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In vitro antimicrobial activity of lemongrass *(Cymbopogon flexuosus)* oil and citral against Methicillin Resistant *Staphylococcus Aureus* (MRSA) from bovine mastitis*

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Abstract

The mounting tide of bovine mastitis by methicillin-resistant Staphylococcus aureus (MRSA) poses serious threat to efficacy of antibiotic treatment. Therefore, plant derived essential oils and their constituents are gaining great importance in the recent times as promising antimicrobial agents. Hence the present study was conducted to evaluate antibacterial activity of lemongrass oil (LGO) and its active principle, citral against methicillin and oxacillin resistant Staphylococcus aureus (MRSA) isolated from bovine mastitis cases. Among the 16 S. aureus organisms isolated, six isolates identified as MRSA by antibiogram against methicillin and oxacillin were selected for further study. The findings of the study indicated significant antimicrobial activity of LGO and citral as evident from their minimum inhibitory concentration (MIC) and diameter of zone of inhibition values.

Keywords : *MRSA, LGO, Antibiogram, MIC*

Mastitis, a multi-etiological composite disease defined as the inflammation of parenchyma of mammary glands is characterised by pathological alterations in glandular tissues in conjunction with physical, chemical and bacteriological changes in milk (Radostits *et al*., 2000). It is a worldwide crisis as it adversely impinges, not only the milk quality and welfare of the animal, but also engenders enormous financial losses to every country; including developed ones by the substantial descend in milk production (Sharma, 2007). According to Guha and Gera. (2011), the incidence of mastitis is a consequence of interplay between three key aspects such as host resistance, infectious agents and environmental factors.

Staphylococcus spp. bacteria are one of the prime causes of clinical and subclinical

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mastitis in dairy cattle, and the animals affected with subclinical mastitis in the herd when left untreated, may act as carriers of the pathogenic bacteria and can transmit the infection to other cattle as well as susceptible people. The widespread use of beta lactam antibiotics against bovine mastitis has led to the development of livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) in cattle. LA-MRSA is mostly associated with multidrug resistance and biofilm formation leading to the persistent infections which are difficult to cure. In recent times, LA-MRSA infections been recognized as a rapidly evolving cause of human infections that may end in fatality.

The combination therapy, especially combining essential oil with the conventional antibiotics represents a vital strategy to ameliorate antimicrobial resistance. Essential oils (EOs) are the aromatic derivatives of plants that are used for centuries to treat infections. Recently these ancient remedies are being continuously explored as a new source of antimicrobial agents as they are active against a wide range of organisms. This is mainly attributed to the complex chemical constituents of these EOs which produces its action through the inhibition or interaction of multiple targets of cells (Boire *et al*., 2013). Moreover, the lipophilic nature of EOs makes it more active as this enables easy penetration into the bacterial cell (Kalemba and Kunicka, 2003).

Materials and methods

Microorganisms

The test microorganisms used in the study included 16 *S.aureus* strains isolated from clinical or subclinical mastitis, presented at Teaching Veterinary Clinical Complex, Mannuthy as well as from University Livestock Farm, Mannuthy. The working cultures of the bacteria were maintained on Brain Heart Infusion (BHI) agar, sub-culturing done weekly for a maximum of three weeks, to maintain viability and colony characteristics.

Essential oil and components

The EOs used in this study were lemongrass (*Cymbopogon flexuosus*) oil procured from Synthite Industries Pvt. Ltd., Kerala and citral, 95 % (C83007) purchased from M/s Sigma Aldrich, India. They were kept tightly closed in a dry ventilated area, protected from light.

Identification of methicillin resistant S. aureus (MRSA)

Antimicrobial susceptibility testing of the *S. aureus* isolates against methicillin (5 µg) and oxacillin (1 µg) was done for the identification of MRSA isolates by Kirby-Bauer disc diffusion method (Bauer *et al*., 1966) as per the Clinical and Laboratory Standards Institute guidelines.

Kirby-Bauer disc diffusion method

A sterile cotton swab on a wooden applicator was dipped into the standardised inoculum. Excess fluid was removed by pressing and rotating the swab against the side of the tube above the suspension. The entire agar surface of the Mueller Hinton agar (MHA) plate was streaked with the swab three times, turning the plate at 60° angle between each streaking to ensure even distribution. The methicillin, oxacillin and cefoxitin discs were placed equidistant from each other on the agar surface and the plates were incubated at 35°C for 18 to 24 h. After incubation, the zone of inhibition was measured and interpreted as either susceptible or resistant to the exposed agent according to Clinical Laboratory Standards Institute criteria. Accordingly, diameter of inhibition zones of ≤10, 11-12, and ≥13 mm was categorized as resistant (R), intermediate (I), or susceptible (S) to methicillin and oxacillin. For cefoxitin disc, a diameter of inhibition zones of ≤21 mm and ≥22 mm to the Staphylococci was considered as resistant and susceptible respectively (CLSI, 2016).

Antimicrobial susceptibility of MRSA against LGO and citral

Disc diffusion assay and Minimum Inhibitory Concentration determination methods were employed to assess the antimicrobial susceptibility of the MRSA isolates against LGO and its primary chemical component citral as per the procedure described by De silva *et al*. (2017) with minor modifications.

Disc Diffusion Assay

Under aseptic conditions, five 6 mm diameter sterile discs were impregnated with 10 µL of different dilutions of LGO/citral in 5 per cent Tween 20 at 1:1, 1:2, 1:5 and 1:10 ratio *i.e.*1 part of the LGO or citral in respective parts of 5 per cent Tween 20 (vehicle) and were placed symmetrically by means of sterile disc holding forceps on the surface of agar plates. One of the discs was moistened with 5 per cent tween 20 served as vehicle control. All Petri dishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were incubated at 35ºC for 24 h and the diameter of the zones of complete inhibition measured as >10 mm was considered significantly inhibitory as denoted by Fu *et al*. (2007).

Determination of Minimum Inhibitory Concentration (MIC)

For the measurement of MIC by microbroth dilution method, a modified resazurin microtitre plate assay was employed as reported by Elshikh *et al*. 2016 with minor modifications. An aliquot (50 µL of Cationated Mueller Hinton Broth (CAMHB) supplemented with 2% NaCl, containing 0.5 % (v/v) Tween 20 was added to wells of a sterile 96-well microtitre plate. Two fold serial dilutions of LGO and citral were made by using a multi-channel pipette, for which 50 µL of test substance was added initially in the first well followed by sequential transferring of 50 µL to the subsequent wells after proper mixing, and finally, 50 µL from the last well was discarded. Next, 50 µL of bacterial suspension was added to each well to achieve a concentration of approximately 5×10^5 CFU/mL in the well. The final concentrations of LGO/citral obtained in the wells were 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156, 0.0078, 0.0039 and 0.0019 % v/v. The growth control (inoculum/positive control) wells contained 50 µL each of CAMHB medium and bacterial cells without test substances (LGO/citral) while the sterility control (media/vehicle/negative control) wells contained 100 µL CAMHB only. The plates were incubated at 35°C for 24 h and subsequently 30 µL of 0.01 per cent resazurin was added in each well, mixed by gentle shaking and the plates were again incubated at 37°C for 3 h. Bacterial growth was monitored visually as colour change from blue to pink, which indicated the presence of viable cells in cultures (Elshikh *et al*., 2016). The MIC was defined as the lowest concentration at which visible growth was inhibited *i.e.,* the lowest concentration that remained blue in colour as the colour change from blue to pink was inhibited (Sanchez *et al.,* 2016). Each test reaction was done in triplicates.

Results and Discussion

Identification of methicillin resistant S. aureus (MRSA)

A total of 16 *S. aureus* isolates were tested for methicillin/ oxacillin resistance by Kirby-Bauer disc diffusion test. Out of the 16 strains, six isolates showed resistance against both methicillin and oxacillin and were identified as methicillin resistant *S. aureus.*

Antimicrobial susceptibility of MRSA against LGO and citral

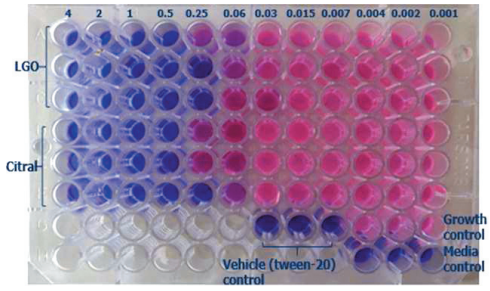
Disc Diffusion Assay

The diameter of zone of inhibition varied from 19.29 ± 1.2 to 9.51 ± 0.38 mm (table 1). The vehicle control disc coated with 5% tween-20 in sterile water did not show any inhibitory zone. Both LGO and citral exhibited significant inhibition zone (>10 mm) against MRSA isolates at all dilutions except for citral at 1:10 ratio. LGO at 1:1 ratio was found to be most effective amongst all the treatment groups with a mean inhibition zone of 19.29 mm followed by citral at 1:1 ratio (18 mm). Moreover, a dose dependent decrease in the diameter of zone of inhibition was noticed for both lemongrass oil and citral.

Determination of Minimum Inhibitory Concentration (MIC)

The vehicle control wells containing 0.5% tween-20 did not show any inhibition, whereas LGO and citral showed MIC values of 0.33 % v/v and 0.67% v/v respectively, indicating that LGO possessed higher activity than citral. Besides, the increase in the sensitivity of

Fig. 1. Determination of MIC of LGO and citral



MRSA isolates towards LGO was found to be significant ($p< 0.01$) as compared to the citral treatment group.

In the study, disc diffusion assay was performed against the MRSA isolates at various dilutions of LGO and citral in 5% tween 20 (1:1, 1:2, 1:5, and 1:10 ratio). The tween 20 used in the experiment serves as a solubilising agent that prevent the possible unequal distribution of the oils through the medium, without interfering bacterial growth and differentiation. Unlike Tween-80 and DMSO, it has little or no effect on the activity of the essential oils. In this method activity of the oils were compared using zone of inhibition around the disc usually expressed in diameter (Kalemba and Kanuka, 2003). Results indicated that both LGO and citral possess significant antimicrobial activity against MRSA with a zone of inhibition greater than 10mm except citral at 1:10 (Fu *et al*., 2007), while the disc coated with 5% tween-20 in sterile water (vehicle control) did not show any inhibitory zone. The maximum zone of inhibition obtained for LGO and citral were 19.29 and 18 mm respectively. Moreover a dose dependent decrease in the diameter of zone of inhibition was noticed for both lemongrass oil and citral.

The MIC values obtained for the LGO using modified resazurin microtitre plate assay against MRSA isolates was significantly higher than that of citral, which could be attributed to the presence of other components of LGO that had antimicrobial activity. This was in consensus with the study conducted by Aiemsaard *et al*. (2012), in which LGO obtained higher inhibition zone when compared to the citral. The higher antimicrobial activity of LGO over citral could be either attributed to the direct antimicrobial effects produced by the major components such as geraniol or to the synergism arising from the potentiating action of minor components (linalool, geraniol acetate and myrcene) of LGO (Aiemsaard *et al*., 2011). This was also regarded as one of the reason for the additive and synergistic effects produced by the combination of these essential oils *C. citratus* with *C. giganteus* (Bassole *et al.*, 2011). However, the LGO elicits the antimicrobial effects by producing distortion of cytoplasmic membrane and swelling of cell wall, whereas citral affects the stability of bacterial cell membrane causing cell leakage (Aiemsaard *et al*., 2011 and Taufik *et al*., 2017). On contrary, there are studies that have obtained same MIC values for both LGO and citral suggesting that the whole essential oils are more suitable than the pure compounds whose isolation is difficult and costly practice in the drug development (Onawunmi, 1989; Christensen and Anderson, 2017).

Table.1 Diameter of zone inhibition of LGO and citral against MRSA isolates, mm

Dilutions	Zone of inhibition (mm)	
	LGO	Citral
1:1	19.29 ± 1.2^{b}	18.0 ± 0.76^{d}
1:2	17.13 ± 1.12^{ab}	15.71 ± 0.79^{c}
1:5	15.25 ± 1.46^{ab}	13.37 ± 0.74^{b}
1:10	13.58 ± 1.42^{aA}	9.51 ± 0.38^{aB}

Values are expressed as Mean ± SE, n=6: Values bearing different superscripts (a-b in column and A-B in row) vary significantly at $p< 0.05$. No inhibition zone was observed with vehicle control; Diameter values of zone inhibition are inclusive of disc diameter of 6 mm.

Table. 2 Minimum inhibitory concentration of lemongrass oil and citral against MRSA isolates, % v/v

Isolate ID	Lemongrass oil	Citral
SA1	0.25	0.5
SA2	0.25	0.5
SA3	0.5	1
SA4	0.25	0.5
SA5	0.25	0.5
SA6	0.5	1
Mean ± SE	**0.33 ± 0.53^{a}**	**0.67 ± 0.10^{b}**

Values of MIC are mean of triplicates, n=6; Values bearing different superscripts vary significantly at $p< 0.05$.

Thus, it could be concluded that the lemongrass oil extracted from *Cymbopogon flexuosus* has marked antimicrobial activity against methicillin resistant *S. aureus* strains isolated from bovine mastitis.

References

Aiemsaard, J., Aiumlamai, S., Aromdee, C., Taweechaisupapong, S. and Khunkitti, W. 2011. The effect of lemongrass oil and its major components on clinical isolate mastitis pathogens and their mechanisms of action on *Staphylococcus aureus* DMST 4745. *Res. Vet. Sci.* **91**:31-37.

Bassolé, I.H.N., Lamien-Meda, A., Bayala, B.O.L.C., Obame, L.C., Ilboudo, A.J., Franz, C., Novak, J., Nebié, R.C. and Dicko, M.H. 2011. Chemical composition and antimicrobial activity of *Cymbopogoncitratus* and *Cymbopogongiganteus* essential oils alone and in combination. *Phytomedicine*. **18**:1070-1074.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.* **45**: 493-496.

Boire, N.A., Riedel, S. and Parrish, N.M. 2013. Essential oils and future antibiotics: new weapons against emerging 'superbugs'. *J. Anc. Dis. Prev. Rem*. **1**:105-111.

Christensen, C.J. and Anderson, K.L. 2017. Characterizing the Antibacterial Properties and Transcriptional Alterations Induced by Lemongrass oil in *Staphylococcus aureus*. *Microbiol. Immunol*. **3:** 57-62.

CLSI. 2016. Performance standards for antimicrobial susceptibility testing. *Clinical Lab Standards Institute*.

De Silva, B.C.J., Jung, W.G., Hossain, S., Wimalasena, S.H.M.P., Pathirana, H.N.K.S. and Heo, G.J. 2017. Antimicrobial property of lemongrass (*Cymbopogoncitratus*) oil against pathogenic bacteria isolated from pet turtles. *Lab. Anim. Res.* **33**: 84-91.

Elshikh, M., Ahmed, S., Funston, S., Dunlop, P., McGaw, M., Marchant, R. and Banat, I.M. 2016. Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. *Biotechnol. Lett.* **38:** 1015-1019.

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z., Sun, S. and Efferth, T. 2007. Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytotherapy Res.* **21:** 989-994.

Guha, A. and Gera, S., 2011. Etio-prevalence of sub clinical mastitis in Holstein X Haryana crossbred cattle. *Expl. Ani. Med. Res*. **1:**75-78.

Kalemba, D.A.A.K. and Kunicka, A. 2003. Antibacterial and antifungal properties of essential oils. *Curr. Med. Chem.* **10:** 813-829.

Onawunmi, G.O., 1989. Evaluation of the antimicrobial activity of citral. *Lett. Appl. Microbial*. **9:** 105-108.

Radostits, O.M., Gay, C.C., Blood, D.C. and Hinchcliff, K.W. 2000. Diseases caused by rickettsia. *Veterinary medicine: a textbook of the disease of cattle, sheep, goats and horses.* (9th Ed.). Philadelphia: WB Saunders Co. 1261p.

Sánchez, E., Rivas Morales, C., Castillo, S., Leos-Rivas, C., García-Becerra, L. and Ortiz Martínez, D.M. 2016. Antibacterial and antibiofilm activity of methanolic plant extracts against nosocomial microorganisms. *Evidence-Based Compl. Alt. Med.* 2016: 1-16.

Sharma, N. 2007. Alternative approach to control intramammary infection in dairy cows-A review. *Asian J. Anim. Vet. Adv*. **2:** 50-62.

Taufik, I., Astuti, D.I., Purwasena, I.A. and Rodiana, W.N. 2018. Effect of Lemongrass (*Cymbopogon citratus*) Essential Oil on Biofilm-biocorrossion in Formation Water Environment. *Asian J. Sci. Res.* **11**: 329-336. ■