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Antimicrobial Effects of Propolis Ethanol Extract Tetragonula laeviceps Against Bacteria Methicillin Resistant Staphylococcus Aureus

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ABSTRACT
Staphylococcus aureus bacteria, especially Methicillin Resistant Staphylococcus aureus (MRSA), is one of the main causes of antibiotic resistance. MRSA is a bacteria that is resistant to the β-lactam class of penicillin antibiotics. MRSA infections can cause a variety of health problems, including skin infections, pneumonia, and bacteremia. This problem requires alternative resources that can be an option. Propolis from the stingless bee species Tetragonula laeviceps can be a potential alternative for treating MRSA infections. This research is classified as a true experiment with a posttest control group design. The concentration of propolis ethanol extract tested was 20%; 10%; 5%; 2.5%; 1.25%; and 0.625% in the diffusion and dilution methods. These two methods provide assessment output in the form of inhibition zone diameter, minimum inhibition concentration, and minimum bactericidal concentration. The data obtained will be tested statistically using One-way Anova and Fisher's exact tests. Test results show that the ethanol extract of Tetragonula laeviceps propolis has an antimicrobial effect against MRSA bacteria. The diffusion test showed that the average diameter of the inhibition zone formed by the ethanol extract of propolis successively from a concentration of 20% to 1,25% with six repetitions was 12.7 ± 1.23 mm; 11.36 ± 0.67 mm; 10.24 ± 0.61 mm; 7.59 ± 1.69 mm; and 6.5 ± 1.14 mm. From these averages, the One-Way Anova test revealed a statistically significant difference in the diameter of the inhibition zone (F(7,40) = [192.79], p = 0.000).

Introduction

Antibiotics as curative chemotherapy to treat bacterial infections have been used since 1907 until now. The continuous use of antibiotics has an impact in the form of developing the ability of microbial resistance to antibiotics, thereby reducing the effectiveness of antibiotic work (Siahaan et al., 2022). Microbial defense mechanisms

have a high speed of adaptability compared to the development of antibiotic drugs. This event is reflected in the bacterium Staphylococcus aureus. These bacteria were identified as one of the major contributors to the global burden of antibiotic resistance and categorized as a level 3 cause of disease mortality in the Global Health metrics standard (Aslam et al., 2018). This is related to the ability of one strain of Staphylococcus aureus bacteria that produces a cell wall protection protein from penicillin class antibiotics as a result of adaptation to the use of antibiotic drugs, namely MRSA.

Methicillin Resistant Staphylococcus aureus is an S. aureus bacterium defined as resistant to penicillin class narrow-spectrum β-lactam antibiotics with a minimum inhibitory concentration of more than 4 μg/mL (Lakhundi & Zhang, 2018). This pathogen is the cause of skin infections, pneumonia and bacteremia by communal or nosocomial transmission (Monaco et al., 2017). With special modalities such as virulence factors and speed of adaptation of bacteria to antibiotics, the incidence of MRSA bacteremia in Southeast Asia has a percentage of 2.3-69.1% in 2014 (Hassoun et al., 2017). ECDC (2020) also mentions that in 2019 invasive S. aureus infections were 25% MRSA isolates in 7 out of 29 EU countries. The impact of these bacterial infections leads to extended hospitalizations, increased costs of care, and increased mortality. This problem also implies the necessity of the use of antibiotic drugs "Drug of Choice" so that it threatens the sensitivity of the drug to MRSA if used intensively on a massive scale. Thus, there is a potential for the development of bacteria that are more resistant to the antibiotics of choice that are able to effectively overcome bacterial infections. This is an indication for resource management in tackling MRSA threats.

Countermeasures against bacterial threats have been carried out with various approaches both preventive and curative and directed and directed. One alternative treatment of bacterial infections is to use propolis. Propolis is a waxy substance produced by honey bees from extracts of plant parts to protect their hives (Wagh, 2013). Propolis has antioxidant, antibacterial, and immunomodulatory properties that can be applied to humans in extract form (Toreti et al., 2013). This property is an effect of the content of propolis, namely saponins, terpenoids, alkaloids, and polyphenols. (Ahangari et al., 2018). The use of propolis extract as a bacterial inhibitor has been done since ancient Egypt as an embalming material to preserve bodies. In the 12th century, Georgian medical care treatise, karabadini, documented the benefits of propolis in treating oral and pharyngeal infections (Kuropatnicki et al., 2013). The implementation of propolis extract as an antibiotic in modern times leads to a supportive or supplemental role, while conventional antibiotics are still the main line in handling infections.

Currently, agricultural resources in the form of bee cultivation are very massive in Indonesia with 2 species of honey bees, 46 species of stingless bees and 2 species of wasps (Kahono et al., 2018). CBD reports that 48% of the agricultural sector of honey production is dominated by stingless beekeepers or Tetragonula laeviceps (Commission On Genetic Resources for Food and Agriculture, 2018). This species has the largest population distributed in Southeast Asia (Buchori et al., 2022). With this condition, the utilization of Tetragonula laeviceps products, especially propolis is more accessible and researched. This species also has special enzymes that function to extract antimicrobial substances such as alkaloids, saponins, and polyphenols in their saliva (Sanpa et al., 2015).

This study aims to test the antimicrobial properties found in propolis extract of Tetragonula laeviceps species against Methicillin Resistant Staphylococcus aureus. To test its antimicrobial properties, propolis must be altered in extract form. The method of

extraction has an influence on the type of substance that can be isolated. Antimicrobial substances such as terpenoids, saponins, alkaloids, and polyphenols can be easily extracted with ethanol-based solvents so propolis must be converted in the form of Ethanolic Extract of Propolis (EEP) (Grecka et al., 2020). Propolis extract testing was carried out by diffusion and dilution methods to determine the minimum inhibitory rate (KHM), minimal kill rate (KBM), and inhibition zone diameter as indicators of antimicrobial effects on MRSA. Thus, the use of ethanol extract of propolis species Tetragonula laeviceps is expected to be a method of overcoming MRSA infection in the rampant development of microbial antibiotic resistance.

The general objective of this study was to analyze the antimicrobial effect of propolis ethanol extract as a treatment of microbial antibiotic resistance problems.

The theoretical benefit of this study is to provide information related to the antimicrobial effect of ethanol extract of propolis Tetragonula laeviceps against Methicillin Resistant Staphylococcus aureus.

Research methods

This study is a purely experimental study to determine the minimum inhibitory rate (KHM), minimal kill rate (KBM), and inhibitory zone of bee propolis ethanol extract without the sting of Tetragonula laeviceps with a posttest control group design. Experiments were carried out by the method of dilution and diffusion.

This study used a population of Methicillin Resistant Staphylococcus aureus (MRSA) bacterial culture stocks in the Laboratory of the Department of Microbiology, Faculty of Medicine, Universitas Airlangga and Tetragonula laeviceps propolis obtained from Trawas District, Mojokerto Regency, East Java in August 2022.

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Results and Discussions

Experiments were conducted with diffusion and dilution test methods to determine the antimicrobial effect of propolis ethanol extract *Tetragonula laeviceps* against *Methicillin Resistant Staphylococcus aureus bacteria*. Repetition was carried out 6 times for each test method. The dilution test experiment provides measurement results in the form of a minimum kill concentration (KBM) measured from the growth of dilution treatment subcultures. Meanwhile, the diffusion test provides measurement results in the form of an inhibitory zone diameter measured using a digital caliper in millimeters (mm). The results of subculturization of propolis ethanol extract *Tetragonula laeviceps* against *Methicillin Resistant Staphylococcus aureus bacteria* are as follower:

Table 1 Results of subculturization of EEP dilution test of Tetragonula laeviceps against MRSA

Repetisi	Perlakuan							
	EEP 20%	EEP 10%	EEP 5%	EEP 2,5%	EEP 1,25%	EEP 0,625%	Control Positive	Control Negative
1	Not Grow	Grow	Grow	Grow	Grow	Grow	Grow	Not Grow
2	Not Grow	Not Grow	Grow	Grow	Grow	Grow	Grow	Not Grow
3	Not Grow	Grow	Grow	Grow	Grow	Grow	Grow	Not Grow
4	Not Grow	Grow	Grow	Grow	Grow	Grow	Grow	Not Grow
5	Not Grow	Grow	Grow	Grow	Grow	Grow	Grow	Not Grow
6	Not Grow	Grow	Grow	Grow	Grow	Grow	Grow	Not Grow

Results of measuring the diameter of the inhibitory zone of propolis ethanol extract Tetragonula laeviceps against Methicillin Resistant Staphylococcus aureus bacteria as follows.

Table 2 Results of measuring the diameter of the EEP inhibitory zone of Tetragonula laeviceps against MRSA

Treatment	Diameter (mm)						
	1st rep	2nd rep	Reps 3rd	Reps 4th	Reps 5th	Reps 6th	Average
EEP 20%	14,98	13,00	11,90	12,32	11,48	12,55	12,70 ± 1,23
EEP 10%	11,59	12,14	10,49	11,16	10,77	12,04	11,36 ± 0,67
EEP 5%	11,16	10,20	9,26	10,12	10,23	10,47	10,24 ± 0,61
EEP 2,5%	5,02	5,92	8,52	8,43	9,20	8,46	7,59 ± 1,69
EEP 1,25%	4,92	5,48	7, 48	7,78	7,1	6,26	6,50 ± 1,14
EEP 0,625%	0	0	0	0	0	0	-
Vankomisin 30 µg	18,48	15,17	17,68	19,51	19,67	19,78	17,88 ± 1,72
Etil Asetat 5%	0	0	0	0	0	0	-

Analysis

The data obtained in the experiment were processed statistically by analysis of the SPSS program. In processing inhibitory zone diameter data, the first test is a normality test to confirm data taken from populations with normal distribution at certain tolerances. The type of test used is the one sample Kolmogorov-smirnov test. Then, statistical analysis is followed by a levene test to determine the homogeneity of the data. The results of the static analysis showed an asymptomatic significance value of more than 0.05 in both tests so that the data were said to be normally distributed and homogeneously varied. This signifies validation for One-Way ANOVA and post-hoc duncan testing. Data from the dilution test method experiment were processed with Fisher's Exact nonparametric test. The following are the results of statistical processing of dilution test and diffusion test.

Table 3 Kolmogorov-Smirnov One-Sample *normality test* of inhibitory zone diameter

		Diameter of the inhibitory zone
N		48
Normal Parameter[a,b]	Mean	8.2858
	Std. Deviation	5.91191
Most Extreme Differences	Absolute	.169
	Positive	.169
	Negative	-.101
Kolmogorov-Smirnov Z		1.174

Asymp. Sig. (2-tailed)	.127
a. *Test distribution is Normal.*	
b. *Calculated from data.*	

Table 4 Test of homogeneity of Levene diameter of the inhibitory zone

Levene Statistic	*df1*	*df2*	*Sig.*
5.968	7	40	.085

Table 5 Test One-Way ANOVA diameter inhibitory zone

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	1595.270	7	227.896	192.279	.000
Within Groups	47.409	40	1.185		
Total	1642.679	47			

Table 6 Post-hoc test of duncan diameter of inhibitory zone

group. Treatment	**N**	***Subset for alpha = 0.05***				
		Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**
0.625%	6	.0000				
etil asetat 5%	6	.0000				
1.25%	6		6.5033			
2.5%	6		7.5917			
5%	6			10.2400		
10%	6			11.3650		
20%	6				12.7050	
vancomycin 30ug	6					17.8817
Sig.		1.000	.091	.081	1.000	1.000
Means for groups in homogeneous subsets are displayed.						
a. Uses Harmonic Mean Sample Size = 6.000.						

Table 7 Fisher *'s Exact* subculturation test dilution test

	Value	**df**	**Asymptotic Significance (2-sided)**	**Exact Sig. (2-sided)**
Pearson Chi-Square	41.310[a]	7	.000	.000
Likelihood Ratio	34.473	7	.000	.000
Fisher's Exact Test	23.898			.000
N of Valid Cases	48			
a. 8 cells (50.0%) have expected count less than 5. The minimum expected count is .88.				

EEP Inhibitor Zone Diameter *Tetragonula laeviceps* Against MRSA

The bacterial inhibition zone is an area that is not overgrown with bacteria and surrounds antibiotics so that the zone is circular. The measurement of the inhibitory zone is based on the size of the diameter of the area that is not overgrown by bacteria. In the diffusion test of this study, the average diameter of the inhibitory zone *of propolis Tetragonula laeviceps* as in table 5.2, with a concentration of 0.625%; 1.25%; 2.5%; 5%; 10%; and 20% respectively is 0 mm; 6.50 ± 1.14 mm; 7.59 ± 1.69 mm; 10.24 ± 0.61 mm; 11.36 ± 0.67 mm; and 12.70 ± 1.23 mm after a duration of 24 hours of incubation. No

previous studies have examined the antimicrobial effect of propolis *Tetragonula laeviceps* against *Methicillin Resistant Staphylococcus aureus* bacteria by diffusion test method so there is no comparison.

The diameter of the inhibitory zone is highly dependent on the type of antibacterial substance and the type of bacteria tested. In this diffusion test, vancomycin 30 μg is used as a positive control. This antibiotic is a *drug of choice* for handling *Methicillin Resistant Staphyloccocus aureus infection*. With the mechanism of inhibition of bacterial cell wall biosynthesis through the prevention of N-acetylaminic acid and N-acetylglucosamine acid bonds, the mechanism of action of vancomycin is not influenced by PBP-2A which inhibits the action of beta-lactam antibiotics (Patel et al., 2019). This mechanism also affects *strains of Staphylococcus aureus* that are not resistant to penicillin and methicillin. Testing of vancomycin discus 30 μg against MRSA bacteria with the result that the diameter of the inhibitory zone is more than 16 mm can be considered sensitive (Maharjan et al., 2021). However, according to CLSI 2020, the diameter of the inhibitory zone in vancomycin diffusion testing against all *strains of Staphyloccous aureus has the same size range except* Vancomycin Resistant Staphylococcus aureus so that minimum inhibitory testing is needed. The average diameter size of vancomycin inhibitory zone 30 μg against MRSA bacteria is 17.88 ± 1.72 mm.

Diffusion test treatment from 0.0625% to 20% concentration with control in the form of vancomycin 30 μg and 5% ethyl acetate solution, produces an inhibitory zone that must be processed meaningfully. The realization of this is statistical testing using the ANOVA One Way Test with data that has been normally distributed and homogeneously varied. This test aims to determine the significance of differences between several independent groups by comparing the average between groups so that statistical evidence is visible (Ostertagová & Ostertag, 2013). The results of SPSS analysis using the One-Way ANOVA method showed a statistically significant difference in the diameter of the inhibitory zone between at least two treatments ($F(7.40) = [\ 192.79]$, $p = 0.000$). These differences in meaning can be clarified by Duncan's post-hoc test which aims to measure specific differences from the average between treatments. In accordance with table 5.5, the difference in diameter of the inhibitory zone based on treatment is divided into five groups. EEP concentration of 0.065% has no inhibitory zone diameter. This makes the concentration grouped with a negative control, namely 5% ethyl acetate. EEP concentrations of 1.25% and 2.5% were grouped in group 2 with an average diameter of the inhibitory zone that did not differ significantly between the two concentrations, namely 6.5 mm and 7.5 mm. EEP concentrations of 5% and 10% in group 3, having an average diameter of the inhibitory zone were not significantly different when compared. For the treatment of 20% EEP concentration, the diameter of the inhibitory zone was the largest among the EEP concentrations tested with an average of 12.7 mm. However, the diameter of the inhibitory zone produced by the 20% concentration EEP treatment is still far from the effect produced by vancomycin 30 μg with an average diameter of the inhibitory zone produced of 17.8 mm. This indicates that the treatment of vancomycin 30 μg was significantly different from the whole group.

After observation of each treatment, the inhibitory zone produced by the interaction between Propolis Ethanol Extract solution and bacteria began to appear at a concentration of 1.25%. The size of the inhibitory zone also expands according to the increase in concentration which tends to be linear. Control treatment with a function as an indicator of the validity of treatment results that meet expectations, provide indications and comparisons. 5% ethyl acetate as a solvent in this experiment only has the function

of dissolving EEP with water so that it can be easily applied to bacterial culture media and used as a negative control. 5% ethyl acetate does not have antimcrode properties in MRSA bacteria so it was chosen as a solvent (Lens et al., 2016). This is reflected by wells containing 5% ethyl acetate having no inhibition zone. As a comparison indicator, 5% ethyl acetate treatment gives an idea to the results of EEP treatment of 0.625% concentration. The EEP concentration gives a response in the form of no inhibitory zone as 5% ethyl acetate. This positive control in the diffusion test also provides a reference in the comparison of the diameter of the inhibitory zone. Vancomycin as a conventional antibiotic in the treatment of MRSA infection responds to the largest average diameter of the inhibitory zone and indicates MRSA's sensitivity to the antibotic. Propolis ethanol extract can respond in the form of the emergence of the diameter of the inhibitory zone according to the increase in concentration. However, the diameter of the resulting inhibitory zone has not reached the size of a conventional antibiotic, namely vancomycin.

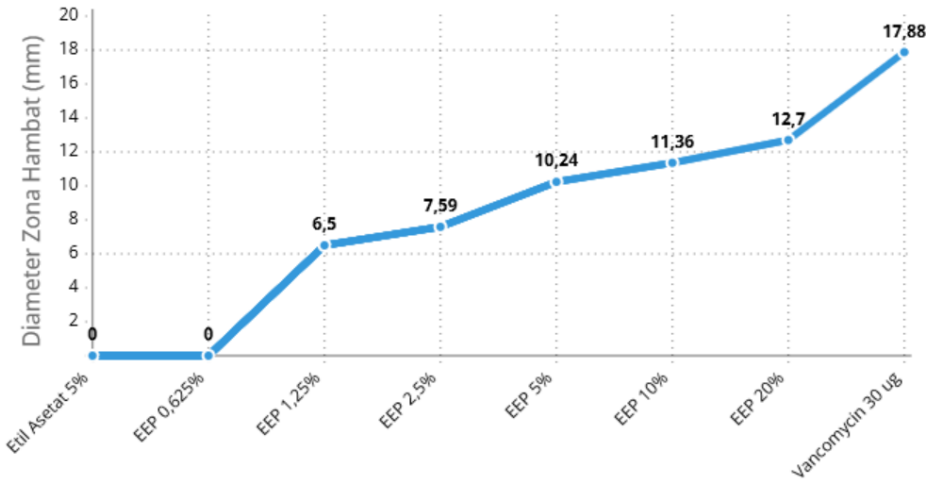

Figure 1 EEP Concentration Against MRSA Bacterial Inhibitory Zone Diameter

Minimal Kill Rate EEP *Tetragonula laeviceps* Against MRSA

Propolis ethanol extract was tested by dilution method serialized from 20% concentration to 0.625% concentration. The propolis content in each successive concentration of concentration was 2 mg/ml; 1 mg/ml; 0.5 mg/ml; 0.25 mg/ml; 0.125 mg/ml; and 0.0625 mg/ml. This serial dilution test provides measurement outputs in the form of minimal inhibitory and minimal kill rates. The degree of minimal inhibition can be visually assessed through the comparison of turbidity with the control treatment. For minimal inhibition, it is necessary to subculture the specimen on agar media. In this test, each concentration of propolis ethanol extract with a volume of 1 ml and given a nutrient broth containing inoculents of 1 ml with a standard of 0.5 mcfarland. After incubating for 24 hours, visually, each tube could not be assessed for turbidity because the characteristics of the dark-colored extract followed the size of the content. It can be seen in figure 6.1, The color gradation of the solution in the tube starts from dark brown to light yellow respectively from the tube EEP concentration of 20% to 0.625%. This causes the minimal inhibitory level cannot be measured.

The dilution test is followed by measuring the minimum inhibitory level. Subculturization is carried out on all tubes with transfer to agar nutrition media. After 24 hours of incubation and 6 six repetitions, the results were obtained as in the picture. Subculture results showed no bacterial growth at 20% EEP treatment. Meanwhile, treatment with a lower concentration of 10%-0.625% there was bacterial growth except for the 10% EEP treatment in the second rep. Bacterial growth from a concentration of

10% to a concentration of 0.625% has a gradation pattern. The 10% concentration had the least MRSA colony growth among the EEP concentrations tested and followed by a 5% concentration with MRSA colony growth still visually calculated. For the treatment of EEP concentration 2.5%; 1,25%; and 0.625%, the growth of MRSA bacterial colonies is very large and can only be distinguished by the density of colonies grown in the media. This can be observed especially in the treatment subculture of 0.625% EEP concentration which is visually comparable with positive controls. Comparisons were also made to the treatment subcultures of 20% EEP concentration and negative control. The comparison results show a similarity in output in the form of no bacterial colony growth. This indicates that the minimum kill rate of Propolis Ethanol Extract is 2 mg / ml or 20% concentration. According to research by (Sanpa et al., 2015), the minimum kill rate found in the dilution test against MRSA bacteria is 16-128 mg / ml. The difference in minimal suicide rates can be caused by regionally different propolis content and extraction methods using 70% ethanol. The study used 96% ethanol.

The results of serial dilution test observations were carried out statistical testing in a non-paramteric manner. The type of test used is Fisher*'s Exact Test*. This test was conducted to determine the independence between EEP concentration and subculture bacterial growth (Kim, 2017). The requirement to use the fisher exact test is that it is valid to find an expected frequency of less than 5 in *cells* of more than 20%. In accordance with table 5.6, the number *of cells* that have an expected frequency of less than 5 is 50% so the fisher's exact test is performed with. The test results showed that there was a significant relationship between EEP concentration and the growth of subculture bacteria with a significance value of less than 0.05 (2-tailed P = 0.000).

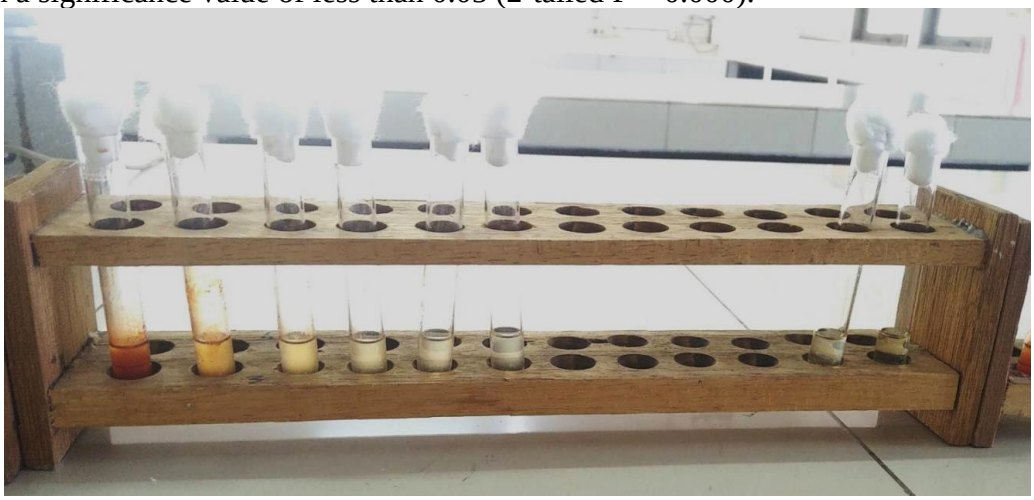

Figure 2 Dilution Test Tube After 24-Hour Incubation

Conclusion

Referring to the formulation of the problem and discussion of data, this experimental study can be concluded that the minimum inhibitory level of ethanol extract of propolis Tetragonula laeviceps against Methicillin Resistant Staphylococcus aureus bacteria cannot be determined because the characteristics of the extract are graded according to the concentration level.

The minimum kill rate of propolis ethanol extract Tetragonula laeviceps against Methicillin Resistant Staphylococcus aureus bacteria is 2 mg / ml or EEP concentration 20%

Ethanol extract of propolis tetragonula laeviceps produced an inhibitory zone at a concentration of 20%; 10%; 5%; 2,5%; and 1.25% with the most significant average diameter of the inhibitory zone owned by EEP concentration of 20%, which is 12.70 ± 1.23 mm.

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