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Inhibition of biofilm formation by Methicillin-resistant *Staphylococcus warneri* using *Citrus limon* oil

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ABSTRACT

Methicillin-resistant coagulase negative *Staphylococcus warneri* (MR-CoNS) is the major source of infective diseases because of its capacity to form biofilm inhabiting the hospital environments or community to regulate the sensitivity of *S. warneri* to lemon oil and also examination of the anti-biofilm activity. Five isolates were taken from urinary tract infection and skin burns, tested by the VITEK-2 compact system (AST and ID) to confirm *S. warneri*. The diagnosis result showed one isolate due to *S. warneri*. The susceptibility of *S. warneri* to the formation of the biofilm was investigated in the presence and absence of glucose, exhibiting greater ability to form the biofilm in its presence. Penicillin G and Mixofloxacin were examined with the concentrations of MIC, sub-MIC and sub-sub-MIC, used to inhibit the formation of biofilm. The synergism activity was tested between lemon oil, penicillin G and moxifloxacin. The result displayed that a synergistic actvitity between the two antibiotics and lemon oil was the most efficient to prevent biofilm formation.

Keywords: Bacteria, *Staphylococcus warneri*, *Citrus limon* oil, biofilm formation.
Article type: Research Article.

INTRODUCTION

Essential oils are substitutional antimicrobials, exhibit capacity to prevent biofilm formation or decrease, and capacity to provide AMR regulator. Little concentrations of lemon oil can prevent gram-positive and gram-negative pathogenic microbe. Some essential oils have demonstrated strong anti-biofilm activities. If essential oils are effective against biofilm forming, especially in bacteria emerging AMR, those may be join into new antimicrobial (El-Tarabily *et al*. 2012). Citrus lemon is highly common for outright consuming also jelly, jam and juices. Beside the nutritional things, citrus lemon show certain anti‑microbial properties through containing terpenoids polymethoxylated steroids, flavones, flavonoids, saponins, alkaloids, reducing sugars etc. (Al-Mamun & Feroz 2017). Limonene exhibits broad spectrum antimicrobial and antifungal activity (Vimal *et al*. 2013). *Staphylococcus warneri* is a gram-positive bacterium combined commensal as portion of the natural flora colonizing mucosal membranes, animals and human skin. *S. warneri* is a non-motile, facultative anaerobic, and coagulase-negative *Staphylococcus* (CNS). It is an opportunistic etiological cause extremely remote of the immunocompromised bacteraemia and bearing sepsis with multiple abscesses, orthopedic diseases, ventricular shunt infections, and vertebral osteomyelitis (Ivić *et al*. 2013). The virulence ability of *S. warneri* has been recommended to be multifactorial, including, adhesins, exo-enzymes, virulence regulators, capsule and iron uptake techniques (zczuka *et al*. 2016). Biofilm is a microbial composition covering manufacture surfaces, commonly joint with organic and mineral layers. Bacterial biofilm found preventative purposes against antimicrobial agents, dehydration and bacteriophage infection. Essentially, biofilms are a main worry in a food manufacture for their potency of food degradation resist, antimicrobial treatments, and helping pathogenic infection (Cunha *et al*. 2019). A modern research on the pathogenesis of *S. warneri* infection has revealed that the

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isolates from blood samples are efficient for attachment to epithelial cells and creating biofilm with numerous expressed antibiotic resistance genes (Dhanaraj *et al.* 2015).

MATERIALS AND METHODS

Preparation of *Citrus lemon* oil

Lemon fruits were obtained from the local market, washed with sterile water, then carefully peeled off in order to preserve the oil glands in them, cut into small portions, then kept frozen until use. Fifty grams of fresh lemon peels were placed in a beaker with a capability of 200 mL, followed by adding sterile distilled water, then placing in the beaker of Clevenger at 45 ºC temperature for 20 min. The lemon oil began to appear, after the water evaporated with oil, separating the oil from the water by pulling the top layer of the oil, then drying oil from the water using anhydrous sodium sulphate, after oil store in 4 ºC (Kırbaşlar *et al*. 2009).

Crystal violet staining

Preventing biofilm creation by bacteria was identified using the crystal violet staining technique. The isolate of *Staphylococcus warneri* was cultured on Nutrient agar medium for 24 hours at 37 °C, then part of the growing colonies was transferred to tubes containing tryptone soy broth, 100 microliter was taken from the tube and added to the well (Microtiter plates (96-well flat-bottom). After 24 h, the supernatant was removed, the wells were washed with physiological normal saline, methanol was added for fixation of the biofilms, then the supernatant was removed again. Thereafter, 0.1% crystal violet (CV) solution was added to wells, then the plates washed under running tap water. Finally, 33% acetic acid was added. The absorbance was measured at 590 nm (Peeters *et al*. 2008).

Testing the effect of oil on the biofilm

The isolates of *S. warneri* were cultured on Nutrient agar medium for 24 hours at 37 °C, then part of the growing colonies was transferred to tubes containing tryptone soy broth. 100 µL was then taken from the tube and added to the wells of Microtiter plates (96-well flat-bottom). Then, the essential oil of lemon was taken in concentrated form. 100 µL of each extract was added to the well, as well as the control well, which included only the medium of plant and the lemon essential oil. It was only incubated for 24 h at a temperature of ٣٧ °C, thereafter it was cleared, washed three times with saline phosphate buffer (PBS) to remove non-adherent cells, then 160 µL of 95% methanol was added and left for 10 min to stabilize adherent cells. 100-µL crystal violet (0.25%) was prepared for each hole in the plate and left for 15 min. The wells were washed using distilled water to remove excess dye. 160-µL glacial acetic acid were added at a concentration of 33% and the absorbance was measured along a 630 nm wavelength with a micro ELISA reader to quantify the bacterial potential for bio-membrane production (Mathur *et al.* 2006).

Statistical analysis

Statistical analysis was performed using One-Way ANOVA test. Significance was considered at $p < 0.05$ **(**Morgan *et al*. 2004).

RESULTS

Finding active compounds in lemon oil

Detection active compounds contained oil using gas chromatography technique (Table 1). The results of the table displayed that the highest ratio was due to limonene (102.34 ppm). It is the major compound in lemon oil followed by alpha-pinene (86.66 ppm), and other oil ingredients in different proportions.

Table 1. Active compounds with lemon oil using Gas Chromatography (GC) method.

Active Compounds	ppm
Camphore	17.01
Camphene	51.01
Teiclene	65.21
Linalool	76.97
Limonene	102.34
Myrcene	70.64
Sabinene	64.68
Alpha-pinen	86.67

Susceptibility of *Staphylococcus warneri* to biofilm formation

The biofilm has the vital elements which significantly provide the capacity of microorganism to cause disease and its readiness to resist many kinds of antibiotics (Schroll *et al.* 2010). The results showed that the significant differences in biofilm formation of *S. warneri* in a presence of glucose was strong, while the absence of glucose decrease its (Table 2). Several factors affect biofilm formation, including genetic factors and environmental conditions, followed by the adhesion efficacy of the PIA supply to the capacity of bacteria for producing biofilm and also type of media used.

Table 2. Biofilm with presence and absence of glucose for isolates of *Staphylococcus warneri* measured by 590 nm.

Biofilm in the existence of glucose (Average ± standard deviation)	Biofilm in the absence of glucose (Average ± standard deviation)	Bacterial isolate
0.517 ± 0.012 *	0.425 ± 0.02	*S. warneri*

*. Significant differences at $p \leq 0.05$

Moxifloxacin and Penicillin G effects on the biofilm of *S. warneri*

Moxifloxacin and Penicillin G was examined by three concentrations of each antibiotic taken from the VITEK-2 compact system. In the case of penicillin G, the concentrations included the lower inhibitory MIC (0.5 μg mL^{-1}) ,Sub-MIC c (0.25 μg mL^{-1}) and Sub-Sub-MIC (0.125 μg mL^{-1}), while in the case of moxifloxacin, (0.25 μg mL^{-1}), Sub-MIC (0.125 μg mL^{-1}) and Sub-Sub-MIC (0.0625 μg mL^{-1}), were examined on the biofilm formation of the *S. warneri* isolates. The statistical results are shown in Table 3, exhibiting the effect of penicillin G in preventing biofilm formation compared to control and also effect of Moxifloxacin with three concentrations (Sub-Sub-MIC, Sub-MIC, MIC). The isolate showed that biofilm was affected by the antibiotic, compared to control.

Effect of lemon oil on the biofilm formation

The lemon essential oil showed a perfect effect on the biofilm when comparing the results with the control, including bacterial culture (Table 3). The preventing effect of the oil on biofilm MRSW was due to its active chemical components.

The results depicted huge efficiency in inhibiting the formation of biofilm. The lemon peel oil has a high acidic property which deforms the protein forming in the cell wall leading to an imbalance osmotic pressure loss of homeostasis. In addition, the terpenoids rates are high in oil, known for their ability to prevent the quorum sensing process that plays a role in the organization of biofilm production (Vikram *et al*. 2011).

The results obtained in Table 3 are in line with those of Kavanaugh & Ribbeck's (2012) reporting the efficiency of essential oils in stopping the formation of *S. aureus* biofilm than that of some antibiotics. The cells in the biofilm as some parts of the biomass, exhibit high resistance to most antibiotics.

Synergistic action between two antibiotics and lemon oil

The medicinal plants have numerous advantages related to the presence of synergistic activity between extract and many kinds of antibiotics. The most protuberant of these advantages include raises in the efficiency of antibiotics, decreasing the undesirable effects and elevating the stability of the antibiotic, as well as the bioavailability of the drug with free factors in the body, along with getting the result of therapeutic treatment of antibiotics at moderately low concentrations.

Inui *et al*. (2007) pointed out that some medicinal plants may not have an effect on pathogenic microorganisms, however, elevates antibiotic efficacy when joint with antibiotics (Kamatou *et al*. 2006). So, prescription of penicillin G with a volatile oil leads to a significant synergistic activity between them. The synergistic effect of the oil with moxifloxacin in three concentrations (MIC– Sub-MIC, Sub-Sub MIC) against *S. warneri* (Table 3) led to better results than effects observed between bacteria and penicillin G.

Table 3. Action between two antibiotics and lemon oil.

(Average ± standard deviation)	µg mL^{-1}		Antibiotics	Samples
0.055 ± 0.007	MIC	0.25	moxifloxacin	*S. warneri*
0.063 ± 0.006	Sub MIC	0.125		
0.086 ± 0.003a	Sub -Sub MIC	0.0625		
0.425 ± 0.020		control		
0.172 ± 0.027	MIC	0.5	moxifloxacin	*S. warneri*
0.178 ± 0.013	Sub MIC	0.25	and Limon Oil	
0.168 ± 0.014	Sub -Sub MIC	0.125		
0.425 ± 0.020		control		
0.143 ± 0.023	Concentrated		Limon Oil	*S. warneri*
0.425 ± 0.020		control		
0.1200 ± 0.013	MIC	0. 5	Penicillin G	*S. warneri*
0.089 ± 0.008	Sub MIC	0.25		
0.151 ± 0.005	Sub -Sub MIC	0.125		
0.265 ± 0.004		control		
0.243 ± 0.0015	MIC	0. 5	Penicillin G	*S. warneri*
0.369 ± 0.0035	Sub MIC	0. 25	and Limon Oil	
0.853 ± 0.0085	Sub -Sub MIC	0.125		
0.425 ± 0.020		control		

Significant differences at $p \leq 0.05$.

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