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IN VITRO INVESTIGATION OF ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

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Abstract: *Staphylococcus aureus* is one of the major causes of both community and hospital acquired infections. Methicillin-resistant *S. aureus* (MRSA) is one of the *S. aureus* strains that resists a variety of antibiotics, especially methicillin and penicillin groups. Therefore, very few antibiotics are effective in treating MRSA infections. Many compounds were developed for the treatment of MRSA infections. Some essential oils are good candidates due to their antimicrobial potentials. In this study, eleven essential oils, i.e., clove, geranium rose, kaffir lime, lavender, lemongrass, mandarin orange, patchouli, plai, rosemary, Siamese rosewood, and vetiver were evaluated. These oils were determined for antimicrobial activities against MRSA and MSSA by the agar disk diffusion method. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were also determined. In addition, the effectiveness of the potent essential oils were analyzed using the time-kill assay. The agar disk diffusion test showed that lemongrass oil possessed the highest antibacterial activity against MRSA and MSSA with inhibition zones of 29.4 ± 3.3 mm and 28.3 ± 4.7 mm, respectively. Clove oil and geranium oil also showed antibacterial activity against MRSA and MSSA with inhibition zones of 15.3 ± 2.1 mm and 15.5 ± 1.7 mm for clove oil and 13.2 ± 2.3 mm. and 12.1 ± 1.3 mm for geranium oil, respectively. Therefore, lemongrass oil, clove oil and geranium oil were selected for further determination of MIC and MBC. The results showed that the MIC and MBC against MRSA were 0.3906 % v/v and 0.3906 % v/v for clove oil, 0.3906 % v/v and 1.5625 % v/v for geranium oil and 0.7813 % v/v and 3.125 % v/v for lemongrass oil. Kinetics of kill studies showed that lemongrass oil at its 1MIC had more killing effect than clove and geranium oil at their 1MIC. Lemongrass oil killed MRSA and MSSA at least 3 logs in 1 minute whereas geranium oil took more than 5 minutes to achieve the same kill.

Keywords: Essential oils, Antimicrobial activity, Methicillin-resistant *S. aureus*

บทคัดย่อ *Staphylococcus aureus* เป็นหนึ่งในสาเหตุสำคัญของการติดเชื้อในชุมชนและโรงพยาบาล Methicillin-resistant *S. aureus* (MRSA) ดื้อต่อยาปฏิชีวนะหลายชนิดโดยเฉพาะกลุ่ม Methicillin และ Penicillin จึงมียาปฏิชีวนะที่มีประสิทธิภาพในการต้าน MRSA จำนวนไม่มากนัก สารหลายชนิดได้ถูกพัฒนาขึ้นมาเพื่อรักษาโรคติดเชื้อจาก MRSA น้ำมันหอมระเหยเป็นหนึ่งในตัวเลือกเพื่อนำมาใช้ เนื่องจากน้ำมันหอมระเหยบางชนิดมีประสิทธิภาพในการยับยั้งจุลชีพได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อตรวจสอบฤทธิ์ของน้ำมันหอมระเหยในการต้านแบคทีเรีย MSSA และ MRSA น้ำมันหอมระเหย 11 ชนิดได้แก่ กานพลู เจอราเนียม มะกรูด ลาเวนเดอร์ ตะไคร้ ส้มแมนดาริน พิมเสน ไพล โรสแมรี่ พะยูง และหญ้าแฝก ถูกนำมาศึกษาฤทธิ์ในการฆ่าเชื้อโดยวิธี Agar disk diffusion และ หาความเข้มข้นต่ำสุดในการยับยั้งการเจริญเติบโต (MIC) และความเข้มข้นต่ำสุดที่ฆ่าเชื้อแบคทีเรีย (MBC) ด้วยวิธี Broth microdilution และศึกษาประสิทธิภาพของน้ำมันหอมระเหยในการลดจำนวนจุลชีพได้ 99.9% ด้วยวิธี Time killing จากการวิจัยพบว่าน้ำมันหอมระเหยตะไคร้สามารถยับยั้งเชื้อทั้ง MRSA และ MSSA ได้ดีที่สุดเมื่อทดสอบด้วย Agar disk diffusion โดยมีขนาด Inhibition zone เท่ากับ 29.4 ± 3.3 มิลลิเมตรและ 28.3 ± 4.7 มิลลิเมตรตามลำดับ น้ำมันหอมระเหยจากกานพลูและเจอราเนียมมีฤทธิ์ยับยั้งเชื้อ MRSA และ MSSA เช่นกันโดยมีขนาด Inhibition zone ของน้ำมันหอมระเหยจากกานพลูเท่ากับ 15.3 ± 2.1 และ 15.5 ± 1.7 มิลลิเมตรและของน้ำมันหอมระเหยจากเจอราเนียมเท่ากับ 13.2 ± 2.3 และ 12.1 ± 1.3 มิลลิเมตร น้ำมันหอมระเหยจากตะไคร้ กานพลู และเจอราเนียม ได้ถูกนำไปทดสอบหาค่า MIC และ MBC พบว่า ค่า MIC และ MBC ของน้ำมันหอมระเหยจากกานพลูมีค่าเท่ากับร้อยละ 0.3906 และ ร้อยละ 0.3906 โดยปริมาตร จากเจอรา

เนียมมีค่าเท่ากับร้อยละ 0.3906 และร้อยละ 1.5625 โดยปริมาตร และจากตะไคร้มีค่าเท่ากับ ร้อยละ 0.7813 และร้อยละ 3.125 โดยปริมาตร จากการศึกษาฤทธิ์ฆ่าเชื้อแบคทีเรียของตะไคร้และกานพลูที่ความเข้มข้นเท่ากับ 1MIC สามารถฆ่าเชื้อแบคทีเรียทั้ง MRSA และ MSSA ได้อย่างน้อย 3log ในเวลา 1 นาที ขณะที่น้ำมันหอมระเหยจากเจอราเนียมต้องใช้เวลามากกว่า 5 นาทีจึงจะฆ่าเชื้อได้เท่ากัน

INTRODUCTION

Staphylococcus aureus colonized in nostrils and skin causes a variety of infections such as bacteremia, bone-joint infections, pneumonia and skin infections, e.g., abscess, carbuncle, cellulitis, erysipelas, folliculitis, furuncle, and impetigo. In addition, more than 90% of *S. aureus* infections are resistant to penicillins (Lowy, 2003). Penicillin is usually the treatment of choice for infection of normal strains of *S. aureus*. Soon after penicillin was introduced for the treatment of *S. aureus* infection, penicillin-resistant *S. aureus* strains have developed in many countries. The semisynthetic β-lactamase-resistant penicillins such as methicillin and oxacillin were introduced in 1959 and brought about a general decline in the resistant *S. aureus*. In 1961, two years after methicillin licensing to treat the penicillin resistant *S. aureus* infections, the first methicillin resistant *S. aureus* (MRSA) strain was found in England. The MRSA strain is also resistant to all β-lactams, sulfonamides, erythromycin, aminoglycosides, tetracyclines, and clindamycin. Vancomycin and teicoplanin are glycopeptides used to treat MRSA infections. The first vancomycin intermediate *S. aureus* (VISA) was found in Japan in 1996. Then the vancomycin resistant *S. aureus* (VRSA) was first found in India in 2011. Now linezolid, daptomycin, and quinupristin / dalfopristin remain effective antibiotics against MRSA infections (Rayner & Munckhof, 2005; Kaur et al., 2012).

Many scientists have tried to search for new antimicrobial agents to combat MRSA. Many natural products have been interested and extensively studied as sources of medicines. The most common traditional plants used for the treatment of many diseases are families of *Lamiaceae/Labiatae, Lauraceae, Myrtaceae, Oleaceae, Piperaceae, Poaceae/Gramineae, Rosaceae, Rutaceae* and *Zingiberaceae*. The essential oil (EO) is composed of numerous active components from plants which have the defensive activity against microorganisms, insects and herbivores. The bioactive molecules of EO are terpenes including monoterpenes, sesquiterpenes, oxygenated terpenes, and phenylpropenes, which possess antimicrobial activities. The non-polar agents of EO are active while the polar agents show inactive antimicrobial effects. The phenolic compounds in the EO have antioxidant activity while other components possessed antimicrobial activity (Tepe, Daferera, Sökmen, Polissiou, & Sökmen, 2004).

EOs such as carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde, and cinnamic acid have possessed powerful activities of antibacteria, antifungi, antivirus, insecticide, and antioxidant (Burt, 2004; Kordali et al., 2005). EO from *Croton flavens* leaf has been used in cancer treatment, which very active against both human lung carcinoma and colon adenocarcinoma cell lines (Sylvestre, Pichette, Longtin, Nagau, & Legault, 2006). Some EOs have been used in food preservation due to their antibacterial activity against coliforms, enterococci, staphylococci, *Salmonella*, and yeasts (Faid, Bakhy, Anchad, & Tantaoui-Elaraki, 1995). Some EOs have been used in aromatherapy and fragrance industries since they act against airborne microbial contamination (Buttner, Willeke, & Grinshpun, 1996; Van de Braak & Leijten, 1999). In addition, antimalarial activity has been found in essential oils extracted from some plants such as *Artemisia vulgaris*, *Eucalyptus globulus, Myrtus communis, Juniperus communis, Lavandula angusti/olia, Origanum vulgare, Rosmarinus officinalis* and *Salvia officinalis.* (Milhau et al., 1997).

Since *Staphylococcus aureus* is one of the major causes of both community and hospital acquired infections, enormous efforts are being made to search for new compounds which can be used as potential antimicrobial agents. Among the potential sources of new antimicrobial agents, essential oils could be an interesting alternative. Therefore, the objectives of this study were to investigate antimicrobial activity of essential oils against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA), to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of essential oils and to analyze the effectiveness of the most potent essential oil against both bacterial strains using the time-kill assay.

MATERIALS AND METHODS

Natural herbal essential oils

Eleven pure natural herbal essential oils used in this study included clove (*Syzygium aromaticum* Linn. Merrill & Perry), geranium rose (*Pelargonium graveolens* L'Hér.), kaffir lime (*Citrus hystrix* DC.), lavender (*Lavandula angustifolia* Mill.), lemongrass (*Cymbopogon citratus* DC. Stapf), mandarin orange (*Citrus reticulate* Blanco), patchouli (*Pogostemon cablin* Blanco Benth.), plai (*Zingiber cassumunar* Roxb.), rosemary (*Rosemarinus officinalis* Linn.), Siamese rosewood (*Dalbergia cochinchinensis* Pierre ex Laness.), and vetiver (*Chrysopogon zizanioides* Linn. Roberty) were kindly provided by the Faculty of Oriental Medicine, Rangsit University. These essential oils were extracted by steam distillation. They were stored in tightly closed glass vials and covered with aluminum foil and kept at 4°C until analysis.

Bacterial strains

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains were from the culture collections of the Microbiology Unit, Department of Medical Sciences, Faculty of Science, Rangsit University.

These isolates were identified as *S. aureus* by standard microbiological method including gram stain, catalase, coagulase and growth on mannitol salt agar (MSA). *S. aureus* ATCC25923 and *S. aureus* ATCC43300 were also included in this study as reference strains of MSSA and MRSA, respectively.

All *S. aureus* isolates obtained were screened for MRSA and MSSA by using a 1 μg oxacillin disk diffusion test. In addition**,** Polymerase Chain Reaction (PCR) was performed to detect the *mecA* gene.

Antimicrobial susceptibility testing

Eleven essential oils were screened for their inhibitory activity against MRSA and MSSA using agar disk diffusion method (Ortez, 2005). The disk diffusion assay was performed by making a lawn culture of approximately10^8 cells/mL of the test bacteria on Tryptic soy agar (TSA) plates. Then sterile 6 mm in diameter filter paper disks loaded with 10 μL of sample solutions were placed on the TSA plates. The plates were then incubated at 35 °C for 24 h and the diameter of the inhibition zone was measured. Zone of inhibition of essential oils was compared with corresponding solvent as controls.

Each susceptibility experiment was performed in triplicates. The diameters of the zone of growth inhibition around the disks measured in millimeter were averaged mean values.

Determination of MIC and MBC

MIC was determined by the microdilution broth susceptibility method (Rankin, 2005). MRSA and MSSA were cultured in 5 mL Tryptic soy broth (TSB) and incubated in a shaker incubator at 35˚C for 18-24 h. The bacteria were adjusted to the 0.5 McFarland standard with TSB solution to achieve a concentration of approximately 10^8 cells/mL. Then this bacterial culture was further diluted in TSB to obtain 10^6 cells/mL suspension of the test bacterial culture.

Essential oils were two-fold serial diluted in 100 µL TSB in 96 well plates. One hundred microliters of 10^6 cells/mL bacterial suspension were added into each dilution and then the plate was incubated at 35˚C for 18-24h. Each dilution was done in duplicate. The MIC was taken as the lowest concentration that inhibited the growth of bacteria. Inhibition of microbial growth in the microtiter plate containing test oil was judged by comparison with growth of growth control inoculated in the microtiter plate, i.e., bacterial culture without the oil. The MIC results were expressed in % v/v or mg/mL.

The Minimal bactericidal concentration (MBC) is defined as the lowest concentration of the compound that kills the bacteria. All wells that showed no growth in the MIC studies were determined for MBC. Five microliters of the clear suspension was transferred and spotted onto TSA that contain no test agent and incubated at 35˚C for 18-24h. The lowest concentration that showed no growth on TSA was taken as MBC.

Kinetics of killing

The effectiveness of the most potent essential oil against bacterial strains of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) was determined by the Time-kill assay.

The 10^6 cells/mL bacterial suspension was mixed with the essential oil at the concentration of 1MIC and incubated at 35 °C. After the incubation period of 1, 3, 5, 10, 20, 30, and 60 min, the mixture was taken out for bacterial count on TSA. The experiments were carried out in triplicate. Time–kill curves were constructed by plotting $\log_{10}$ CFU/mL of survivals against time. The effectiveness of the most potent essential oil against MRSA is when the viable cells reduced $3\log_{10}$ meaning that the bactericidal activity reached 99.9%.

RESULTS

Antimicrobial activity of essential oils against MRSA and MSSA

The antibacterial activities of eleven essential oils were tested against 27 strains of Methicillin resistant *Staphylococcus aureus* (MRSA) and 25 strains of Methicillin susceptible *Staphylococcus aureus* (MSSA) by the agar disk diffusion method. The results showed that both MRSA and MSSA strains were susceptible to almost all tested essential oils except mandarin orange oil. In addition, lemongrass oil possessed the highest antibacterial activity against MRSA and MSSA with inhibition zones of 29.4 ± 3.3 mm and 28.3 ± 4.7 mm, respectively (Table 1). Clove oil and geranium oil also showed antibacterial activity against MRSA and MSSA. From the screening result of the antimicrobial activity lemongrass oil, clove oil and geranium oil were selected for further determination of MIC and MBC. The results showed that lemongrass oil and geranium oil possessed higher antibacterial activity against MRSA than MSSA.

Determination of MIC and MBC

The MIC and MBC of tested essential oils against type strains of MRSA (*S. aureus* ATCC25923) and MSSA (*S. aureus* ATCC43300) showed in Table 2. The values of MIC

and MBC of clove oils against MRSA and MSSA were the same while the MICs and MBCs of geranium and lemongrass against MSSA were higher than MRSA.

Kinetics of killing

Kinetics of kill studies showed that lemongrass oil at concentration of 1MIC has more killing effect than clove and geranium oil. Lemongrass oil killed MRSA and MSSA at least 3logs in 1 minute whereas geranium oil took more than 5 minutes to achieve the same kill as shown in Figures 1-2. In addition, lemongrass, clove, and geranium oils showed bactericidal effect against MRSA and MSSA. Lemongrass and clove oils at concentration of 1MIC reduced the growth of MRSA and MSSA after 1 minute of incubation whereas geranium at 1MIC reduced both strains after 10 minutes of incubation.

Table 1. Inhibition zone of eleven essential oils against MRSA and MSSA

Essential oils	**Zones of inhibition (mm) mean ± SD**	
	MRSA	**MSSA**
Mandarin Orange	No inhibition	No inhibition
Clove	15.3 ± 2.1	15.5 ± 1.7
Plai	8.9 ± 1.3	8.7 ± 1.4
Siamese Rosewood	9.7 ± 1.4	11.5 ± 2.0
Rosemary	7.5 ± 0.4	7.7 ± 0.6
Patchouli	10.8 ± 1.4	10.0 ± 2.1
Vetiver	8.4 ± 1.0	7.9 ± 1.0
Lemongrass	29.4 ± 3.3	28.3 ± 4.7
Kaffir Lime	11.2 ± 2.9	10.1 ± 2.1
Geranium Rose	13.2 ± 2.3	12.1 ± 1.3
Lavender	10.4 ± 1.1	11.5 ± 1.8

Table 2. MIC and MBC of essential oils against MRSA (ATCC43300) and MSSA (ATCC25923)

Essential oils	MIC (%v/v)		MBC (%v/v)	
	MRSA ATCC43300	MSSA ATCC25923	MRSA ATCC43300	MSSA ATCC25923
Clove	0.3906	0.3906	0.3906	0.3906
Geranium	0.3906	3.125	1.5625	3.125
Lemongrass	0.7813	3.125	3.125	6.25

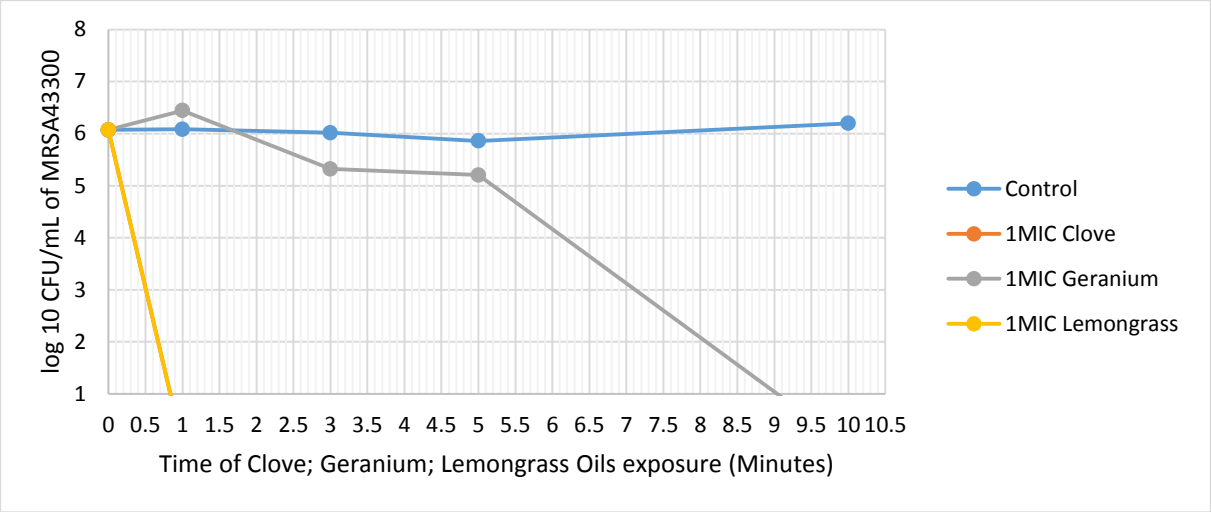

Figure 1. Time-Kill curve assay of essential oils against MRSA (ATCC43300)

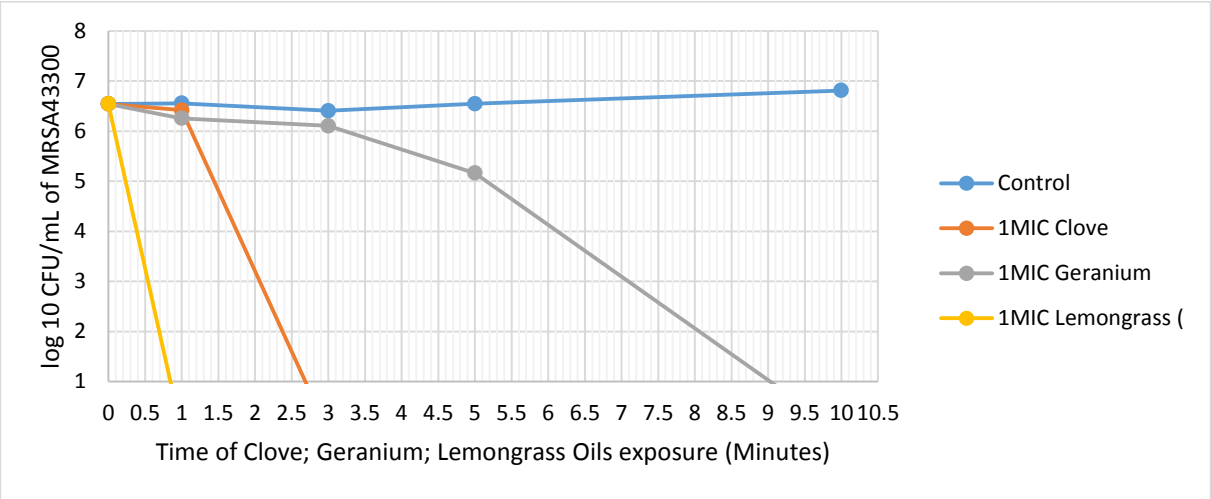

Figure 2. Time-Kill curve assay of essential oils against MSSA (ATCC25923)

DISCUSSION AND CONCLUSION

The most important problem in the treatment of infections is the increasing rate of antibiotic resistance, which results in the failure of antimicrobial usage. Documented essential oils have altered antimicrobial activity profiles against bacteria. Studies on antimicrobial properties of essential oils against microorganisms are limited. Essential oils were extensively used to treat infections due to their antimicrobial activities while mechanisms of action were not well implicit.

In this study eleven essential oils were verified for inhibitory activity against MRSA and MSSA by the disk diffusion method. Clove, geranium, and lemongrass were selected for further study in broth microdilution method due to their best inhibition activities. Among tested essential oils lemongrass oil showed highest antibacterial activity against MRSA and MSSA while mandarin oil was not effective against either strains. In the disk diffusion method the essential oils were dotted on paper disk and placed on agar to interact against MRSA and MSSA. Lack of inhibition zone could be as a result of incapability of the active chemical constituents to diffuse freely. Subsequently some essential oils are hydrophobic, they could not diffuse optimally in aqueous surroundings, such as is present in the disk diffusion test (Chao, Young, Oberg, & Nakaoka, 2008). The volatility as well as miscibility of the essential oils may affect difficulties while testing their activity (Carson & Riley, 2003). According to the broth microdilution method, investigation in clove, geranium, and lemongrass oils confirmed the results obtained with the disk diffusion method. MBC was ranging from 0.3906 to 6.25 % v/v. Results from in vitro studies in this work exhibited that the essential oils inhibited bacterial growth and good time-kill effectiveness in 0.5- 7.1 minutes.

Significant characteristic of essential oils and constituents is hydrophobicity, which empower to partition the lipids of the bacterial cell membrane, disturbing the cell structures and execution more leaky (Knobloch, Weigand, Weis, & Vigenschow, 1986; Sikkema, de Bont, & Poolman, 1994). Widespread leakage from bacterial cells or the exit of life-threatening molecules and ions will lead to cell death (Denyer & Hugo, 1991). It is well-known that many essential oils kill bacteria by damaging the cell membrane's structure and inhibiting cell membrane function (Gibbons, 2004; Sikkema, de Bont, & Poolman, 1995).

The antibacterial activity against MRSA and MSSA of selected essential oils studied in this research may due to the major compositions of these oils; for example, clove oil contained a variety of bioactive composites such as eugenol and phenolics. Eugenol has strong properties in antimicrobial activities while phenolic composites are also responsible for the antibacterial and antifungal activity (Verma, Karkun, & Siddiqui, 2015). Lemongrass oil contained very high amount of α-citral (geranial), β-citral (neral) and myrcene. The α-citral (geranial) and β-citral (neral) have antibacterial activity against Gram positive and Gram negative bacteria but myrcene possesses no antibacterial activity alone. However, myrcene showed increased bacterial activity when combined with other components in lemongrass oil (Onawunmi, Yisak, & Ogunlan, 1984). Citronellol, nerol and geraniol which are the major constituents of geranium oil have antimicrobial activity against bacteria and fungi (Sharopov, Zhang, & Setzer, 2014).

This study established that various essential oils have antibacterial activities. Clove and lemongrass oils that have the greatest prospective in vitro bactericidal activities against both MRSA and MSSA could be used as the new developed antibacterial agents. However, in vivo studies and clinical trials must be carried on to validate and evaluate the prospective of them as external and internal antibacterial submissions. For the prospective significance of essential oils as healing antimicrobial agents, future investigations must focus on challenging the clinical safety and efficacy of the essential oils.

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