van, Linz, Teico, Nx, Nit, Lz
5	VRSA 298	Pen, Ery, Clind, Cipro, Tey	Gen, Cotri, Tetra, Ctx, Van, Linz, Teico, Nx, Nit, Lz
6	VRSA 212	Pen, Fox, E, Cd	Cn, Sxt, Cip, Tey, Van, Ctx, G, Teico, Lz, Cr
7	VRE1	Amc, Az, Le, Ro, Fox	Cot, Cn, Te, Ctx, Lz, G, Cz, Nt, Cs, At, Van,Rf
8	VRE2	Amc, Az, Ctx, Le, G, Cs, Rf, Fox	Cot, Cn, Te, Cip, Lz, Ro, Cz, Nt, At, Van
9	VRE3	Amc, Az, Ctx, Le, Cs, Fox	Cot, Cn, Te, Cip, Lz, Ro, G, Cz, Nt, At, Van,Rf

Abbreviations –

Pen – Penicillin, Cx – Cefoxitin, E –Erythromycin, Cd - Clindamycin ,Cn- Cefoxitin, Sxt- Cotrimazole, Cip- Ciprofoxacin, Tcy- Tetracyclin, Van- Vancomycin,teico- Teicoplanin, Cz- Cefazolin, G- Gentamicin, Cr- Carbenicillin , Amc- Amakacin, Az- Azithromycin, Cot- Ceftolozane, Cn- Tazobactum, Le- Levofloxacin,Lz- Linezolid, Rf- rifampicin, Ctx- Cefotaxime.

3.6 Antimicrobial activity of LGEO against MRSA, VRSA and VRE

The lemon grass essential oil showed antibacterial activity against all nine pus isolates of MRSA, VRSA and VRE by disc diffusion assay. Zone of Inhibition ranged from 49.5mm to 11.5mm for pure essential oil (Table 5). Antibacterial activity of lemongrass oil is high for pure essential oil and gradually decreases with dilutions (Fig 2). Each line in graph represents the pattern of antimicrobial activity of LGEO at different concentrations. VRSA 212, MRSA 304 and VRE 3 showed sharp decline in antimicrobial activity at 6.25% concentration, at which there was no inhibition of growth. VRE 2 and MRSA 299 growth was inhibited at 3.25% concentration while VRSA 302 at 1.50% concentration. However, VRE1, MRSA 93 and VRSA 298 are not inhibited even at 1.50% concentration indicating lowest MIC values. MIC for test organisms ranged between 12.5 to < 1.50 % of lemon grass oil (Table 6). It was highest for VRE 3, MRSA 304 and VRSA 212 while lowest for VRE 1, MRSA 93 and VRSA 298.

Table 5: Zone of Inhibition in mm

Conc. Of LGEO→ ---------- **Isolates↓**	100%	50%	25%	12.50%	6.25%	3.25%	1.50%
MRSA 304	39.16± 0.28	31.5 ± 0.5	25.83 ± 0.28	17.16 ± 0.28	0 ± 0	0 ± 0	0 ± 0
MRSA 299	11.5 ± 0.5	9.83 ± 0.28	10.33 ± 0.57	9.16 ± 0.28	9.66 ± 0.76	0 ± 0	0 ± 0
MRSA 93	44.83 ± 0.28	38.83 ± 0.28	38.16 ± 0.28	17 ± 0.5	12.16 ± 0.28	9.83 ± 0.28	10.33 ± 0.57
VRSA 302	26.83 ± 0.76	20.83 ± 0.76	14.5 ± 0.5	11.83 ± 0.28	10.16 ± 0.28	8.83 ± 0.28	0 ± 0
VRSA 298	31.83 ± 0.28	26.83 ± 0.76	22.83 ± 0.76	16.33 ± 0.57	12.5 ± 0.86	8.66 ± 0.28	9.33 ± 0.28
VRSA 212	49.5 ± 0.5	45.16 ± 0.28	39.83 ± 0.28	23.16 ± 0.28	0 ± 0	0 ± 0	0 ± 0
VRE 1	35.33 ± 0.28	32 ± 0	27.5 ± 0.5	24.83 ± 0.28	17.33 ± 0.28	11.83 ± 0.28	9.83 ± 0.76
VRE 2	23.33 ± 0.57	22 ± 0.5	12.16 ± 0.28	11.16 ± 0.28	9.66 ± 0.57	0 ± 0	0 ± 0
VRE 3	17.16 ± 0.28	15.16 ± 0.28	12.83 ± 0.28	11.33 ± 0.28	0 ± 0	0 ± 0	0 ± 0

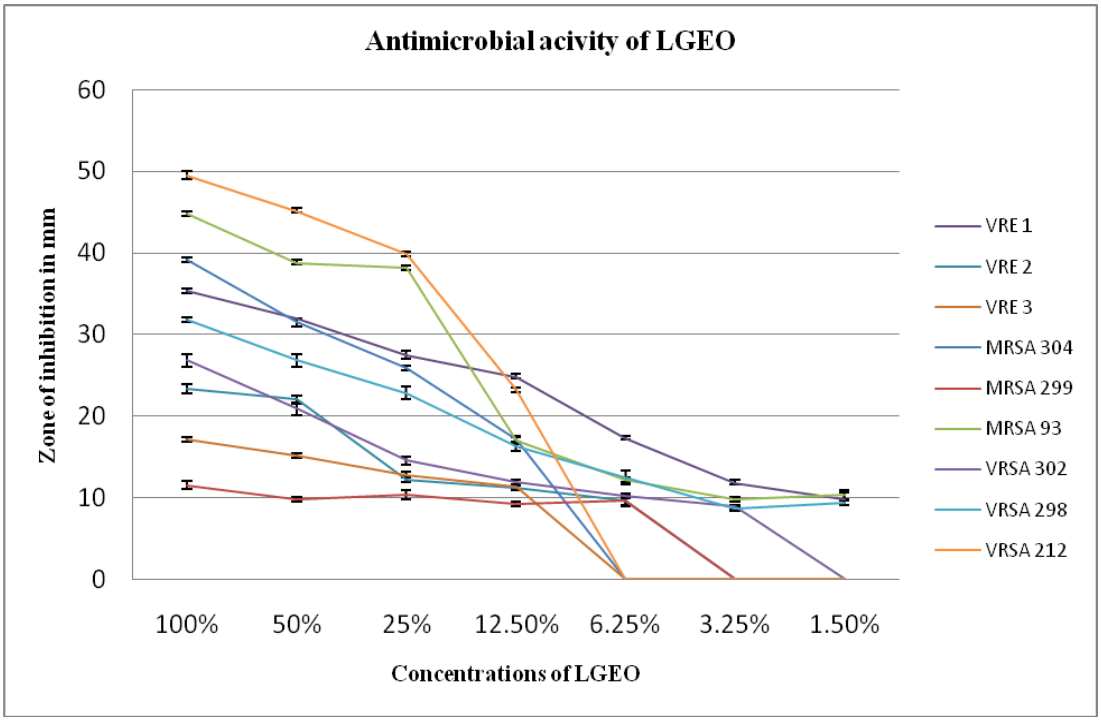

Fig 2. Graph showing antibacterial activity of lemon grass essential oil showed against nine MDR isolates

3.6.1 Minimum Inhibitory Concentration

Table 6: Minimum Inhibitory Concentration (MIC) values of each isolates

S. No	Isolates	MIC value
1	MRSA 304	Between 12.50 to 6.25%
2	MRSA 299	Between 6.25 to 3.25%
3	MRSA 93	<1.50%
4	VRSA 302	Between 3.25 to 1.50%
5	VRSA 298	<1.50%
6	VRSA 212	Between 12.50 to 6.25%
7	VRE 1	<1.50%
8	VRE 2	Between 6.25 to 3.25%
9	VRE 3	Between 12.50 to 6.25%

DISCUSSION

Health care-acquired MRSA infections happen frequently in hospitals, rehab facilities, nursing homes and have been increasing at alarming rates for decades. MRSA is becoming more prevalent at healthcare settings due to lapses in infection control. Treatment of MRSA frequently involves the use of Vancomycin, often in combination with other antibiotics given by intravenous. VRSA infected patients are left with a choice of very few drugs/antibiotics like Linezoid, Trimethoprime and Daptomycin (1). Hence, test organisms chosen represent hospital isolates of MRSA, VRSA and VRE which are difficult to treat.

A traditional system of medicine in India, Ayurveda, offers a distinct advantage in terms of efficacy and overall effect over the current approach for treatment of infectious diseases. *C. citratus* is a medicinal plant with antimicrobial properties. One of the main constituents of the many different species of lemongrass (genus *Cymbopogon*) is citral (3,7-dimethyl-2,6-octadien-1-al) (18). In present study the major components identified were α- Citral (35%), and β- Citral (27%). Limonene (6%) citronellal (2%) and limonene oxide (4%) were minor but significant components (Table 1). Lemongrass oil contains citral at concentrations of approximately 65-85% w/w (19). Our analysis shows that the total citral content (62%) obtained during GC–MS analysis.

According to Onawunmi (1989),(20) the essential oil of *C. citratus* has three main components in its composition, which are: α-citral (geranial), β-citral (neral) and myrcene. The study showed that α and β citral exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria. However, the component myrcene showed no antimicrobial activity alone. Barbosa *et al.* (2008) evaluated the concentration and the chemical composition of the essential oils obtained from 12 different samples of *C. citratus* and the following compounds were found: neral, geranial, limonene, citronellal, myrcene, and geraniol. In present study also we found the presence of the above compound during GC–MS analysis (Table 1).

Essential oil from lemon grass contains active constituents which show potent antibacterial activity against oral microflora (21). The present work demonstrates antimicrobial activity of lemongrass (*C.citratus, Poaceae*) against pus forming clinical isolates. An important characteristic of essential oils and their components is their hydrophobicity, which allows them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (22),(23). Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death (24).

Earlier studies showed that *in vitro*, plant-derived antiseptic oils may represent a promising and affordable topical agent to support surgical treatment against multi-resistant and hospital-acquired infections (25). The use of lemon grass oil

against multi drug resistant (MDR) pathogen can be a viable alternative to other antimicrobial agent as these offers cheap and effective module use in control of bacteria isolated from pus. Our studies have clearly demonstrated possible application of essential oil of lemon grass to control wound infection by pus forming MDR bacteria. There is need to conduct *in vivo* studies to ascertain the safety and acceptability of the product.

CONCLUSION

The results of this study revealed that, essential oil may be suggested as a new potential source of natural antimicrobial for the prevention, treatment and control of bacterial infections of multidrug resistant clinical isolates of MRSA, VRSA and VRE with reference to skin and wound infections. LGEO based formulation can be used for topical application to control pus forming MDR.

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Conflict of Interest:

None declared.

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