Original Article

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Antimicrobial Effect of Clove and Lemongrass Oils against Planktonic Cells and Biofilms of *Staphylococcus aureus*

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Abstract

The aim of this study was to investigate the antimicrobial activity of clove and lemongrass oils against 10 clinical isolates and the reference strains *S. aureus* ATCC 29213 and ATCC 43300. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of clove and lemongrass oils against all tested isolates were performed by standard broth microdilution assay. Minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC) were also investigated. The kinetics of the essential oils was performed by time-kill assay. The results showed that the MIC and MBC values of clove oil against planktonic cell ranged from 2.0 % to 3.0 % and 2.4 % to 5.0 %, respectively. MIC and MBC of clove and lemongrass oils against cell within biofilm were raised up to 3.0 % to 5.0 % and 4.0 % to >5.0 %, respectively. The MBEC of clove and lemongrass oils were usually 1.00 to 2.08 and 2.00 to 4.00 times higher than the MBC. Synergistic effect between clove and lemongrass oils was demonstrated by time-kill assay on *S. aureus* ATCC 43300. These data show that combined clove and lemongrass essential oils efficiently kills *S. aureus* within biofilm and is therefore an alternative method for *S. aureus* eradication.

Keyword: Biofilm, Clove oil, Lemongrass oil, *Staphylococcus aureus*

INTRODUCTION

Biofilm is the community of microorganisms living together in amorphous extracellular matrix composed of polysaccharides, extracellular DNA, and proteins. In the nature, we have found that biofilm can develop both on abiotic and biotic surfaces. Because of their complexity, biofilm makes microbial cells inside the matrix confer high level of antibiotic resistance. Biofilm is not only a key factor for survival in diverse environments but also a way of microorganisms to colonize the new sites.[1]

Biofilm affects human life in different ways, such as food, environment, and public health. It is the important problem that disturbs the efficiency of antibiotics and disease treatment, because it makes the pathogen more tolerate to antibiotics and the host immune system defense, leading to persistent and chronic infection.[2-5] Among the biofilm-producing pathogens, staphylococci are one of the most important bacteria that used for studying on biofilm due to the frequency of disease prevalence and their pathogenic characteristics such as antibiotic resistance, the pathogenicity in many organs, and their ability to form biofilm on different types of medical device surfaces.[3,6-8]

Nowadays, most of the medical treatments of staphylococci-causing diseases are using various antibiotics depends on the causative strains.[6,9] In addition to antibiotics, herbal or medicinal plant extracts become the new interesting choices.[10-11] There are many researches reported that essential oils from clove and lemongrass exert the potential antimicrobial activity.[10,12-22]

Thailand is the agricultural country that has many kinds of aromatic plants, therefore the efficacy and potential of these plants should be evaluated in order to develop the utilization of Thai medicinal plants. In this study, we evaluate the antibacterial efficacy of clove and lemongrass essential oils, including the kinetics and combined effect of the oils, against biofilm of the reference and clinical strains of *S. aureus*.

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MATERIALS AND METHODS

Essential oils

Clove oil (CO) (Batch no. 5306613/2306 Manufactured date: 22/06/2010) and lemongrass oils (LO) (Batch no. 5306613-1/2306 Manufactured date: 22/06/2010) were purchased from Thai-China Flavours and Fragrances Industry Co., Ltd.

Bacterial strains and growth conditions

The following strains of *S. aureus* were used in this study. Twelve clinical isolates originated from the collection of clinical microbiology laboratory of Srinakarind Hospital, Faculty of Medicine, Khon Kaen University, and the reference strain ATCC 29213 and ATCC 43300. All isolates were maintained at -70°C in Trypticase soy broth (TSB, Becton, Dickinson and company) with 10 % glycerol. Prior to inoculation, all isolates were subcultured at 37°C for 24 h on Trypticase soy agar (TSA, Becton, Dickinson and company) and TSB respectively.

Quantification of biofilm formation

The biofilm formation of all tested isolates was quantified as mentioned by Stepanovic *et al.*[23] with slightly modified. Briefly, *S. aureus* cell suspensions (200 µLof suspension containing $1.5x10^{8}$ CFU/mL in TSB + 2.5% glucose) were seeded into 96-well microplates. After aerobically incubated at 37°C for 24 h, the medium was gently removed and the wells were washed three times with potassium phosphate buffer pH 7.5. The biofilm fixation was done by adding 200 µL of methanol and left for 15 min. After the methanol was removed and the microtiter plate was air dried, each well was stained with 200 µL of 2 % crystal violet for 5 min and was wahsed in tap water. When the microtiter plate was completely air dried, 200 µL of 33 % glacial acetic acid was added and the OD_{570nm} was measured using microtiter plate ELISA reader (Spectrostar nano, BMG labtech).

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of planktonic cell.

MIC and MBC of clove and lemongrass oils were performed by standard microbroth dilution assay[24,25]. Overnight cultures of *S. aureus* were adjusted to McFarland standard no. 0.5 and then diluted with sterile distilled water (1:300) to give a final concentration of $1.5x10^{5}$ CFU/mL, confirmed by viable counts. Serial dilutions of clove oil (from 5.0 % to 0.5 % v/v) and lemongrass oil (from 2.0 % to 0.125 % v/v) in Mueller Hinton broth (MHB, Difco) + 15 % Tween 80 were prepared in 96-well flat-bottomed microtiter plate (Cellstar, Greiner bio-one) (100 µL per well). Ten microliters of diluted bacterial suspension were added into each well to give a final concentration of 1.5×10^{4} CFU/mL, confirmed by viable counts. The wells with no clove or lemongrass oils added were used as positive growth control wells, and the well with 15% Tween80 were used as diluents control well. The plate was aerobically incubated aerobically at 37◦c for 24 h. All tests were performed in triplicate. The MICs were defined as the lowest concentration of essential oils inhibiting visible growth after 24 h of incubation. Ten microliters from the invisible growth were inoculated onto TSA and incubated at 37◦c for 24 h. MBCs were determined from the lowest concentration of essential oil that inhibited growth on TSA.

Determination of minimum biofilm inhibition concentrations (MBICs) and minimum biofilm eradication concentration (MBECs).

The protocol for biofilm formation in 96-well tissues culture plates (Cellstar, Greiner bio-one) according to Karpanen *et al.*[25] and Stepanovic *et al.*[23] was used. After aerobically incubated at 37◦c for 24 h, the medium was gently removed and the wells were washed three times with phosphate buffer saline pH 7.4 Serial dilution of clove and lemongrass oils were added. Essential oils-free wells and biofilm-free wells were also included as positive and negative controls, respectively. MBICs were defined as the lowest concentration of essential oils inhibiting visible growth after 24 h incubation. MBECs were determined from the lowest concentration of essential oils that inhibited growth on TSA. All tests were performed in triplicate.

Time-kill assay

The effect of clove oil (0.5xMIC), lemongrass oil (0.5x MIC) and their combination (0.5xMIC CO + 0.5xMIC LO) on viability

of *S. aureus* ATCC 43300 (1.5x10 ^{6} CFU/mL) was studied. For this purpose, 10 microliters of the content of each test tube after 4, 10, and 24 h of incubation at 37°C was subculture onto TSA. The tube with 15 % Tween80 was used as diluents control. Each experiment was performed in duplicate.

RESULTS

Quantification of biofilm formation

Figure 1 showed the OD_{570nm} of 12 clinical isolates and 2 reference strains. All tested isolates were capable of biofilm formation in various amounts. Nearly all clinical isolates were higher biofilm-producer than the reference strains (ATCC 29213 and ATCC43300). Five clinical isolates, SA+2, SA+5, SA+6, SA+11, and SA+12 had two time higher OD_{570nm} than *S. aureus* ATCC 43300. Ten clinical strains that showed higher biofilm formation were selected for the next experiments.

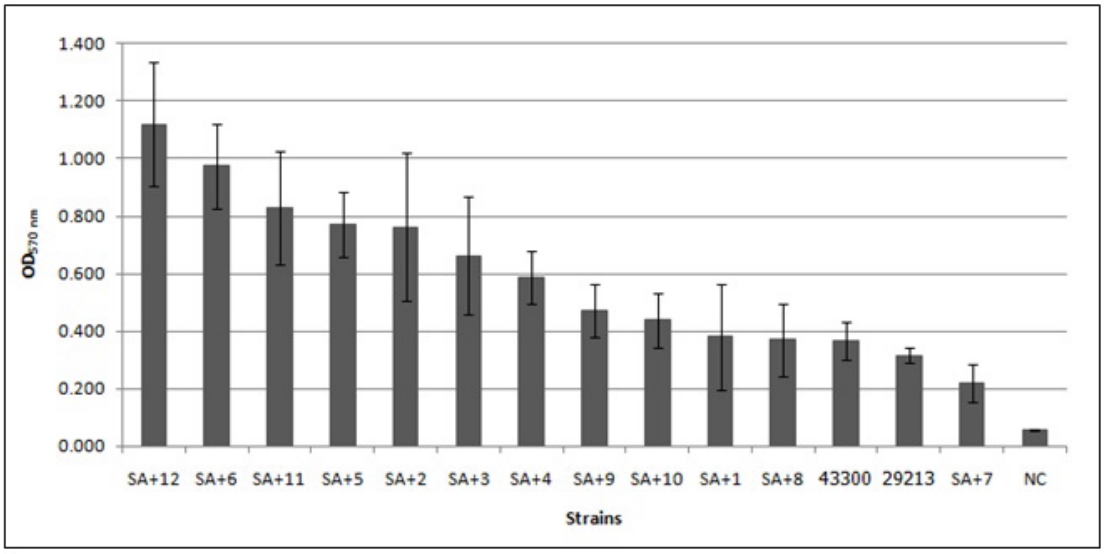

Figure 1. OD_{570mm} of 12 tested isolates and 2 reference strains (SA+ = Staphylococcus coagulase positive; 29213 = *S.aureus* ATCC 29213; 43300 = *S.aureus* ATCC 43300; NC = negative control)

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of planktonic cell.

After 24 h of incubation with clove and lemongrass oils, the range of MIC and MBC of clove oil against all tested organisms were 2.0 % to 3.0 % and 2.4 % to 5.0 % (v/v), respectively. All isolates had their MBCs higher than MICs (Figure 2). Unlike clove oil, nearly the MICS of LO of all isolates were equal to their MBC and the values were lower than 1 %. (The MIC and MBC of lemongrass oil were between 0.125 % to 0.5 % and 0.125 % to 0.5 %, respectively.)

Determination of the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) of biofilm.

Differ from planktonic cell, the concentrations of essential oils for growth inhibition of selected isolates were higher (Figure 3). The range of MBICs and MBECs of clove oil were 3.0 % to 5.0 % and 4.0 % to >5.0 % (v/v), respectively. The MBECs of eleven isolates were equal to or higher than 5.0 % but their MBICs were lower than 5.0 % (except one isolates, SA+6). Interestingly, lemongrass oil exhibited a good activity on biofilm similar to the planktonic cell. Although the MIC and MBC values of biofilm (MBIC and MBEC, respectively) were higher than that of cells in planktonic phase, they were equal to or lower than 1.0 % (The ranges were between 0.5 % to 1.0 % both MBICs and MBECs).

Time-kill assay

The time-kill curves showed the sub-MIC and combination effects against

S. aureus ATCC 43300. Figure 6 showed the remarkably reduction of the log CFU values when the tested strain exposed to the combination of clove and lemongrass oils. Especially after 24 h, the combination had obviously reduced over 4 times the log CFU value which lower than positive control while clove and lemongrass oils alone were not different. (Figure 4).

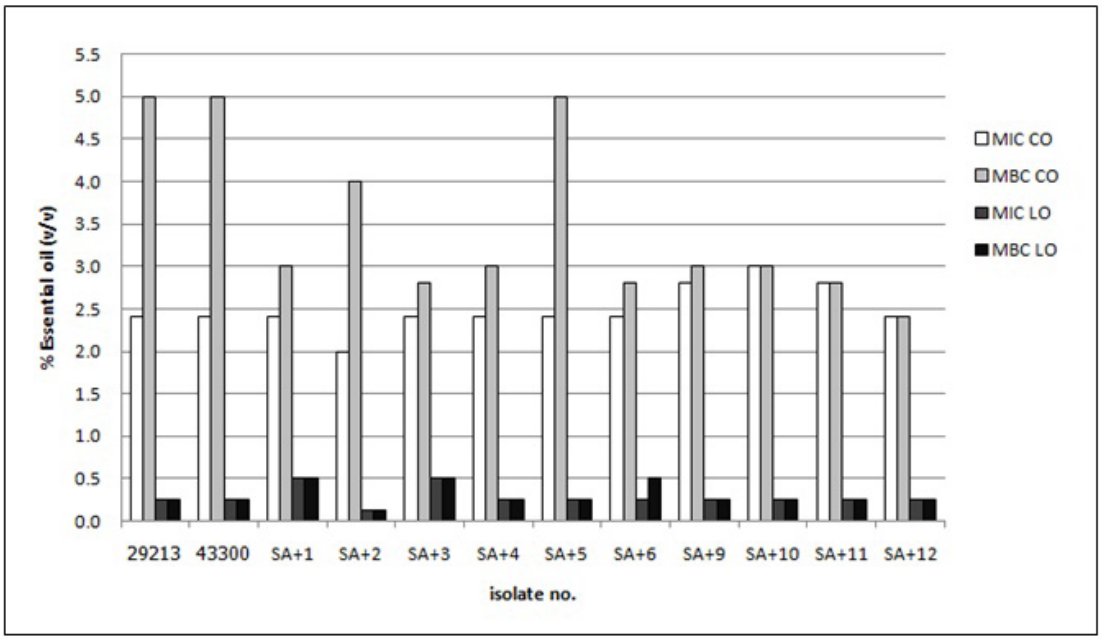

Figure 2. Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs) of clove (CO) and lemongrass (LO) essential oils agaist planktonic cell of clinical isolates and reference strains determined by broth microdilution technique (SA+ = Staphylococcus coagulase positive; 29213 = *S. aureus* ATCC 29213; 43300 = *S. aureus* ATCC 43300; NC = negative control)

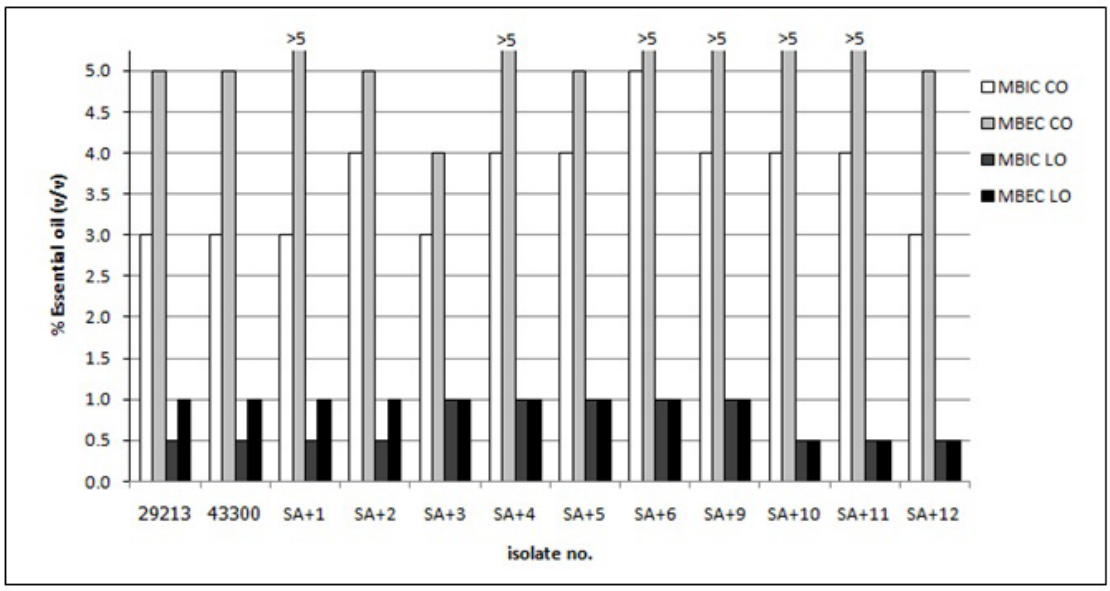

Figure 3. Minimum biofilm inhibitory concentrations (MICs) and Minimum biofilm eradication concentrations (MBCs) of clove (CO) and lemongrass (LO) essential oils agaist clinical isolates and reference strains determined by broth microdilution technique (SA+ = Staphylococcus coagulase positive; 29213 = *S. aureus* ATCC 29213; 43300 = *S. aureus* ATCC 43300; NC = negative control)

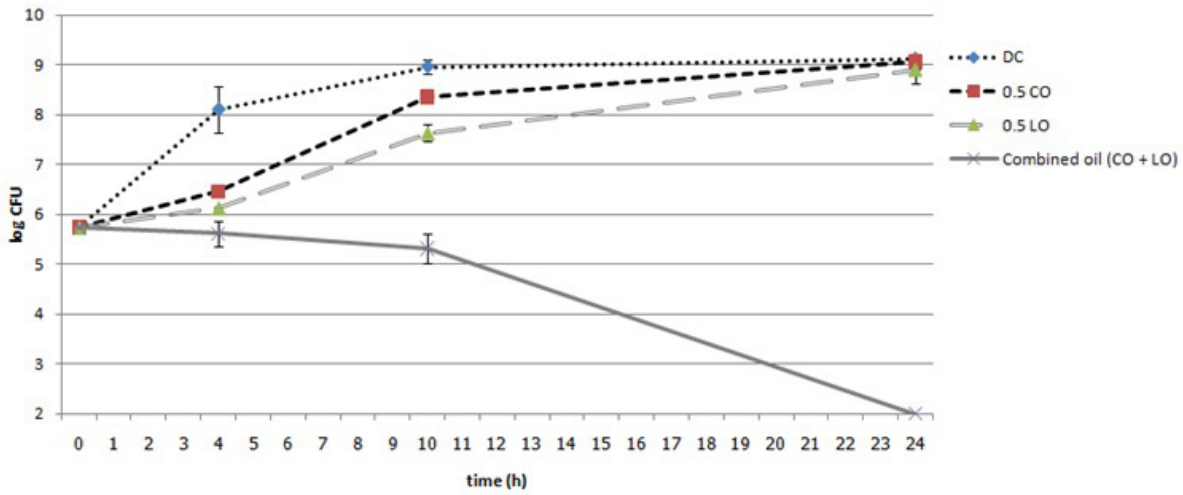

Figure 4. The time-kill assay of clove and lemongrass essential oils against *S.aureus* ATCC43300 (DC = diluents control; 0.5 CO = 0.5xMic of clove oil; 0.5 LO = 0.5xMIC of lemongrass oils;) Combined oil (CO+LO) = the combination of 0.5xMIC clove and lemongrass oils)

Discussion

Biofilm formation of *S. aureus* was evaluated by crystal violet staining technique. After the $OD_{570\,nm}$ was measured, we found that all tested isolates produced various amounts of biofilm (fig 1). According to Stepanovic *et al*.[23], the ability of biofilm formation can be classified into 4 categories: non-adherent, weakly, moderately, and strongly adherent based on the OD_{570nm}. All tested isolates in this experiment, were categorized as strongly adherent organisms based on Stepanovic *et al*. criteria[23]. In our study, the tested isolates were cultured in TSB with 0.25 % glucose for the markedly result because the glucose supplementation may support biofilm formation due to staphylococcal biofilm which is composed of polysaccharide called Poly-N-acetylglucosamine (PNAG) (also called Polysaccharide intercellular adhesion, PIA)[7, 26, 27]. In addition, crystal violet resolubilized with 33 % glacial acetic acid resulted the high optical density because it resolubilized the dye bound to the cells attached to the wall of the microtiter-plate well[23].

Although only 10 clinical isolates and 2 reference strains were used in this study, all tested *S.aureus* are biofilm-producing isolates. Biofilm formation affects the treatment because biofilm not only makes bacteria more resistant to antimicrobial agents, but also help the organisms spreading to another site[1].

Using broth microdilution technique, the MICs of clove and lemongrass oils are in the ranges of 2.0 % to 3.0 % and 0.125 % to 0.5 % (v/v), respectively. The MICs in this study are higher than the previous reports, 0.008 % to 0.25 % for clove oil and 0.008 % to 0.2 % for lemongrass oil.[13, 28-32] The difference may be due to many factors, such as plant culture condition, part of plant material, essential oil extraction method, and type of the solvent.[13, 33-35] In addition, the difference of *S. aureus* strains is one of the affected factors. Fluit *et al*.[36] found that antimicrobial susceptibility of 3,051 *S. aureus* isolates exhibited wide range of MIC level of gentamicin (0.12 to >8 $mg.L^{-1}$).

In this study, both MICs and MBCs of biofilm are higher than that of the planktonic cell (Figure 5 and 6). This indicates that biofilm are more resistant than planktonic cell. Biofilm, not only make the microorganism more resists to antimicrobial agents, there are many advantages of the cells in biofilm have been reported, such as protection from host immune system and increasing of pathogenicity.[1, 9] However, the essential oils still showed the same effect against both planktonic cell and biofilm, most of MBCs of clove oil for each isolate are higher than their MICs while MICs and MBCs of lemongrass oil are usually the same values. This result shows the effectiveness of clove and lemongrass oils

against both planktonic cell and biofilm of *S. aureus*.

Time-kill assay was performed as mention by Lee *et al.*[37] At the half of MIC, the single oil fails to inhibit growth of *S. aureus* ATCC 43300. When clove and lemongrass oils are simultaneously tested, approximately 7 log CFU reduction, compared with positive control, is observed after 24 h. This indicates the synergistic effect between them. Both clove and lemongrass oils have been reported about their likely mode of action, they affect cell membrane permeability and disrupt the metabolisms occurred on cell membrane. [13, 20, 28, 38, 39]

Further study is needed to determine their effectiveness and their possibility for *S. aureus* treatment. However, our data indicated that clove oil and lemongrass oil efficiently inhibited and killed both planktonic cell and biofilm of *S. aureus* therefore possibility an alternative method for *S. aureus* eradication.

Acknowledgements

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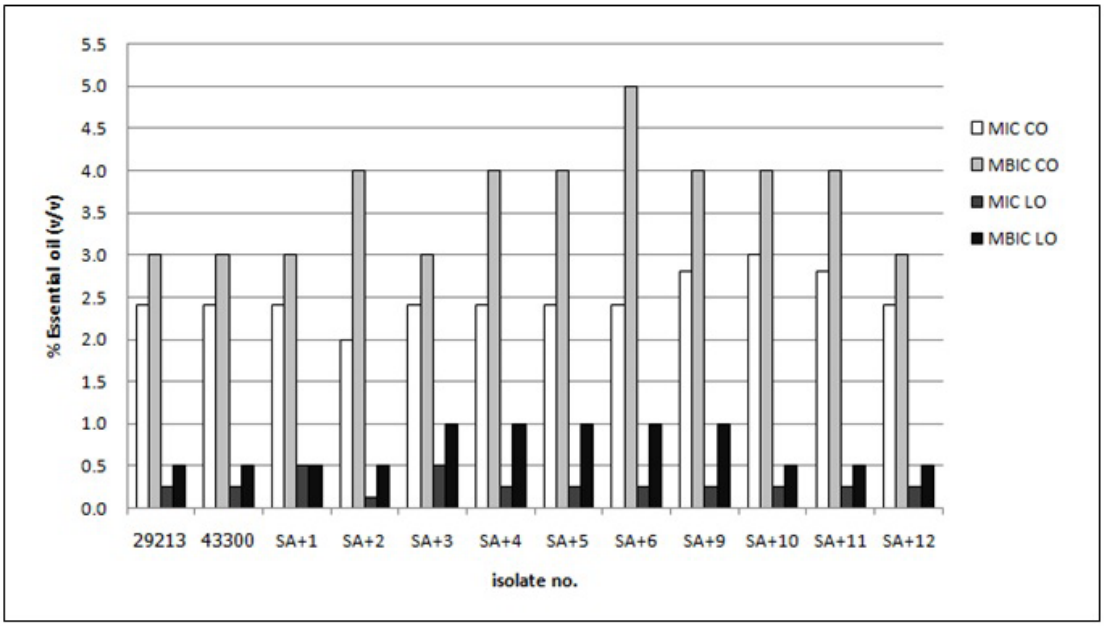

Figure 5. Minimum inhibitory concentrations (MICs) and Minimum biofilm inhibitory concentrations (MBCs) of clove and lemongrass essential oils agaist planktonic cell and biofilm of clinical isolates and reference strains determined by broth microdilution technique

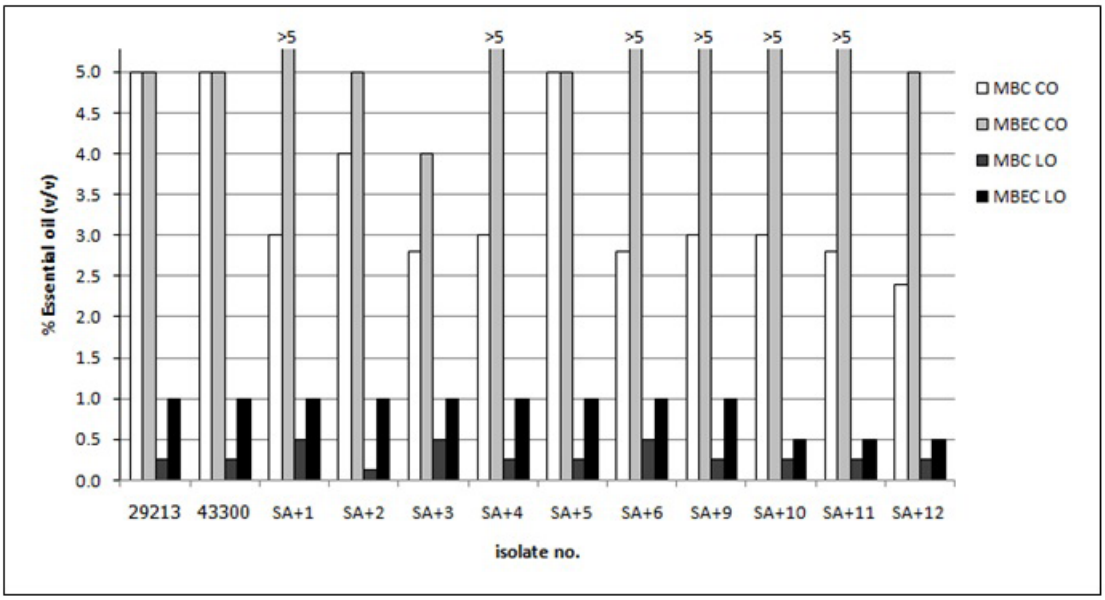

Figure 6. Minimum inhibitory concentrations (MICs) and Minimum biofilm eradication concentrations (MBECs) of clove and lemongrass essential oils agaist planktonic cell and biofilm of clinical isolates and reference strains determined by broth microdilution technique

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