

## CORRELATION BETWEEN THE BACTERIOSTATIC AND BACTERICIDE EFFECT WITH ANTIBIOFILM AND ANTICOLONY SPREADING FROM JAVANESE CITRONELLA OIL ON METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA)

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### ABSTRACT

*Methicillin-resistant Staphylococcus aureus (MRSA) is a pathogenic bacterium that has been resistant to various types of antibiotics, so it is not easy to be treated with antibiotics and needs other solutions. Javanese citronella oil distilled from the Cymbopogon nardus plant is proven to function as an antibacterial agent (bacteriostatic and bactericidal), fungicide and repellent. This study aimed to prove that there is a positive correlation between bacteriostatic and bactericidal effects with antibiofilm and anticolony spreading from Javanese citronella oil on MRSA. The intended antibiofilm is a barrier to biofilm formation and eradication. Bacteriostatic and antibiofilm effects were tested using microtiter plates assay, bactericidal effect test with subculture into the media and anticolony spreading effect test with spot inoculation in Tryptic Soy Broth media supplemented with 0.24% agar. The bacteriostatic effect test data were analyzed using paired t-test, bactericidal effect using the Friedman test, antibiofilm effect test using Kruskal-Wallis and the results of all the tests correlated using Pearson and Spearman correlation. The statistical significance used was  $p < 0.05$ . The results showed that Javanese citronella oil had a bacteriostatic concentration of 0.02% (v/v) and bactericidal concentration of 0.78% (v/v). The Pearson correlation test showed that there was a negative correlation between bacteriostatic and bactericidal effects on biofilm formation with  $r = -0.956$  ( $p = 0.000$ ), but the correlation was positive for biofilm eradication with  $r = 0.918$  ( $p = 0.000$ ) and anticolony spreading with  $r = 1.000$  ( $p = 0.000$ ).*

**Keywords:** *Methicillin-resistant Staphylococcus aureus; Javanese citronella oil; bacteriostatic effect; bactericidal effect; antibiofilm effect; anticolony spreading effect*

### ABSTRAK

*Methicillin-resistant Staphylococcus aureus (MRSA) merupakan bakteri patogen yang telah resisten terhadap berbagai jenis antibiotik, sehingga tidak mudah diterapi menggunakan antibiotik dan membutuhkan solusi lain. Minyak sereh Jawa disuling dari tanaman Cymbopogon nardus terbukti dapat berfungsi sebagai agen antibakteri (bakteriostatik dan bakterisida), fungisida dan repelen. Penelitian ini bertujuan untuk membuktikan adanya korelasi positif antara efek bakteriostatik dan bakterisida dengan antibiofilm dan anticolony spreading dari minyak sereh Jawa pada MRSA. Antibiofilm yang dimaksud adalah hambatan pembentukan dan eradikasi biofilm. Efek bakteriostatik dan antibiofilm diuji menggunakan microtiter plates assay, uji efek bakterisida dengan subkultur ke media agar dan uji efek anticolony spreading dengan inokulasi spot di media Tryptic Soy Broth yang disuplementasi 0,24% agar. Data uji efek bakteriostatik dianalisa menggunakan uji t-berpasangan, efek bakterisida menggunakan uji Friedman, uji efek antibiofilm menggunakan Kruskal-Wallis dan hasil semua uji dikorelasikan menggunakan korelasi Pearson dan Spearman. Signifikansi statistik yang digunakan adalah  $p < 0,05$ . Hasil penelitian menunjukkan bahwa minyak sereh Jawa memiliki konsentrasi bakteriostatik 0,02% (v/v) dan bakterisida 0,78% (v/v). Uji korelasi Pearson menunjukkan bahwa terdapat korelasi negatif antara efek bakteriostatik dan bakterisida terhadap pembentukan biofilm dengan  $r = -0,956$  ( $p = 0,000$ ), namun korelasi bernilai positif terhadap eradikasi biofilm dengan  $r = 0,918$  ( $p = 0,000$ ) dan anticolony spreading dengan  $r = 1,000$  ( $p = 0,000$ ).*

**Kata kunci:** *Methicillin-resistant Staphylococcus aureus; minyak sereh Jawa; efek bakteriostatik; efek bakterisida; efek antibiofilm; efek anticolony spreading*

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## INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* or MRSA is *Staphylococcus aureus* which has been resistant to various types of antibiotics. The pathogenicity of MRSA apart from being caused by antibiotic resistance can be caused by the ability to form biofilms and colony spreading. The ability to form biofilms causes infection to be chronic as indicated by persistent inflammation, tissue damage, slowing healing and difficult to determine the type of antibiotic that is appropriate for therapy (Taraszkievicz et al 2013). Colony spreading on the surface of the media is supported by the ability to produce phenol soluble modulins (PSM) toxins (Pollitt et al 2015).

Antibacterial agent innovation (bacteriostatic and bactericidal), inhibition and eradication of biofilms and colony spreading MRSA from effective, inexpensive and easily obtained natural ingredients are needed to accelerate infection recovery without resistance. One way that can be done is by utilizing Indonesia's abundant natural resources, producing essential oils and has been proven to be an antibacterial agent (bacteriostatic and bactericidal), antifungi and repellent, which is Javanese citronella plants (*Cymbopogon nardus*). Javanese citronella is a plant that is widely distributed throughout the world and most uses are in the fields of culinary, herbal medicine and repellent (Lucas et al 2012, Wei & Wee 2013, Sritabutra & Soonwera 2013, Avoseh et al 2015).

Essential oils can damage the cell wall through suppression of genes that play a role in the biosynthesis of peptidoglycan, an enzyme that regulates the acidization of teichoic acid and PBP (penicillin binding protein) (Bir 2000). Damage to the cell wall is a gap for essential oils to penetrate into the second barrier, the cell membrane. Hydrophobic essential oils do not easily pass through and absorb the phospholipid head which is hydrophilic (Bir 2000).

Javanese citronella oil is hydrophobic, so it does not easily penetrate cell membranes. Javanese citronella oils, such as geraniol, carvacrol, eugenol and limonene, can increase membrane fluidity. Increased membrane fluidity will cause cell damage (Tsuchiya 2015). Based on literature studies, no studies have shown a correlation between bacteriostatic and bactericidal effects with antibiofilm and anticolony spreading from Javanese citronella oil on methicillin-resistant *Staphylococcus aureus* (MRSA), so this study needs to be done.

## MATERIALS AND METHODS

The tools used in this study included autoclave, incubator, petridish, osse, bunsen, refrigerator, pH meter, ELISA reader, electric scales, calipers, erlemeyer, Beaker glass, aluminum foil, test tubes, test tube racks, vortex, microtiter plate 96 wells, laminar air flow, micropipette and micropipette tips. The materials used included Javanese citronella oil, methicillin-resistant *Staphylococcus aureus* (MRSA), Mueller-Hinton agar (MHA) and liquid (MHB) isolates, Tryptic-soy broth (TSB) supplemented with 0.24% agar, 70% alcohol, aquades, 0.5% (v/v) Tween 20, 1 M NaOH, 0.5 McFarland standard solution, 1% (w/v) glucose, phosphate buffer saline, crystal violet 0.1% (w/v) and glacial acetic acid 10% (v/v).

### Javanese citronella distillation and GC-MS (Gas Chromatography-Mass Spectrometry) test

Javanese citronella distillation was done using the water-steam method. Fresh Javanese citronella was cut into pieces and then placed in a distillation container filled with water. Distillation devices were arranged in such way that they were interconnected. The Javanese citronella oil produced was separated from water but had not been completely free from water content, so anhydrous sodium sulfate was added to bind the remaining water. Javanese citronella oil was filtered using filter paper and stored in glass bottles. The phytochemical content of Javanese citronella oil was measured using GC-MS conducted at PT. Gelora Djaya, Surabaya.

### Javanese citronella oil screening test uses disc diffusion

Screening tests were carried out in MHA media planted with McFarland standard MRSA 0.5 suspension. Five mm Whatmann discs were placed on the media and 10 mL of Javanese citronella oil were concentrated at 100, 50 and 25% (v/v). The media was then incubated for 24 hours at 37°C. Barriers to MRSA growth and diameter of the resistance zone were measured using calipers.

### Test for resistance re-identification of MRSA isolates

The cefoxitin (Fox) and oxacillin (Ox) antibiotics used for re-identification were placed on MHA media planted with MRSA 0.5 McFarland and incubated for 24 hours at 37°C. Barriers zones were measured using calipers. The measurement results were compared with the CLSI standard. If the cefoxitin inhibition zone =22 mm and oxacillin =13 mm, they were sensitive. However if they were less, they were resistant.

### **Test for MRSA isolate biofilm formation on 96-well microtiter plate qualitatively**

The suspension of 0.5 MRSA of the McFarland standard in MHB media supplemented with 1% (b/v) glucose was channeled to the test well and incubated with variations of time 1, 2, 3 and 10 days at 37°C. The contents of the well were then discarded and washed using sterile PBS twice, dried and stained using crystal violet for 30 minutes. Violet crystals were then removed and washed using sterile PBS 3 times and then dried. The formed biofilms were observed visually and documented.

### **Javanese citronella oil bacteriostatic test on MRSA**

Javanese citronella oil with a concentration of 12.5% was obtained by dissolving Javanese citronella oil on MHB media containing 0.5% (v/v) Tween 20. Variation in concentration in wells 6.25; 3.13; 1.56; 0.78; 0.39; 0.20; 0.10; 0.05 and 0.02% (v/v) was obtained by microdilution. Positive controls contained MHB and MRSA media while negative control wells contained MHB media. Optical density (OD) value of 595 nm was measured using ELISA reader before and after incubation.

### **The bactericidal test of Javanese citronella oil on MRSA**

A total of 10 µl of liquid from each well in the bacteriostatic test was streaked on MHB media and incubated for 24 hours at 37°C. The concentration that shows no MRSA growth in the media was called bactericidal concentration.

### **Barrier test for MRSA biofilm formation**

The test procedure was based on the Adukwu et al's (2012) method. A total of 50 mL of MRSA culture in MHB media supplemented with 1% (w/v) of glucose was adjusted to 0.5 McFarland and distributed to each test well. 50 µl of Javanese citronella oil bacteriostatic and bactericidal concentrations were added to each well. Negative control was MHB media supplemented with 1% (w/v) glucose and positive control was liquid Mueller-Hinton media with a supplement of 1% (w/v) glucose and MRSA 0.5 McFarland without Javanese citronella oil. The well was incubated for 24 hours at 37°C. The contents of the well were then removed, washed twice using sterile 300 µl phosphate buffer saline (PBS) and dried for 30 minutes. The wells were stained using crystal violet for 30 minutes at room temperature and washed three times using sterile and dried PBS. Violet crystals were then dissolved using

10% (v/v) glacial acetic acid and OD was measured at 595 nm using an ELISA reader.

### **MRSA biofilm eradication test**

The MRSA biofilm eradication test procedure was carried out based on the Adukwu et al's (2012) method. Fifty µl of suspension of MRSA 0.5 McFarland on MHB media with a supplement of 1% (w/v) of glucose was channeled to each test well and incubated for 48 hours at 37°C. The contents of the well were then discarded and each well was washed twice using 300 µl phosphate buffer saline (PBS). Fifty µl of Javanese citronella oil bacteriostatic or bactericidal concentrations were added to each well and re-incubated for 24 hours at 37°C. The wells were then washed using sterile PBS and stained using crystal violet. Positive control was MRSA biofilm without Javanese citronella oil.

### **Anticolony spreading MRSA test**

The colony spreading test procedure was carried out based on the studies of Kaito and Sekimizu (2006) and Omae et al (2012). Mixing TSB supplemented with 0.24% agar with Javanese citronella oil was done to obtain bacteriostatic and bactericidal concentrations. The mixture was then poured immediately into a plate with a diameter of 60 mm and allowed to solidify for 20 minutes at 37°C. A 2 µl suspension of MRSA 0.5 McFarland (from overnight culture) was inoculated in the middle of the medium and dried for 15 minutes then incubated at 37°C for 8 hours or more.

## **RESULTS**

Javanese citronella oil used in this study was known to contain several components of phytochemicals as shown in Table 1. Phytochemical components that can act as bacteriostatic agents are limonene, citral, carvacrol, citronellal, eugenol and citronellol (Escheverrigaray et al 2008, Brugnera et al 2011, Lopez -Romero et al).

Based on the results of the study, Javanese citronella oil may inhibit MRSA growth at concentrations of 100, 50 and 25% (v/v) with a decrease in the average zone of resistance. The mean inhibition zone of MRSA growth at a concentration of 100% (v/v) was 18.97 mm, at a concentration of 50% was 14.77 mm and at a concentration of 25% was 9.79 mm. The average obstacle zone can be seen in Table 2.

Based on the results of the re-identification of MRSA isolates used in the study, it showed resistance to

cefotixin and oxacillin antibiotics. The results of the reidentification can be seen in Fig. 1.

Resistance to the two antibiotics betalactam showed an affinity change in penicillin-binding proteins (PBP). Cefoxitin and oxacillin are  $\beta$ -lactam group antibiotics that can interfere with bacterial cell wall biosynthesis (Soleha 2015). The test results showed that MRSA isolates can form biofilms in 24, 48 and 72 hours of incubation. The results of the preliminary test can be seen in Fig. 2.

Table 1. Components and percentage (%) of Javanese citronella oil phytochemical compounds

No.	Component	Percentage (%)
1.	Cyclohexane	0.08
2.	1-Vinylcyclohexane	0.05
3.	2-Thiopyridine	0.04
4.	Myrcene	0.78
5.	D-Limonene	3.28
6.	3-Carene	0.06
7.	2,6-Dimethyl Hept-5-en-1-al	0.19
8.	1,3-Cyclohexadiene	0.10
9.	Linalool	1.07
10.	Citronellal	13.26
11.	Cyclohexanol	0.53
12.	Baros Camphor	0.31
13.	4-Methyl-1,4-heptadiene	0.20
14.	Decanal	0.30
15.	Citronellol	9.92
16.	Z-citral	2.11
17.	Geraniol	12.40
18.	E-citral	2.84
19.	(R)-(+)-Pulegone	0.15
20.	Geranyl acetate	3.60
21.	7-(1-methylethylidene)	0.33
22.	Citronellyl propionat	3.77
23.	Eugenol	2.28
24.	1-ethenyl-1-methyl-2	3.56
25.	Alpha-Caryophyllene	0.31
26.	1,6-Cyclodecadiene	4.73
27.	Isoledene	1.29
28.	Naphthalene	13.86
29.	Cyclohexanemethanol	8.88
30.	Farnesol	0.54
31.	Delta-Selinene	0.50
32.	Tau-cadinol	2.15
33.	(-)-isoledene	3.20
34.	1,6,10-Dodecatrien-3-ol	0.64
35.	Geranyl linalool isomer	0.18
36.	Carvacrol	0.46
37.	Isopathulenol	0.51
38.	1-Methoxy-3-(2-hydroxyethyl)nonane	0.44
39.	1,4,9-Triazaphenoxathiin	0.13
40.	2-Methyl-6-methylene-1	1.20

Source: Laboratory of PT. Gelora Djaja, Surabaya (2017)

According to Adukwu et al (2012), bacteriostatic concentration is the inhibition of MRSA growth indicated by the value of OD595 24 hours=0 hours in citronella oil with a concentration of 12.5; 6.25; 3.13; 1.56; 0.78; 0.39; 0.20; 0.10; 0.05 and 0.02% (v/v). The test results of the bacteriostatic effect of Javanese citronella oil on MRSA can be seen in Table 3.

Table 2. Average Java citronella oil resistance zone in MRSA growth

Concentration (% v/v)	Mean obstacle zone (mm) $\pm$ SE
100	18.97 $\pm$ 1.85
50	14.77 $\pm$ 1.17
25	9.79 $\pm$ 0.67

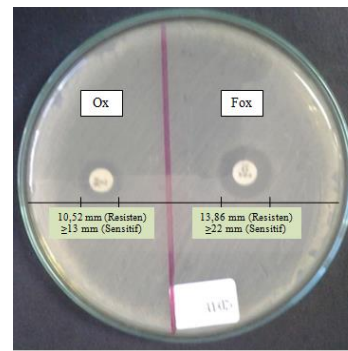


Fig. 1. Reidentification of the resistance of MRSA isolates to cefoxitin and oxacillin.

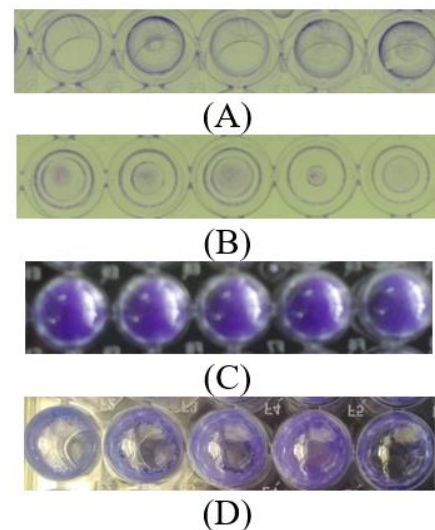


Fig. 2. Preliminary results of MRSA biofilm formation: 24 hours (A), 48 hours (B), 72 hours (C) and 240 hours or 10 days in microtiter plate 96 well.

Based on Table 4, it can be seen that at concentrations of 12.5% (v/v) to 0.78% (v/v) there are no MRSA

colonies while at concentrations of 0.39% (v/v) to 0.02% (v/v) there are colonies. Based on Adukwu et al (2012), bactericidal concentrations were selected based on the lowest concentration which showed no colonies on subculture media so that based on the results of the study it was determined that bactericidal concentrations were 0.78% (v/v). Based on Table 3, at the lowest concentration of 0.02% (v/v) the value of OD595 24 hours is less than the value of OD595 at 0 hours, so the

bacteriostatic concentration is set at 0.02% (v/v). Bacteriostatic and bactericidal concentrations of Javanese citronella oil on MRSA are concentrations used to test the inhibition of MRSA biofilm formation. This study used different negative and positive controls for bacteriostatic and bactericidal concentrations, this was due to different research times.

Table 3. Average OD595 test for the bacteriostatic effect of Javanese citronella oil on MRSA at 0 and 24 hours

Concentration (% v/v)	Mean OD595 0 hour ± SE	Rerata OD595 24 hour ± SE
12,5	0.275 ±0.012	0.133±0.009
6.25	0.237 ±0.021	0.140±0.015
3.13	0.194 ±0.013	0.130±0.009
1.56	0.198 ±0.039	0.137±0.007
0.78	0.184 ±0.016	0.145±0.003
0.39	0.162 ±0.015	0.124±0.008
0.20	0.146 ±0.003	0.125±0.004
0.10	0.140 ±0.004	0.124±0.002
0.05	0.134 ±0.006	0.123±0.000
0.02	0.136 ±0.007	0.123±0.004
Positive control	0.115 ±0.002	0.212±0.021
Negative control	0.096 ±0.001	0.090±0.000

Table. 4 Test results for the bactericidal effect of Javanese citronella oil on MRSA

Concentration (% v/v)	Colony (any/none)
12.5	None
6.25	None
3.13	None
1.56	None
0.78	None
0.39	Any
0.20	None
0.10	Any
0.05	None
0.02	Any
Positive control	Any
Negative control	None

Table 5. Average OD595 bacteriostatic and bactericidal concentrations of Javanese citronella oil in inhibition of MRSA biofilm formation

OD595 ± SE					
Bacteriostatic concentration	Positive control of bacteriostatic concentration	Negative control of bacteriostatic concentration	Bactericidal concentration	Positive control of bactericidal concentration	Negative control of bactericidal concentration
0.239 ± 0.005	0.177 ± 0.005	0.070 ± 0.002	0.109 ± 0.004	0.153 ± 0.009	0.082 ± 0.007

The test results can be seen in Table 5. Javanese citronella oil bacteriostatic and bactericidal concentrations were used for MRSA biofilm eradication tests. The test results can be seen in Table 6. Whereas, bacteriostatic and bactericidal concentrations of Javanese citronella oil were used for anticolony spreading MRSA tests. The test results can be seen in Table 7.

Table 6. Average OD595 effect of bacteriostatic and bactericidal concentrations of Javanese citronella oil on eradication of MRSA biofilms

Group	OD595 ± SE
Positive control	0.193 ± 0.024
Bacteriostatic concentration	0.109 ± 0.002
Bactericidal concentration	0.181 ± 0.007
Negative control	0.071 ± 0.003

Javanese citronella oil is known in bacteriostatic and bactericidal concentrations which can cause MRSA biofilm eradication which is indicated by the average OD595 value less than positive control OD595. The

results of the data analysis mean that the bacteriostatic concentration of Javanese citronella oil plays a role in the eradication of MRSA biofilms, whereas there is no eradication of MRSA biofilms at bactericidal concentrations.

According to Kaito and Sekimizu (2006), one of the factors that plays a role in colony spreading is the water content in the media. Based on the results of the study, Javanese citronella oil at bactericidal concentrations can play a role in inhibiting biofilm formation and colony spreading MRSA.

According to Kaito and Sekimizu (2006), the water content in the media affects colony spreading. This is in accordance with the results of the study, namely the water content in media containing Javanese citronella oil bactericidal concentrations were relatively less compared to bacteriostatic concentrations, so that colony spreading MRSA would experience resistance.

Table 7. Test results for bacteriostatic and bactericidal effects of Javanese citronella oil on colony spreading MRSA

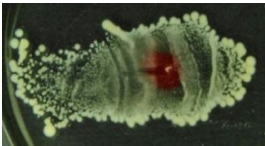
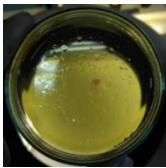

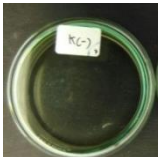
No.	Group	Colony spreading	Shape	Note
1.	Bacteriostatic concentration	Yes		A red sign indicates the location of MRSA inoculation
2.	Bactericidal concentration	No		
3.	Positive control	Yes		
4.	Negative control	No		MRSA inoculation is not performed

Table 8. Correlation results of bacteriostatic and bactericidal effects of Javanese citronella oil with antibiofilm and anticolony spreading on MRSA

Group	Significance correlation (r)		
	OD595 barriers to MRSA biofilm formation	OD595 eradication of MRSA biofilms	Colony spreading of MRSA
Javanese citronella oil (bacteriostatic and bactericidal concentration)	- 0.956 (0.000)	0.918 (0.000)	1.000 (0.000)

Table 8 shows that the citronella oil of bacteriostatic and bactericidal concentrations has a negative correlation with the inhibition of MRSA biofilm formation, ie  $r=-0,956$  with  $p=0.000$  which means the higher the concentration, the lower the OD595 inhibition of biofilm formation and the lower the concentration OD595 inhibits the formation of biofilms.

## DISCUSSION

In this study, Javanese citronella oil may inhibit MRSA growth at concentrations of 100, 50 and 25% (v/v) with a decrease in the average zone of resistance. The Brugnera et al's (2011) study showed that Javanese citronella oil had a mean zone of resistance to the growth of *Staphylococcus aureus* at a concentration of 50% (v/v) was 5.37 mm and a concentration of 25% was 6.51 mm. When compared, the average inhibition zone of Javanese citronella oil in MRSA in this study was higher than that of Brugnera et al (2011). This may be caused by differences in the type and phytochemical composition of the Javanese citronella oil produced.

The MRSA isolates used in this study were isolated from the throat, according to Mirani et al's (2013) study which showed that MRSA isolated from food can form biofilms after 24 hours of incubation. Fig. 2 shows that biofilm visually formed is thicker in 72 hours after incubation than 24 and 48 hours after incubation. Biofilms are thick enough to be seen without using a microscope when they are in stable environmental conditions and a relatively long time (Phillips et al 2012). Bacteria have the ability to escape from the substrate if the repulsive force is greater than the attraction (Garrett et al 2008). Figure 2 shows that biofilms undergo dispersion after 240 hours of incubation.

Javanese citronella oil has a bacteriostatic effect on MRSA at a low concentration of 0.02% (v/v). According to Pankey and Sabath (2004), bacteriostatic concentration showed that Javanese citronella oil at a concentration of 0.02% (v/v) could inhibit growth by maintaining MRSA growth in the stationary phase. The

results of the study by Espina et al (2015) showed that *Staphylococcus aureus* strains SC-01, USA300, UAMS-1 and Newman planktonik were sensitive to limonene with bacteriostatic concentrations of 0.5% (v/v) and citral 0.05% (v/v). Based on Yadav et al. (2015), the bacteriostatic concentration of eugenol ranged from 0.01% to 0.04%.

MRSA bacteria are gram-positive bacteria that have thick cell walls and cell membranes, so it is not easy for antibacterial agents to penetrate. Javanese citronella oil which shows a role as an antibacterial agent (bacteriostatic and bactericidal) can be associated with hydrophobic properties and the role of phytochemical content in MRSA cells. Javanese citronella oil is naturally hydrophobic and the surface of bacterial cells is the same, so Javanese citronella oil can be firmly attached to the cell surface (Gupta et al 2015).

Javanese citronella oil used in the study had citronellate content of 9.92%. Citronelol is known to cause changes in the surface of *S. aureus* cells to be hydrophilic (Lopez-Romero et al 2015). Changing the surface properties of the cell to be hydrophilic facilitates citronella oil in bacteriostatic and bactericidal concentrations to penetrate cell walls because the water content in the mixture is greater than that of Javanese citronella oil.

Biofilm formation was considered to have resistance when the average OD595 value was less than the mean positive control OD595 value. The meaning of the results of the study, namely Javanese citronella oil at bactericidal concentrations can inhibit MRSA biofilm formation while bacteriostatic concentrations cannot inhibit. This is consistent with the results of research on subculture media for determination of bactericidal concentrations that no colony was found in all replications. Javanese citronella oil at bactericidal concentrations can kill =99.9% MRSA so that attachment to biofilm formation does not occur.

Obstacles in biofilm formation show obstacles in growth that affect cell density, quorum sensing and attachment of bacterial cells to the substrate. Quorum

sensing is influenced by an increase in bacterial cell density, production of signaling molecules and recognition of signal molecules by receptors and this can affect biofilm formation (Podbielski & Kreikemeyer 2003, Le & Otto 2015). The barriers to attaching bacterial cells to the substrate are one of the strategies to inhibit bacterial pathogenesis.

Phytochemicals contained in natural antibacterial agents play a role in changes in cell morphology and membrane dysfunction leading to bacterial cell death (Renner and Weibel, 2011; Trentin et al., 2013). Javanese citronella oil used in the study is known to have an eugenol content of 2.28%. Based on research by Kim et al. (2016), eugenol has a bacteriostatic concentration > 0.1% and the bacteriostatic concentration is greater than the concentration to inhibit *Escherichia coli* O157 biofilm formation: H7. The research of Yadav et al. (2015) showed that eugenol can inhibit the formation and eradication of MRSA and MSSA biofilms.

The results of the study can be explained by hydrogen bonds in a mixture that affect the ability to be adsorbed by certain substances. Hydrogen bonds in water-water or alcoholic mixtures are weaker than alcohol-water mixtures (Krishna & van Baten 2010). Biofilm attachment to the substrate is influenced by hydrogen bonds, van der Waals forces and electrostatic forces (Lembre et al 2012).

Javanese citronella oil used in the study was known to contain eugenol 2.28%. The Yadav et al. (2015) showed that eugenol can inhibit and eradicate MRSA and MSSA biofilms. Eugenol at bacteriostatic concentrations (0.01% -0.04%) can significantly cause a decrease in MRSA and MSSA biofilm biomass.

Javanese citronella oil is an essential oil that is naturally hydrophobic and cannot blend with water (Lopez-Romero et al 2015). Based on the results of observations, Javanese citronella oil at bactericidal concentrations was seen visually on the surface of the media, whereas bacteriostatic concentrations could not be observed visually. Javanese citronella oil is known to contain phytochemical components that can change the hydrophobic nature of the bacterial cell wall to be hydrophilic, which is citronellol.

Colony spreading is influenced by teicoic acid on the cell wall surface (Kaito & Sekimizu 2006). Damage to cell walls by citronellol can cause MRSA barriers to colony spreading. Colony spreading on semi-solid media is influenced by cell growth. Cell growth will force outermost cells in the colony to move out and if supported by surfactant production such as PSM

(phenol soluble modulins) will prevent bacteria from being trapped in the media resulting in movement (Pollit et al 2015).

The correlation between bacteriostatic and bactericidal concentrations with an average OD595 value of MRSA biofilm eradication showed a positive correlation that was  $r=0.918$  with  $p=0.000$  which means the higher the concentration, the higher the mean OD595 biofilm eradication and the lower the concentration, the lower the mean OD595 biofilm eradication.

The correlation between bacteriostatic and bactericidal concentrations from Javanese citronella oil with colony spreading showed a value of 1.000 with  $p=0.000$ . The absence of colony spreading in this case was likened to an increase in concentration and the presence of colony spreading was equated with a decrease in concentration. The meaning of the results of the correlation test on colony spreading MRSA was colony spreading MRSA did not occur at bactericidal concentrations and occurs in bacteriostatic concentrations.

## CONCLUSION

Based on the results of the study, there was no positive correlation between bacteriostatic and bactericidal effects of Javanese citronella oil with barriers to MRSA biofilm formation but it was negatively correlated, ie  $r=-0.956$  and  $p=0.000$ . There was a positive correlation between the bacteriostatic and bactericidal effects of Javanese citronella oil with MRSA biofilm eradication of  $r=0.918$  and  $p=0.000$ . There was a positive correlation between bacteriostatic and bactericidal effects of Javanese citronella oil with colony spreading MRSA, ie  $r=1.000$  and  $p=0.000$ .

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