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Research Article

Growth Inhibition Effect of *Syzygium aromaticum* Ethanol Extract on *Methicillin-Resistant Staphylococcus aureus*

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ABSTRACT

Background: MRSA is the leading cause of death caused by antimicrobial resistance. The distribution of MRSA occurs globally, and the incidence rate is around 30 per 100,000 people per year. Cloves had antimicrobial properties that were tested in both resistant and susceptible clones. **Purpose**: To determine the growth inhibition effect of clove flower ethanol extract on the growth of Methicillin-Susceptible *Staphylococcus aureus* (MSSA) and Methicillin-Resistant *Staphylococcus aureus* (MRSA). **Methods**: This research uses an experimental design. Clove extract will be analyzed using gas mass chromatography-spectrometry (GC-MS). MSSA isolate was taken from *S.aureus* ATCC 25913, and MRSA was taken from nasal swabs. The concentrations of the ethanol extract of cloves used are 20%, 40%, 60%, 80%, and 100%. All ethanol extract concentrations from cloves will be tested using the Kirby-Bauer disc diffusion technique. The magnitude of the barrier zone determines the antibacterial properties. **Results**: The results of the GC-MS analysis showed three main antibacterial compounds, including eugenol, phenol, and caryophyllene. The average inhibitory zones of the antibacterial test at concentrations of 20%, 40%, 60%, 80%, and 100% clove flower ethanol extract against MSSA were 16 mm, 16.33 mm, 17.67 mm, 18.33 mm, and 18.33 mm, respectively. The results of the average calculation of the inhibition zone in MRSA according to each concentration of clove flower ethanol extract were 11 mm, 12 mm, 13 mm, 14.33 mm, and 15.67 mm. **Conclusion**: Cloves have antibacterial properties, as confirmed by studies showing a zero zone in the MSSA and MRSA antibacterial sensitivity test, with the best concentration being 100% ethanol extract. The study's results show that the antibacterial effect of cloves affects MSSA more than MRSA.

Keywords: *clove flower ethanol extract, Methicillin-Resistant Staphylococcus aureus (MRSA), Methicillin-Susceptible Staphylococcus aureus (MSSA)*

INTRODUCTION

Staphylococcus aureus (S. aureus) is a bacterium that can cause oputurnistic infections. This bacterium is one of the organisms that are resistant to many antibiotics (1). One form of resistance of *S.aureus* to antimicrobials is *Methicillin-Resistant Staphylococcus aureus* (MRSA). MRSA is one of the main causes of infections in hospitals (2).

Over the past two decades MRSA has become an 'epidemic' in many hospitals around the world (3). MRSA is the leading cause of death caused by antimicrobial resistance. The

distribution of MRSA occurs globally and the incidence rate is around 30 per 100,000 people per year (4). *S. aureus* bacteremia has an incidence rate of between 20 to 50 cases/100,000 people per year, with 10% to 30% of patients dying (5). In the latest study in 2017, the number of deaths per year due to *S. aureus* bacteremia in the United States was reported to be 20,000 cases of *S. aureus* bacteremia is known to cause a greater number of deaths compared to immune deficiency syndrome (AIDS), tuberculosis and viral hepatitis (6).

In the treatment of resistant bacteria, first-line drugs are often replaced with second-line drugs which of course are more expensive and difficult to obtain (7). Therefore, it is necessary to provide alternative treatment using natural ingredients to be more efficient, effective and safe in inhibiting and killing the growth of *Staphylococcus aureus* bacteria. One of the natural ingredients that can be used is cloves. Cloves (*Syzygium aromaticum*) are spices that originated in Indonesia and have been used as traditional medicine for centuries by people (8).

Cloves have an essential oil content (8). Clove essential oil has phenolic compounds such as flavonoids, eugenol, hydroxybenzoic acid, hydroxycinnamic acid, eugenol and hydroxyphenyl propyl (9). This plant has biological activity as an antiseptic, analgesic, antifungal, antibacterial, antioxidant, and anticarcinogenic (10).

The eugenol contained in cloves is hydrophobic, it is this hydrophobic property that allows it to penetrate the lipopolysaccharides on the plasma membrane of bacteria and modify the cell structure, thus causing the infiltration of intracellular components (11). In general, the antimicrobial effectiveness of a clove can be influenced by a number of factors, such as the characteristics of the targeted microorganism, temperature, pH, concentration of antimicrobial substances and the presence of other ingredients. In the study of Maggini et al, it was observed that the antimicrobial activity of cloves was twenty times higher at a temperature of 37°C. This is because the fluidity of the membrane lipid layer is affected by temperature so that at higher temperatures the cell membrane function is impaired and permeability will increase which results in cells becoming more susceptible to antimicrobial compounds (12).

In the research, Sofia et al. (13), conducted antimicrobial activity tests of various Indian spice plants. From the results of the study, the only sample that showed bactericidal effects on gram-positive and gram-negative bacteria was clove water extract of 3% (9). Alanzi et al., conducted a study on the effect of antibacterial activity of clove extract on MRSA that infects wounds in rats. As a result of this study, rat wounds treated with clove extract showed faster healing and reduced the number of microbes in the wound, compared to rat wounds that were not treated (1).

The many benefits of cloves in the health sector, one of which is as an antimicrobial, make cloves one of the plants that can be used as an antimicrobial alternative. Based on previous research, given the research mentioned above, the use of products of natural origin as antimicrobial is proven disease control, resulting in the development of models that make sustainable production systems and the conservation of biodiversity and natural resources feasible. Thus, this study aims to study the antibacterial properties of clove flower ethanol extract by conducting disc diffusion tests and using different concentrations from the previous study.

METHODS

Clove flower extraction

The clove ethanol extract used in this study was obtained from the results of previous research (unpublished). Clove ethanol extract is processed by maceration technique. Fresh flowers that have been picked will be washed with clean running water, and dried. The drying process is carried out in an open space that is not exposed to direct sunlight. Fresh flowers that were initially yellowish-green will turn brown after drying, and the size of the flowers will shrink. Furthermore, it is crushed by pounding to form a dry powder. Dry powder from cloves as much as 1 kg will be mixed with 96% ethanol and form a solution of 1 liter. This solution will be macerated for 72 hours at room temperature (28-30ºC). Next, the extract is filtered and the filtrate is collected which then the residue is re-macerated with 96% ethanol for 72 hours at room temperature until the filtrate is colorless (unpublished).

Gas Chromatography-Mass Spectrometry (GC-MS)

GCMS in this study was used to analyze the chemical compounds and the concentration of ethanol extract of cloves used. The GCMS in this study was analyzed by Agilant which followed the previous research procedure. The type of compound present in the ethanol extract of cloves was determined by gas chromatography combined with the Shimadzu QP5000 mass spectrometer (Shimadzu, Kyoto, Japan), equipped with a ZB-5ms (5% phenyl arilene) capillary column 95% dimethylpolylocane, with an HP 5MS 70 eV (40-500 Da) electronic impact detector and a transfer temperature of 280°C. Aliquot is injected in splitless mode with a volume of 0.3 μL in ethyl acetate (CP-8410 automatic injector), by establishing the following conditions: high-purity helium as a carrier gas; the injector temperature is maintained at 280°C, split mode (1: 10); followed by an initial temperature of 40°C and a final temperature of 300°C, a start time of 5 minutes and a final time of 7.5 minutes at 8/minute (14).

Bacterial isolate

Methicillin-Susceptible Staphylococcus aureus (MSSA) in this study used *Staphylococcus aureus* Isolate ATCC 25913. While Methicillin-Resistant Staphylococcus aureus (MRSA) uses specimens taken directly through nasal swabs, MRSA in this study was obtained from previous research (unpublished). These two bacteria will be made into an isolate suspension by adding NacL 0.9% and achieving a Farland turbidity of 0.5.

Antimicrobial sensitivity test

The sensitivity test was performed using the Kirby Bauer disc diffusion method. The sterile disc will be soaked in clove flower ethanol extract with concentrations of 20%, 40%, 60%, 80% and 100% for 30 minutes and then it will be put into the Mueller Hinton Agar (MHA) media which was previously applied with a bacterial suspension with mac farland 0.5 turbidity using sterile cotton, the application is carried out by a three-sided technique. This test used tetracycline as the positive control and the blank disk as the negative control .MHA will be incubated for 24 hours at a temperature of 37◦C, followed by an inhibition zone that can be identified and included in the antibacterial category based on research Cimanga et al (14).

RESULTS

Chemical content in clove flower ethanol extract

Based on the results of the GC-MS analysis (table 1), 7 compounds were detected and 9 peaks were seen. There are three compounds that are elevated and have antibacterial activity, namely eugenol with a retention time of 23.3 minutes and a concentration of 100% followed by phenol (retention time of 27.6 minutes and concentration of 11.51%) and caryophyllene (retention time of 24.9 minutes and concentration of 3.48%).

Table 1. GC-MS analysis results

Peak	Component name	Retensio time (menit)	Formula	Molecular weight (g/mol)	Peak area (%)
1	Caryophyllene	22.7	$C_{15}H_{24}$	204.35	1.56
2	Eugenol	23.3	$C_{10}H_{12}O_2$	164.2	100
3	Bicyclo[7.2.0]undec-4 ene, 4,11,11-trimethyl 8-methylene-,[1R (1R*,4Z,9S*)]	24.6	$C_{15}H_{24}$	204.35	1.64
4	Caryophyllene	24.9	$C_{15}H_{24}$	204.35	3.48
5	Bicyclo[7.2.0]undec-4 ene, 4,11,11-trimethyl 8-methylene-,[1R (1R*,4Z,9S*)]	25.8	$C_{15}H_{24}$	204.35	1.21
6	Naphthalene	27.5	$C_{15}H_{24}$	204.35	2.25
7	Phenol, 2-methoxy-4 (1-propenyl)-, acetate	27.6	$C_{12}H_{14}O_4$	206.24	11.51
8	2’, 3’,4’ Trimethoxy acetophenone	31.4	$C_{11}H_{14}O_4$	210.23	1.37
9	Cyclotetrasiloxane, octamethyl	54.7	$C_8H_{12}O_4Si_4$	296.61	1.09

Antimicrobial activity

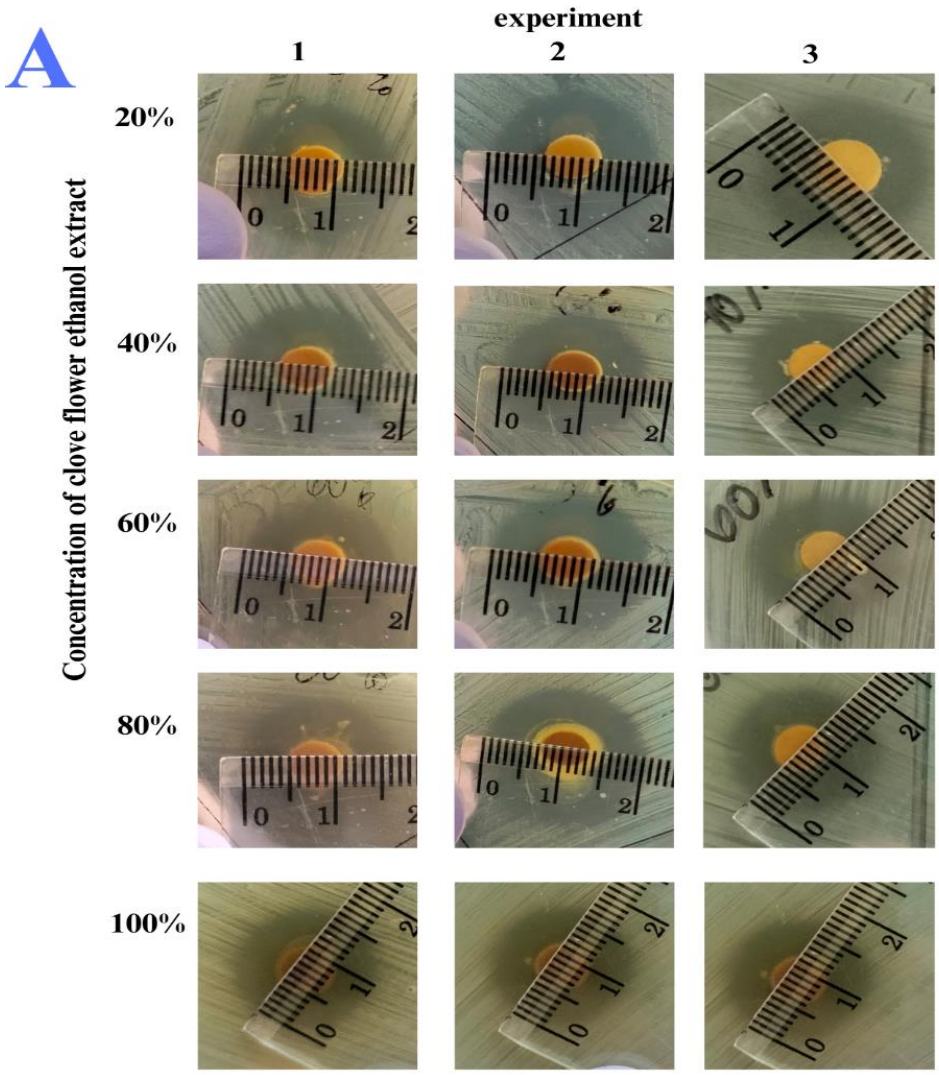
Figure 1 shows that there are inhibition zones from antibacterial sensitivity tests that prove that cloves can affect the growth of MSSA and MRSA. In all concentrations, there were inhibition zones in the antibacterial sensitivity test of clove flower ethanol extract to MSSA and MRSA. The measurement of the inhibition zone was measured using a ruler which then looked at the average of the inhibition zone in each sample tested.

The results of data analysis were obtained from the average and standard deviation described in table 2. The effect of clove flower ethanol extract with a concentration of 20% on MSSA was obtained with an average inhibition zone of 16 mm with a standard deviation of 1.00. At a concentration of 40% clove flower ethanol extract, an average inhibition zone of 16.33 mm was obtained with a standard deviation of 1.15. At a concentration of 60% clove flower ethanol extract, an average inhibition zone of 17.67 mm and a standard deviation of

0.57 were obtained. In the concentration of 80% clove flower ethanol extract, the average value of the inhibition zone was 18.33 mm with a standard deviation of 1.52. At 100% concentration of clove ethanol extract, an average inhibition zone of 18.33 mm was obtained with a standard deviation of 0.57 which is the strongest concentration in inhibiting *S. aureus*.

Table 2. Average and *S.aureus* inhibition zone deviation

Bacterial Names	Average ± *S.aureus* inhibition zone deviation based on the concentration of ethanol extract of cloves				
	20%	40%	60%	80%	100%
MSSA	16±1.00	16.33±1.15	17.67±0.57	18.33±1.52	18.33±0.57
MRSA	11±1.00	12±1.00	13±1.00	14.33±1.15	15.67±0.57



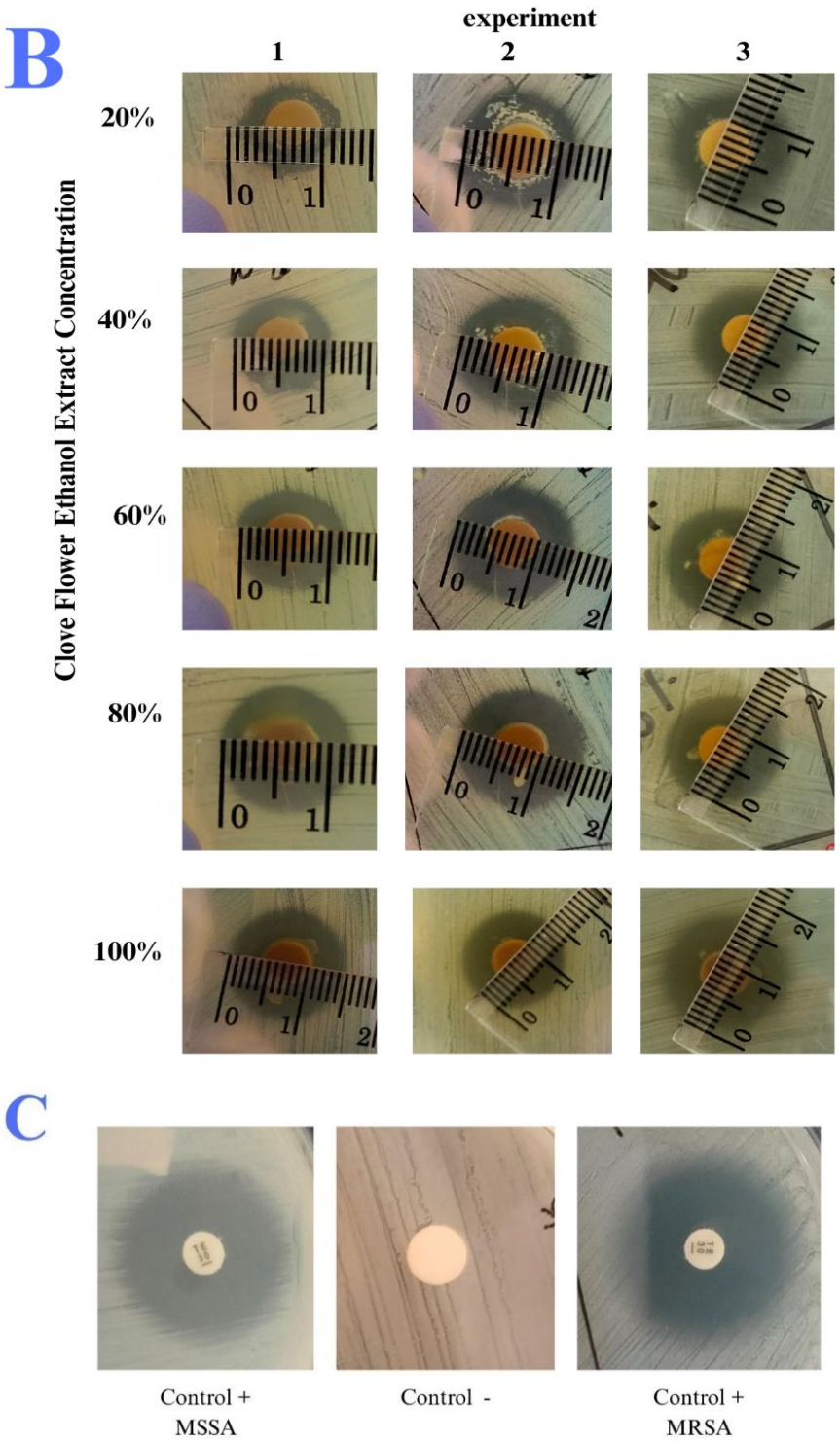


Figure 1. Results of antibacterial tests of clove flower ethanol extract in MSSA and MRSA. (A) he inhibition zone was the result of the antibacterial test of the concentration of ethanol extract of cloves of 20%, 40%, 60%, 80% and 100% on MSSA with a three-time test repeat method. (B) Inhibition zones resulting from antibacterial tests of clove flower ethanol extract with concentrations of 20%, 40%, 60%, 80% and 100% on MRSA with a three-time test repeat method**.** (C) Positive control of MSSA and MRSA with tetracycline antibiotics as well as negative control using blank disks

Cloves can inhibit the growth of MRSA, this is proven based on the results of the calculation of the inhibition zone from the antibacterial sensitivity test by the disc diffusion method. At a concentration of 20% clove flower ethanol extract, the average inhibition zone against MRSA was obtained of 11 mm with a standard deviation of 1.00. At a concentration of 40% clove flower ethanol extract, an average inhibition zone of 12 mm was obtained with a standard deviation of 1.00. At a concentration of 60% clove flower ethanol extract, an average inhibition zone of 13 mm was obtained with a standard deviation of 1.00. At a concentration of 80% clove flower ethanol extract, an average inhibition zone of 14.33 mm was obtained with a standard deviation of 1.15. Meanwhile, at a concentration of 100% clove flower ethanol extract, the highest average inhibition zone was obtained which was around 15.67 mm with a standard deviation of 0.57.

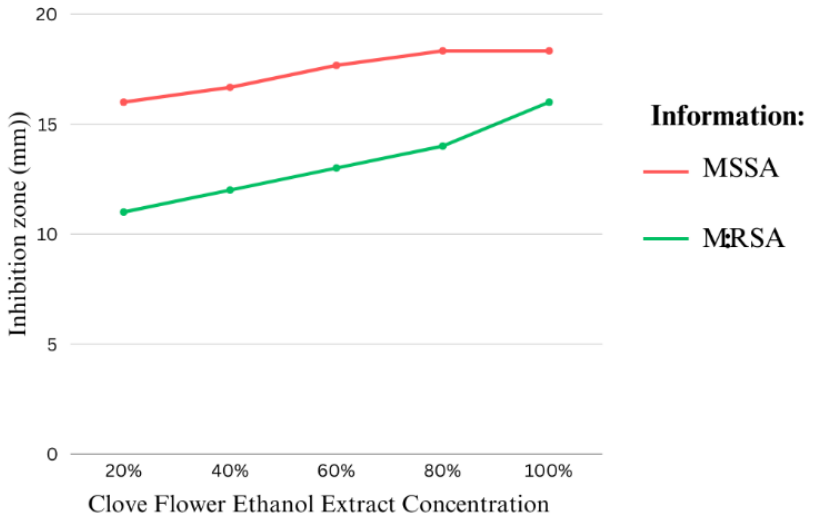


Figure 2. Diagram of the average line of inhibition zones of antibacterial test results in MSSA and MRSA

Based on Figure 2, it shows the average difference in the inhibition zones of *Methicillin-Susceptible Staphylococcus aureus* (MSSA) and *Methicillin-Resistant Staphylococcus aureus* (MRSA) presented in the form of a line diagram. MSSA has a larger inhibition zone compared to MRSA.

DISCUSSION

In this study, GC-MS was used to analyze the chemical content of ethanol extract of cloves flowers, the number of compounds detected in GC-MS was seen based on the number of molecules that arise, the more molecules that arise, the higher the concentration of the chemical. From the results of the analysis of the ethanol content of clove extract using the GC-MS method, there were seven compounds detected and three of them had antibacterial activity. The most dominant compound and has antibacterial activity is eugenol with a concentration of 100% The most dominant compound with antibacterial activity is eugenol with a concentration of 100% followed by phenol 11.51% and Caryophyllene 3.48%. Eugenol is identified as 4-allyl-2 methoxyphenol which has characteristics in the form of a slightly yellow color, oily

texture, and has a pungent aroma (15). Previous studies have shown that the antibacterial mechanism of eugenol in clove extract causes plasma membrane disruption of microorganisms. Eugenol's hydrophobic nature allows it to penetrate lipopolysaccharides in the plasma membrane of bacteria so that death can occur in the bacteria (11).

The second compound that has the highest content in clove flower ethanol extract is phenol with a concentration of 11.51%. Phenolic compounds are metabolites derived from plant sources (plants, vegetables and fruits) that play an important role in growth, reproduction, and as a form of self-protection in plants. One of phenol derivatives, flavons, has a function as an antibacterial (16). Next is caryophyllene which occupies the third highest position in the composition of clove flower ethanol extract with a concentration of 3.48%. Caryophyllene has ethanol-soluble properties but is insoluble in water. The function of caryophyllene is as an anticancer, antioxidant and antimicrobial. In a study conducted by Ullah et al., it was shown that the administration of caryophyllene can inhibit the growth of microbes found in nutritious foods (17).

The research conducted by Teles et al, examined the antimicrobial activity of clove extract using the disc diffusion method. This study showed the results that cloves can inhibit the growth of gram-positive and gram-negative bacteria. The highest inhibition zone of clove extract in gram-positive bacteria is 25 mm, and in gram-negative bacteria is 22 mm (14). Another study on the effect of clove extract on microbes in meat shows that cloves can inhibit the growth of bacteria that colonize raw meat (18).

A compound that has a great effect on inhibiting the growth of bacteria in cloves is eugenol. Eugenol is the largest composition in cloves, this can be proven from the results of the analysis of the extract with GCMS in this study. The results of the GCMS show that eugenol has the highest concentration compared to other compounds. The lipophilic properties of eugenol are an advantage that can kill bacteria. With its lipophilic nature, eugenol easily penetrates the plasma membrane of bacteria which will result in lysis or death of the bacteria. The lowest antibacterial concentration based on the results of GCMS analysis in this study is Caryoophyllene with a concentration of 3.48% which is the lowest concentration compared to eugenol and phenol.

The results of the data processing carried out showed that there was a real difference between the concentration of clove flower ethanol extract of 20%, 40%, 60%, 80% and 100%. This study showed that clove flower ethanol extract could inhibit the growth of *Methicillin-Susceptible Staphylococcus aureus* (MSSA) *and Methicillin-Resistant Staphylococcus aureus* (MRSA) bacteria at all concentrations of clove flower ethanol extract tested during this study. Chimaga et al., made a classification of antibacterial properties based on their inhibition zones, namely <10 mm of weak antibacterial, 10-15 mm of medium antibacterial, and >15 mm of strong antibacterial (14).

Tetracycline was decided to be used as a positive control in this study, this was decided based on Kimberly et al's research on antibiotics that are sensitive to MRSA. Of the antibiotics tested, only three were sensitive to MRSA, including Linezolid, rimethoprim, sulfamethoxazole and tetracycline (19). In this study, tetracycline was used because it is easy to obtain compared to Linezolid and Trimethoprim sulfamethoxazole. The results of the

antibacterial sensitivity test showed the presence of an inhibitory zone around the tetracycline disc which proved that tetracycline was positive against MSSA and MRSA.

In this study, the inhibition of clove flower ethanol extract with a concentration of 20% against MSSA was obtained with an average inhibition zone of about 16 mm which was included in the category of strong antibacterial based on Chimanga et al. In clove flower ethanol extract, the concentration of 40% inhibition against MSSA was obtained with an average inhibition zone of 16.33 mm, which was categorized as a strong antibacterial based on Chimanga et al. In clove flower ethanol extract, the concentration of 60% inhibition against MSSA was obtained with an average inhibition zone of 17.67 mm, which is included in the category of strong antibacterial based on Chimanga et al. Meanwhile, in clove flower ethanol extract, the concentration of 80% and 100% inhibitory power against MSSA was obtained with an average of the same inhibition zone of 18.33 mm, which is the largest inhibition zone and is included in the category of strong antibacterial based on Chimanga et al.

Clove flower ethanol extract has an inhibitory effect on the growth of MRSA which can be seen in the results of this study. At a concentration of 20% clove ethanol extract, the inhibition against MRSA growth was obtained with an average inhibition zone of 11 mm, which was included in the category of moderate antibacterial based on Chimanga et al. At a concentration of 40% ethanol extract of clove flowers, the inhibitory power against the growth of MRSA was obtained with an average inhibition zone of 12 mm, which is included in the medium antibacterial compound of Chimanga et al. At a concentration of 60% clove flower ethanol extract, the inhibitory power against the growth of MRSA was obtained with an average inhibition zone of 13 mm, which is included in the category of moderate antibacterial based on Chimanga et al. At a concentration of 80% clove ethanol extract, the inhibitory power against the growth of MRSA was obtained with an average inhibition zone of 14.33 mm, which was categorized as moderately antibacterial based on Chimanga et al. Furthermore, at a concentration of 100% clove ethanol extract, the inhibition against MRSA was obtained with an average inhibition zone of 15.67 mm, which is included in the strong antibacterial based on Chimanga et al.

Salnus and Islawati conducted research on the effect of clove flower extract on the growth of *S. aureus*. The lowest concentration of clove flower ethanol extract used is 20% and the highest is 100%, from the results of this study the smallest inhibition zone was obtained as 17.5 mm and the largest was 24.5 mm (20). So far there has been no research proving that cloves cannot inhibit the growth of MSSA and MRSA bacteria.

Based on the results of this study, it is proven that cloves can inhibit the growth of MSSA and MRSA, this is based on the existence of an inhibition zone in the antibacterial sensitivity test of ethanol extract of cloves with concentrations of 20%, 40%, 60%, and 100% against MSSA and MRSA. This study shows that the greater the concentration of clove flower ethanol extract, the greater the resistance to bacteria. The concentration that has the best inhibitory power is in clove flower ethanol extract with a concentration of 100%.

The study also showed that there was a difference in the average barrier zone in MSSA and MRSA (Figure 2) even though they were from the same species and used the same concentration of clove flower ethanol extract. In MSSA, the results of the inhibition zone of all concentrations of ethanol extract of cloves in this study were included in the category of strong

antibacterial, with the average of the smallest inhibition zone being 16 mm and the largest being 18.33 mm. Meanwhile, MRSA, which is categorized as a strong antibacterial, is only at a concentration of 100% ethanol extract of cloves flowers, with an average of the smallest inhibition zone of 11 mm and the largest 15.67 mm. From these results, it can be seen that there is a significant difference between the effect of clove flower ethanol extract on the growth of MSSA and MRSA. Clove extract is more potent at inhibiting the growth of MSSA compared to MRSA, this can be influenced by genetic changes in MRSA that make self-protection against antibakery compounds in cloves stronger (21). Ower antibacterial sensitivity of cloves to MRSA compared to MSSA, likely because MRSA has antibiotic resistance genes carried by mobile genetic elements (MGEs) (22). This antibiotic re gensitene makes MRSA resistant to the most antibiotics of the Beta-Lactam group and even other antibiotics, this may be one of the factors that make the antibacterial from cloves less sensitive to MRSA.

Although cloves have been shown to have antibacterial activity, cloves still cannot replace antibiotics made specifically to kill MSSA and MRSA, because antibiotics are already in drug form and have passed clinical trials while clove extract is still in herbal form still in preclinical trials and has not been directly tested in humans. There are many shortcomings in this study, such as not using the Minimum Inhibitory Concentration (MIC) test so that it is still not possible to determine the minimum concentration of clove ethanol extract that can inhibit MSSA and MRSA. The sample used to conduct this study also still needs to be improved. Therefore, there may be differences in research results if a MIC test is carried out and the research sample is added or even replaced with animals, so it is expected that there will be further research on this.

CONCLUSION

There are three compounds in cloves that have antibacterial activity, including eugenol, phenol and Caryophyllene. Clove flower ethanol extract can have antibacterial activity against *Methicillin-Susceptible Staphylococcus aureus* (MSSA) and *Methicillin-Resistant Staphylococcus aureus* (MRSA). The antibacterial properties of cloves were confirmed from the results of antibacterial sensitivity tests which showed the presence of inhibition zones in MSSA and MRSA. In MSSA, all concentrations of clove flower ethanol extract were antibacterial is strong, with an average of the smallest inhibition zone of 16 mm and the largest 18.33 mm. Meanwhile, in MRSA, clove flower ethane extract which is antibacterial is strong at only 100% concentration, with an average of the smallest inhibition zone of 11 mm and the largest 15.67 mm. From this study, there is a difference in the antibacterial effect of cloves on MSSA and MRSA, where the antibacterial properties of cloves are stronger in preventing the growth of MSSA than MRSA, this may be the influence of MRSA which carries the antibacterial resistance gene so that there is more protection in antibacterial materials.

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CONFLICT OF INTEREST

The author has no conflict of interest and has no affiliation with or relationship with any organization or entity that may give rise to biased questions or statements in the discussion and conclusion sections of this paper.

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