ORIGINAL RESEARCH REPORT

In Vitro Antibacterial Activity of Eucalyptus (*Melaleuca leucadendra*) Oil against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Background: Eucalyptus (*Melaleuca leucadendra*) oil is used by Indonesians as an herbal medicine. Eucalyptus containing 1,8-cineol at 72.30% is expected to be used as an antibacterial. **Objective:** The study aimed to assess the antibacterial activity of eucalyptus oil against Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria in vitro. **Material and Method:** The materials used were eucalyptus oil (*M. leucadendra*) in various concentrations with ethyl acetate as solvent. The research method used was the agar-well diffusion assay. The MRSA was suspended to 0.5 McFarland turbidity. The MRSA suspension was thoroughly swabbed onto the surface of the Mueller-Hinton agar plate. The wells were made with a diameter of 0.6 mm on Muller-Hinton agar aseptically and 100 µl of eucalyptus oil was put into the well using a micropipette and incubated for 24 hours at 37°C. The diameter of the inhibition zone was measured with a caliper. Statistical analysis using the SPSS software edition 23. **Result:** Eucalyptus oil solution starting from a concentration of 10% to 100% had an antibacterial response, which could be seen by the formation of a bacterial inhibition zone around the eucalyptus oil (*M. leucadendra*) wells. The inhibition ability of eucalyptus oil against MRSA bacteria was greatest at a concentration of 90% with an inhibition zone of 31.26 mm. It was found that the concentration of eucalyptus oil affected the diameter of the bacterial inhibition area. **Conclusion:** Eucalyptus oil has been shown to have antibacterial activity against MRSA and was influenced by the concentration of the oil.

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Highlights

1. The use of eucalyptus oil is natural and risk-free. It has a lengthy history of use as a traditional medicine in Asia, including Indonesia.
2. It has been demonstrated that eucalyptus oil possesses antibacterial activity against MRSA, and this activity was controlled by the oil's concentration.

BACKGROUND

Staphylococcus aureus is a Gram-positive bacterium that is part of the Staphylococcus group. It has a spherical shape and is usually arranged in grape-like irregular clusters. *S. aureus* is catalase- and coagulase-positive which differentiates it from other Staphylococci species (Riedel, et al., 2019). *S. aureus* can cause opportunistic infections if it enters sterile sites in body tissues or the bloodstream because of trauma or abrasion. Staphylococci are non-motile and non-spore-forming organisms (Tille, 2017). *S. aureus* is a part of the normal flora of the skin and mucous membranes of both humans and animals. It is one of the most clinically important Staphylococci bacteria because of its high frequency of infection. *S. aureus* is able to cause a wide range of diseases in humans, starting from superficial skin lesions to potentially life-threatening septicemia (Pal, et al., 2020; Guo, et al., 2020; Pollitt, et al., 2018). Infections caused by this species include scalded skin syndrome (toxin-mediated): Ritter disease, pemphigus neonatorum; toxic shock syndrome; food poisoning; localized skin infections: folliculitis, furuncles, and carbuncles, impetigo, tissue, and systemic infections: wounds, bacteremia; endocarditis; osteomyelitis; cerebritis; and pyelonephritis (Tille, 2017).

Inappropriate use of antibiotics can lead to resistance in bacteria. To kill bacteria, antibiotics need to pass through the surface of the bacteria and attach to structures or biochemical mechanisms necessary for bacteria to multiply or survive (Bautista-Silva, et al., 2020). Beta-lactam antibiotics inhibit bacterial cells by binding to penicillin-binding protein (PBP), which functions in bacterial cell wall synthesis (da Costa, et al., 2018).

S. aureus can become resistant to methicillin antibiotics and other beta-lactam antibiotics through the expression of an altered penicillin-binding protein (PBP) with decreased affinity for most semisynthetic penicillin, namely PBP2a, which causes it to become resistant to methicillin (Lakhundi & Zhang, 2018). Methicillin-resistant *Staphylococcus aureus* (MRSA) is divided into three types: health-care-associated MRSA (HA-MRSA) originating from patients who are in hospitals or other health services; community-acquired MRSA (CA-MRSA) originating from healthy individuals with no history of going to health services; and livestock-associated MRSA (LA-MRSA) originating from livestock (Trisnawati, et al., 2020; Boswihi, et al., 2020). In the past, MRSA was mostly related to health care but now it has expanded to community and livestock. This shows that MRSA bacterial colonization events are becoming increasingly worrisome. The increasing number of MRSA bacteria in various countries, including Indonesia, indicates that it is necessary to find new antibiotics, which can be developed from various types of herbal plants. One alternative is eucalyptus oil (*Melaleuca leucocadendra*) (Hidayah, et al., 2019; Silva, et al., 2022).

M. leucadendra is part of the Myrtaceae family. The alternate name of *M. leucadendra* is *M. cajuputi* subsp. cajuputi. The leaves and branches of the tree are harvested because they contain 1,8-cineol, which is an important essential oil. The leaves and branches are distilled to produce eucalyptus oil. Eucalyptus oil is natural and safe to use. It has been used for a long time as a traditional medicine in Asian countries, including Indonesia. The leaves of *M. leucadendra* possess antibacterial, anti-inflammatory, and anodyne properties and are used traditionally against pain, burns, colds, influenza, and dyspepsia (Rimbawanto, et al., 2021). *M. leucadendra* essential oil contains a major component which is the 1,8-cineol. GC-MS analysis of eucalyptus leaf oil extraction from *M. leucadendra* clone 71 showed that besides 1,8-cineol, eucalyptus oil also contained α-terpineol, D-limonene, terpinene-4-ol, β-pinene, aromandendrene, α-pinene, δ-terpineol, caryophyllene, camphene, β-springene, β-cymene, α-selinene, β-selinene, humulene, γ-terpinene, β-thujene, and linalool (Monzote, et al., 2020; Ismanto, 2018). α-Terpineol, D-limonene, and terpinene-4-ol also have antimicrobial activity that targets the bacterial cell wall membrane and can cause cell death (Han, et al., 2019; Cordeiro, et al., 2020; Huang, et al., 2021). 1,8-cineol has anti-inflammatory and anti-bacterial properties that are effective against human pathogenic bacteria. 1,8-cineol increases membrane permeability, which

causes leakage of proteins and nucleic acids. In addition, 1,8-cineol can also trigger oxidative stress, which causes disruption of the bacterial membrane through lipid peroxidation and leakage of intracellular materials and consequently causes cell death (Moo, et al., 2021).

"Sendang Arum" is a small and medium enterprises (SMEs) in Lamongan, Indonesia, that produced eucalyptus oil which had been subjected to a gas chromatography test carried out by the Perhutani Laboratory in Surabaya, Indonesia. It was found that the oil had a high concentration of 1.8-cineol, namely 72.3%. Therefore, it is important to carry out tests to prove the antibacterial effect of "Sendang Arum" SMEs eucalyptus oil on MRSA, which is expected to have potential as an alternative treatment, so that it can be used as a solution to overcome the problem of antibiotic resistance. In this context, we evaluated the antibacterial effect of eucalyptus oil (*M. leucadendra*) against MRSA with an agar-well diffusion assay.

OBJECTIVE

This study aimed to study the antibacterial activity of eucalyptus oil (*Melaleuca leucadendra*) against Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria in vitro.

MATERIAL AND METHOD

The method used in this research was an agar-well diffusion assay. Agar-well diffusion assay is a method by which the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 0.6 mm is punched aseptically with a sterile cork borer or a tip, and a volume (100 µL) of the antimicrobial agent or extract solution at the desired concentration is introduced into the well. The media used in this study is Mueller-Hinton (OXOID CM0405, Basingstoke, Hampshire, England). This research was an experimental study with a post-test control group design. The research was conducted in February-June 2022 at the Microbiology Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia.

Preparation of Mueller-Hinton Media

As much as 38 grams of Muller-Hinton Agar (OXOID CM0337, Basingstoke, Hampshire, England) media was weight and then put into an Erlenmeyer and add 1 liter of sterile distilled water was added, and then was heated to boiling to dissolve the medium. The Erlenmeyer was covered with aluminum foil and sterilized with an autoclave (TOMY SX-500, TOMY KOGYO CO., LTD. 3-14-17 Tagara, Nerima-ku, Tokyo 179-0073, Japan) at 121˚C for 15 minutes. After that, the medium was cooled to a lukewarm temperature (45-50˚C). The liquid was poured into the petri dish and it was left to solidify in a flat position.

Cultivation of MRSA bacteria

MRSA bacteria from existing stocks were cultured in petri dishes with Mueller-Hinton Agar media that had solidified, then they were incubated at 37˚C for 24 hours in an incubator.

Preparation of eucalyptus oil (*M. leucadendra)*

The eucalyptus oil (*M. leucadendra*) sample used in this study was taken from the “Sendang Arum” Eucalyptus Oil Small and Medium-sized Enterprises (SMEs) in Candisari Village, Sambeng District, Lamongan Regency, East Java Province, Indonesia (7°18'55.2"S 112°18'00.7"E). The concentrations of eucalyptus oil (*M. leucadendra*) used were 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. The solvent used to make the concentration series was ethyl acetate (109623 Catalog Number: 205-500-4, Merck, Darmstadt, Germany). Ethyl acetate had also been tested for its sterility and antibacterial effects. The results showed that ethyl acetate was sterile and did not have any bacterial effects.

Eucalyptus oil contamination test (*M. leucadendra)*

A contamination test was carried out to ensure that eucalyptus oil (*M. leucadendra*) was not contaminated by bacteria so that the research results were valid. The test was carried out by stroking

eucalyptus (*M. leucadendra*) oil on Mueller-Hinton agar medium and then incubating it at 37°C for 24 hours. In this study, the results showed that the eucalyptus (*M. leucadendra*) oil samples used were sterile.

Preparation of MRSA bacterial suspension

MRSA bacteria were cultured on Mueller-Hinton's medium and incubated at 37°C for 24 hours. The bacterial inoculum was taken from the incubated MRSA culture and then put into Mueller-Hinton broth. MRSA suspension was made up to 0.5 McFarland turbidity. The inoculum standard of 10^8 CFU ml^{-1} was used for diffusion.

Experimental stage

The antibacterial activity test method used in this study was diffusion to determine the diameter of the inhibition zone against MRSA bacteria in vitro. This experiment consisted of two controls (K+ and K-) and ten treatments (P1-P10). The positive control (K+) used in this study was the antibiotics trimethoprim-sulfamethoxazole (TMP/SMX) (Catalog Number CT0052B, OXOID, Basingstoke, Hampshire, England), which is an alternative to vancomycin antibiotics for the treatment of severe *S. aureus* infections. TMP/SMX works by inhibiting the synthesis of folic acid, a cofactor in amino acid and nucleotide synthesis (Vestergaard, et al., 2019). In vitro, TMP/SMX appears to have the most potent bactericidal activity against MRSA compared with linezolid, rifampin, clindamycin, vancomycin, and minocycline, thus making it a suitable substitute for vancomycin (Lewis, et al., 2018). The negative control (K-) used in this study was ethyl acetate. Ethyl acetate is able to dissolve eucalyptus oil and had been proven to have no antibacterial activity in prior testing. The ten treatments (P1-P10) used eucalyptus oil (*M. leucadendra*) from "Sendang Arum" SMEs in concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% with three replications.

The MRSA bacterial suspension was taken using a swab and swabbed on a petri dish. Then, the wells were made and the samples were inserted into each well with different concentrations, and incubated at 37˚C for 24 hours.

The inhibition zone formed is the area around the wells that is not overgrown with bacteria. The inhibition zone formed was measured using a digital caliper in mm. Data from the results of this experiment, i.e., the diameter of the MRSA bacterial inhibition zone, were analyzed using IBM SPSS statistics for Windows, Version 23.0 (Armonk, NY: IBM). The data normality test used Shapiro-Wilk statistical analysis, the homogeneity test used Levene statistical analysis, and the significance test used Kruskal-Wallis statistical analysis.

RESULT

A diffusion test was conducted to determine the antibacterial activity of eucalyptus oil (*M. leucadendra*) against MRSA. From the diffusion test, the measurement results obtained are the diameter of the inhibition zone. The results of measuring the diameter of the inhibition zone of eucalyptus oil (*M. leucadendra*) on the growth of MRSA can be seen in Table 1.

Table 1. Results of inhibition zone diameters of various concentrations of eucalyptus (*M. leucadendra*) oil against MRSA bacteria.

Treatment	Diameter (mm)			
	R1	R2	R3	Mean (SD)
K+	36.65	33.77	33.52	34.65 ± 1.74
K-	0	0	0	0
P1	14.06	10.63	10.78	11.82 ± 1.94
P2	16.27	12.49	11.94	13.57 ± 2.36
P3	13.18	11.31	15.22	13.24 ± 1.96
P4	13.66	13.13	16.94	14.58 ± 2.06
P5	15.15	12.83	13.45	13.81 ± 1.20
P6	28.28	19.17	19.78	22.41 ± 5.09

P7	25.57	21.15	23.39	23.37 ± 2.21
P8	35.34	31.07	20.31	28.91 ± 7.75
P9	41.20	28.33	24.26	31.26 ± 8.84
P10	29.56	26.99	36.55	31.03 ± 4.95

The obtained data were analyzed statistically using the SPSS 23 program. First, the test performed was the Shapiro-Wilk normality test. The significance value obtained for all the MRSA bacteria groups was α >0.05, so it can be said that the data were normally distributed. The next test was the homogeneity test. The data showed a significance value of 0.001 for MRSA bacteria. Because the value obtained was α <0.05, the data were not homogeneous.

Table 2. Mann-Whitney test on inhibition zone diameters of various concentrations of eucalyptus (*M. leucadendra*) oil against MRSA bacteria.

Treatment	K+	K-	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
K+	-											
K-	0.037	-										
P1	0.050	0.037*	-									
P2	0.050	0.037*	0.275	-								
P3	0.050	0.037*	0.275	0.827	-							
P4	0.050	0.037*	0.275	0.275	0.513	-						
P5	0.050	0.037*	0.275	0.513	0.827	0.513	-					
P6	0.050	0.037*	0.050	0.050	0.050	0.050	0.050	-				
P7	0.257	0.037*	0.050	0.050	0.050	0.050	0.050	0.513	-			
P8	0.500	0.037*	0.050	0.050	0.050	0.050	0.050	0.127	0.513	-		
P9	0.513	0.037*	0.050	0.050	0.050	0.050	0.050	0.127	0.127	0.827	-	
P10	0.275	0.037*	0.050	0.050	0.050	0.050	0.050	0.127	0.050	0.827	0.827	-

Because the data were normally distributed but not homogeneous, a Kruskal Wallis non-parametric test was carried out. The Kruskal-Wallis test showed an asymptotic significance value of 0.001 for MRSA. Because the significance value was <0.05, it means that the concentration of eucalyptus oil had an influence on the diameter of the bacterial inhibition area. Then, the Mann-Whitney test was carried out, and the negative control was obtained compared to the positive control. All treatments (P1-P10) were found to have asymp. sig. <0.05, so there was a significant difference, while the positive control and all treatments (P1-P10) had asymp. sig. >0.05, so there was no significant difference between the positive control and the eucalyptus (*M. leucadendra*) oil treatment (P1-P10).

Table 3. Diameter of inhibitory TMP/SMX based on The Clinical & Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2021).

Antibiotics	Staphylococcus type	Category interpretation and zone diameter limits		
		Sensitive	Intermediate	Resistant
Trimethoprim-sulfamethoxazole	All Staphylococci	>16	11-15	<10

Treatments P1 to P5 had an inhibition zone diameter of 11-15 mm which means that MRSA was intermediate to eucalyptus oil (*M. leucadendra*) with a concentration of 10-50%. For treatments P6 to P10 or concentrations of 60% to 100%, the diameter of the eucalyptus oil inhibition zone against MRSA was more than 16 mm, which means that eucalyptus oil can be interpreted as sensitive to MRSA at concentrations of 60%-100%.

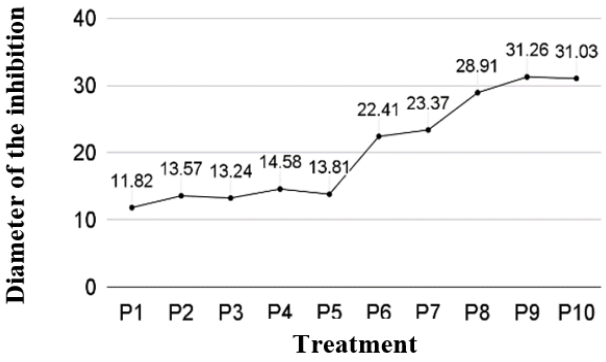

Figure 1. Diameter of the inhibition zone for various concentrations of eucalyptus (*M. leucadendra*) oil against MRSA bacteria.

From the results of the SPSS analysis, the data were not homogeneous, so the Kruskal Wallis test was carried out. The results of the SPSS analysis using the Kruskal-Wallis method on MRSA bacteria showed a significance value of <0.05, which means that the concentration of eucalyptus (*M. leucadendra*) oil influences the diameter of the inhibition of the bacteria. Mann-Whitney follow-up analysis showed no significant difference between TMP/SMX antibiotics and eucalyptus (*M. leucadendra*) oil at a concentration of 10-100%.

DISCUSSION

The eucalyptus oil (*M. leucadendra*) that was used comes from eucalyptus trees of the species *M. leucadendra* clone 71, which grows in Candisari Village, Sambeng District, Lamongan Regency, East Java Province, Indonesia. Eucalyptus oil is obtained by distilling the leaves and branches of the eucalyptus plant. “Sendang Arum” Small and Medium-sized Enterprises (SMEs) perform traditional distillation to get eucalyptus oil, which is a product of these SMEs. Eucalyptus oil, which is used in commercial business, is usually an oil that has a high 1,8-cineol content (Rimbawanto, et al., 2021).

The Perhutani Surabaya Indonesia Laboratory, using gas chromatography, found that the eucalyptus of "Sendang Arum" SMEs had 72.3% of the compound 1,8-cineol. The cineol content is higher than Hakim's research, which has a cineol component of 26.28% (Hakim, et al., 2019). Apart from 1,8-cineol, other compounds are contained in eucalyptus oil (*M. leucadendra*) based on the results of the GC-MS analysis of clone 71 conducted by other studies, α-terpineol, D-limonene, terpinene-4-ol, β-pinene, aromandendrene, α-pinene, δ-terpineol, caryophyllene, camphene, β-springene, β-cymene, α-selinene, β-selinene, humulene, γ-terpinene, β-thujene, and linalool (Ismanto, 2018). 1,8-cineol has anti-inflammatory and anti-bacterial properties that are effective against MRSA. 1,8-cineol increases membrane permeability, which causes leakage of proteins and nucleic acids. In addition, 1,8-cineol can also trigger oxidative stress, which causes disruption of the bacterial membrane through lipid peroxidation and leakage of intracellular materials, and consequently causes cell death (Moo, et al., 2021). α-Terpineol increases the permeability of the bacterial cell membrane (Huang, et al., 2021). D-Limonene has an effect on cell membrane permeability by attacking cell integrity and cell wall structure in Gram-positive bacteria. (Han, et al., 2019). Terpinene-4-ol is bactericidal against *S. aureus*, namely by interfering with bacterial cell wall synthesis, PBP2a becomes a target for anti-biofilm activity and is able to inhibit biofilm formation even at sub-inhibitor concentrations (Han, et al., 2019). All the actions of essential oils contribute to the inhibition and cell death of MRSA.

In the preliminary test, ethyl acetate solvent could not dissolve SMEs eucalyptus oil and bacterial suspension, so the researchers did not use the dilution method for this study. When eucalyptus oil (*M. leucadendra*) was poured into the wells on Mueller Hinton's medium, it was highly likely that some would evaporate, which would affect the amount of inhibition formed against MRSA bacteria.

The bacterial inhibition zone is the circular area around the antibiotic in which bacteria do not grow. The zone of inhibition can be used to measure the sensitivity of a bacterium to an antibiotic. The diameter of the inhibition zone is influenced by the type of antibiotic and bacteria used. Based on The

Clinical & Laboratory Standards Institute, all types of *Staphylococcus* sp. are said to be sensitive if they have a diameter of >16 cm (Clinical and Laboratory Standards Institute, 2021). Eucalyptus oil in this study had the inhibition power shown in Table 1, the concentrations of 50%, 60%, 70%, 80%, 90%, and 100% were 13.81 mm; 22.41 mm; 23.37 mm; 28.91 mm; 31.26 mm; and 31.03 mm. The diameter of the inhibition zone at a concentration of 60% - 100% was found to be larger compared to previous studies. In the research conducted by Hakim, et al., (2019), the diameter of the eucalyptus oil inhibition zone against MRSA at eucalyptus oil concentrations of 50%, 60%, 70%, 80%, 90%, and 100% successively was 17.2 mm; 18.1 mm; 19.1 mm; 19.4 mm; 19.7 mm; and 20.1 mm (Hakim, et al., 2019).

The antibiotics used in this study were TMP/SMX, which is an alternative to vancomycin antibiotics for the treatment of severe *S. aureus* infections. TMP/SMX works by inhibiting the synthesis of folic acid, a cofactor in amino acid and nucleotide synthesis (Vestergaard, et al., 2019). In vitro, TMP/SMX appears to have the most potent bactericidal activity against MRSA compared with linezolid, rifampin, clindamycin, vancomycin, and minocycline, thus making it a suitable substitute for vancomycin (Lewis, et al., 2018). The following are the interpretation categories and diameter limits of the TMP/SMX inhibition zone. Eucalyptus oil solution starting from a concentration of 10% to 100% has an antibacterial response, which can be seen by the formation of a bacterial inhibition zone around the eucalyptus oil (*M. leucadendra*) wells. There was an increase in the diameter of the inhibition zone with increasing concentration, but not at all concentrations. At a concentration of 50%, there is a decrease in the inhibition of MRSA bacteria. This is different from previous research by Hakim, et al., (2019) which showed that the increase in concentration was proportional to the increase in the diameter of the MRSA bacterial inhibition zone. There are many factors that affect the diameter of the bacterial inhibition zone. Differences can occur due to different types of antibiotics, so the inhibition of eucalyptus oil (*M. leucadendra*) used will have different antibacterial potential against various types of antibiotics (Hakim, et al., 2019).

Strength and limitations

This study can contribute data for future studies, especially in Eucalyptus (*Melaleuca leucadendra*) Oil and Methicillin-Resistant *Staphylococcus aureus* (MRSA). This research has limitations because it is difficult to dissolve eucalyptus oil into the medium of Mueller Hinton Agar. Further research should use other media that can be absorbed well or use the paper disk method.

CONCLUSION

The diameter of the eucalyptus oil inhibition zone can be said to be equivalent to the size of TMP/SMX antibiotics so there is a possibility that eucalyptus oil can replace TMP/SMX antibiotics because it has the same good inhibitory power. It is suggested to conduct a toxicity test on eucalyptus oil.

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Conflict of Interest

All authors have no conflict of interest.

Ethic Consideration

The research protocol has been declared ethically feasible by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga (No. 44/EC/KEPK/FKUA/2022) issued on 14-02-2022.

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This study does not receive any funding.

Author Contribution

CLT contributes to conception and design, analysis and interpretation of the data, drafting of the article, and final approval of the article. WR contributes to conception and design, critical revision of the article for important intellectual content and final approval of the article. MFQ contributes to final approval of the article. MRW contributes to final approval of the article.

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