Antibacterial Effects of Eucalyptus Oil (Melaleuca leucadendra) Made in Lamongan against Escherichia coli Bacteria In vitro Study

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Abstract

Diarrhea is a health problem that occurs throughout the world, in Indonesia the prevalence of diarrhea is 11%. Diarrhea is one of the food borne diseases. The most common bacteria that causes diarrhea is Escherichia coli. Diarrhea can be prevented by disinfection using 70% alcohol hand sanitizer, but this material often causes irritation and itching, so alternative materials were needed. One of the potential ingredients is eucalyptus oil (Melaleuca leucadendra) made in Lamongan, because it contains 1,8-Cineol compounds. This study aimed to analyze the antibacterial potential of eucalyptus oil as a solution to health problems. This study was a true experimental study with a posttest-only control group design. Sampling used a simple random sampling technique. Data were obtained by measuring the diameter of the inhibition zone using the diffusion method antibacterial test. The data would be processed using IBM SPSS Statistics 23 software. The results of the measurement of the diameter of the inhibition zone in positive control (31.53 ± 0.6 mm), negative control (0.00 ± 0.00 mm) 20% concentration (19.00 ± 1.17 mm), 30% concentration (21.53 ± 1.33 mm), 40% concentration (24.08 ± 0.28 mm), and 50% concentration (24.76 ± 1.56 mm). Based on the One-Way ANOVA test, it was found that there was a significant difference between the control group and the treatment group (p = 0.000). Based on the Pearson test, it was found that there was a very strong relationship (r = 0.888), positive (r = 0.888) and significant (p = 0.000) between various concentrations of eucalyptus oil (Melaleuca leucadendra) made in Lamongan against Escherichia coli bacteria. There was a significant antibacterial effect of eucalyptus oil (Melaleuca leucadendra) made in Lamongan against Escherichia coli bacteria.

Keywords: antibacterial, eucalyptus oil, Melaleuca leucadendra, Escherichia coli

1. Introduction

Diarrhea is a symptom in the form of defecation with a frequency of 3 times in 24 hours with liquid or shapeless stools [1]. Based on data from WHO and UNICEF every year there are about 2 million cases of diarrhea worldwide. Based on the Basic Health Research 2018 data in Indonesia, the prevalence of diarrhea is 11%, especially in East Java the prevalence is 9.9% [2].

Diarrhea is one of the food borne diseases [3]. The most common bacteria that causes diarrhea is Escherichia coli (15%-20%) [3]. One way to prevent diarrhea is to disinfect hands using a hand sanitizer with 70% alcohol content. However, this type of hand sanitizer can have a negative impact on the skin such as causing irritation and dry skin [4]. In addition, it is proved that most essential oil-based hand sanitizers do not cause effects such as itching and do not cause stinging effects [5]. Therefore, it is necessary to develop hand sanitizers made from essential oils as an alternative to alcohol-based hand sanitizers.

One ingredient that has potential for antibacterial effect is eucalyptus oil. One region in Indonesia that produces eucalyptus oil is Lamongan which is not yet well known in the community. Therefore, the researchers chose eucalyptus oil made in Lamongan which was extracted from the Melaleuca leucadendra plant so that this product was better known to the public. Eucalyptus oil contains the bioactive substance Eucalyptol or 1,8-Cineol which is proven to have various benefits such as anti-inflammatory, antibacterial, and antiviral [6].

Previously there have been studies examining the antibacterial effect of eucalyptus oil, such as in one study at a concentration of 10% there was an inhibition zone against S. aureus 8.3±0.3 mm and 7.1±0.3 mm against Escherichia coli [7]. The results of another study showed that the antibacterial activity of eucalyptus oil was low against Staphylococcus aureus and Escherichia coli bacteria [8]. Therefore, this study aims to provide a third opinion about the antibacterial effect of eucalyptus oil (Melaleuca leucadendra) against Escherichia coli bacteria because there are still 2 contradictory research results.

2. Methods

This study is a true experimental study with a posttest only control group design. The independent variable used in this study was the eucalyptus oil (Melaleuca leucadendra) made in Lamongan, Indonesia with concentrations of 20%, 30%, 40%, and 50%. The dependent variable is the antibacterial effect against Escherichia coli bacteria which in the diffusion method is the diameter of the inhibition zone.

2.1 Sample Preparation

The sample used in this study was a stock culture of Escherichia coli in the Microbiology Laboratory of the Faculty of Medicine, Airlangga University with inclusion criteria, the sample was a homogeneous colony of Escherichia coli bacteria whose species had been identified and then selected by simple random sampling. The sample size in this study was determined using Arifin's formula and obtained 4 repetitions for 4 types of treatment so that the total sample is 16 samples [9].

2.2 Eucalyptus oil preparation

The eucalyptus oil used in this study was obtained from steam distillation of the Melaleuca leucadendra plant made in Lamongan, Indonesia. The stems and leaves of the Melaleuca leucadendra plant are put into a high pressure and temperature distillation furnace, then the steam from the distillation furnace is condensed so that the results are oil and water [10]. The oil obtained was then made into concentrations of 20%, 30%, 40%, and 50% in the following way: (1) Prepare 6 test tubes measuring 13 x 100 mm then labeled with numbers 1 to 6, (2) Tube number 1 is filled with 10 ml of 100% eucalyptus oil (without dilution), (3) Tube number 2 is filled with 10 ml of 100% Ethyl Acetate, (4) Mix 800 µl of liquid in tube number 2 and 200 µl of liquid in tube number 1 using a micropipette then put into tube number 3 and then homogenized with a vortex to get a final concentration of 20%, (5) Mix 700 µl of liquid in tube number 2 and 300 µl of liquid in tube number 1 using a micropipette then put into tube number 4 and then homogenized with a vortex to get a final concentration of 30%, (6) Mix 600 µl of liquid in tube number 2 and 400 µl of liquid in tube number 1 using a micropipette then put into tube number 5 and then homogenized with a vortex to get a final concentration of 40%. (7) Mix 500 µl of liquid in tube number 2 and 500 µl of liquid in tube number 1 using a micropipette then put into tube number 6 and then homogenized with a vortex to get a final concentration of 50%.

2.3 Medium culture preparation

In this study, there were two culture media used, namely Nutrient Broth and Mueller Hinton Agar. The Nutrient Broth medium in this study was made in the following way: (1) Mixed 13 g Nutrient Broth with 1 L sterile distilled water into an Erlenmeyer tube, (2) The mixture in the Erlenmeyer tube is then homogenized, (3) The Erlenmeyer tube is heated on a hot plate until it boils, (4) The mixture in the Erlenmeyer tube is then sterilized by placing it in an autoclave at 121ºC and for 15 minutes. Then, the making of Mueller Hinton Agar in this study was made in the following way: (1) Mixed 28 g Mueller Hinton Agar with 1 L sterile distilled water into an Erlenmeyer tube, (2) The mixture in the Erlenmeyer tube is then homogenized, (3) The Erlenmeyer tube is heated on a hot plate until it boils, (4) The mixture in the Erlenmeyer tube is then sterilized by placing it in an autoclave at 121ºC and for 15 minutes, (5) After cooling, the mixture in the Erlenmeyer tube is poured into a petri dish.

2.4 Preparation of bacterial suspension

The preparation of bacterial suspension was in the following way: (1) Implanting bacteria from culture stock into EMB so that they are then incubated at 37ºC for 24 hours, (2) Take the colonies of Escherichia coli bacteria with ose and then put them into Nutrient Broth, (3) The bacterial suspension was then homogenized with a vortex at room temperature, (4) Equalize the bacterial suspension to 0.5 Mc. Farland (1.5 × 108 cfu/ml).

2.5 Antibacterial effect test

The antibacterial effect test in this study used the diffusion method in the following way: (1) Prepare Mueller Hinton Agar in a petri dish, (2) Prepare a disc of Sulfamethoxazole-Trimethoprim (1.25 µg) as a positive control and 100% Ethyl Acetate as a negative control, (3) Prepare a standardized suspension of Escherichia coli bacteria with 0.5 Mc. Farland (1.5 × 108 cfu/ml), (4) Dip a sterilized cotton swab into the bacterial liquid culture, (5) Wipe a cotton swab over the entire surface of the Mueller Hinton Agar, repeat twice while rotating the plate 60º, (6) Leave the petri dish for 3-5 minutes at room temperature, the petri dish should not be left for more than 5 minutes so that the bacteria are completely dry before applying the antibiotic disc, (7) Prepare 5 wells with a diameter of 6 mm, (8) Drop 100 µl of 100% Ethyl Acetate into one of the wells, (9) Drop 100 µl of eucalyptus oil with different concentrations (20%, 30%, 40%, and 50%) in 4 other wells, (10) Take the Sulfamethoxazole-Trimethoprim disc with tweezers, then place the antibiotic disc on the surface of the agar medium with a little pressure, so that it can adhere completely, (11) Steps number 1 - 10 were repeated 4 times, (12) Incubate at 37ºC for 24 hours.

2.6 Inhibition zone diameter measurement

The Inhibition zone diameter was measured in this following way: (1) Place the petri dish on a flat and dark surface, (2) Look at the petri dish vertically with the naked eye, (3) Measure the diameter of the inhibition zone using a calliper, (4) Measure the diameter from edge to edge of the clear zone without obvious bacterial growth, (5) Record the measurement results to the nearest millimetre.

3. Results

3.1 Eucalyptus Oil Quality

The eucalyptus oil used in this study was analysed for its quality by the PERHUTANI Laboratory based on the Indonesian National Standard No. 3954 of 2014 and it can be concluded that the eucalyptus oil (Melaleuca leucadendra) made in Lamongan, Indonesia meets Indonesian National Standard and have super quality because it meets 6 of 7 criteria (Table 1). Optical rotation measurement cannot be performed because the instrument is faulty.

Table 1. Quality analysis of eucalyptus oil (Melaleuca leucadendra) made in Lamongan, Indonesia.

Parameter	Indonesian National Standard No. 3954:2014	Result	Measuring Device
Specific gravity in 25ºC (g/ml)	0.900-0.930	0.911	Pycnometer
Refractive index in 20ºC	1.450-1.470	1.465	Refractometer
Optical rotation (º)	-4º until 0º	-	Polarimeter
Solubility in ethanol 80%	Clear	Clear	Ethanol 80%
1,8-Cineol concentration (%)	Super:> 60% Major: 55%-60% Primary: 50%-<55%	72,30	Gas Chromatography
Colour	Colourless, Greenish/Yellowish and Clear	Clear and Greenish	Visual
Odor	Typical Eucalyptus odor	Typical Eucalyptus odor	Sense of smell

3.2 Inhibition Zone Diameter

The antibacterial result used in the diffusion method by measuring the diameter of the inhibition zone, which is a transparent area where there is no bacterial growth measured using a calliper (Fig 1). In the control group, the inhibition zone could be seen in the well of the positive control and not in the well of the negative. In the treatment group the inhibition zone could be seen in all treatment wells (20%, 30%, 40%, and 50%), the inhibition zone began to be seen at a concentration of 20%. All treatments were repeated four times and then the diameter of the inhibition zone was averaged (mean) (Table 2). Based on these mean data, the treatment with 50% eucalyptus oil concentration had the largest diameter of the inhibition zone (24.76 ± 1.56 mm) compared to other concentrations, although it was still smaller than the positive control (31.53 ± 0.6 mm).

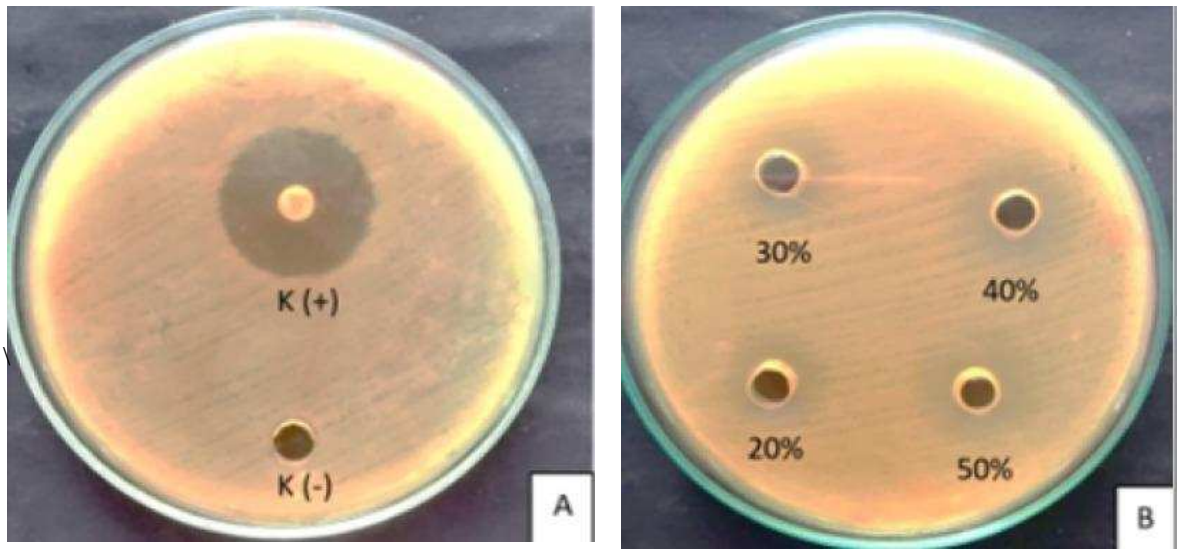

Fig 1. The diameter of the inhibition zone of eucalyptus oil against Escherichia coli.

Note: A : Control group plate
K (+) : Sulfamethoxazole-Trimethoprim 1.25 µg
K (-) : Ethyl Acetate 100%
B : Treatment group plate

Table 2. The average value of the inhibition zone diameter of the antibacterial activity of eucalyptus oil against Escherichia coli bacteria.

Group	1st Repetition (mm)	2nd Repetition (mm)	3rd Repetition (mm)	4th Repetition (mm)	Mean + STD (mm)
Positive Control	32.35	31.02	31.15	31.61	31.53 ± 0.6 [a]
Negative Control	0.00	0.00	0.00	0.00	0.00 ± 0.00 [a]
20%	17.68	19.92	20.06	18.37	19.00 ± 1.17 [a]
30%	19.80	21.37	22.98	21.97	21.53 ± 1.33 [a]
40%	24.02	24.13	23.75	24.42	24.08 ± 0.28 [a,b]
50%	24.76	26.68	24.75	22.85	24.76 ± 1.56 [a,b]

Note: a: There is a significant difference
b: There is no significant difference

3.3 Normality Test and Homogeneity Test

Homogeneity tests and normality tests need to be carried out to determine whether the data obtained can be carried out by using parametric or non-parametric statistic. The normality test used is the Shapiro-Wilk test because the amount of data obtained is less than 50 [11]. Based on the results of the normality test for all variables, p value > 0.05, which mean the data obtained are normally distributed [11]. The negative control was not tested for normality test because the data obtained was constant. The homogeneity test used is the Lavene test. By comparing the data of the control group and the treatment group, the obtained p value was 0.309 (> 0.05) which mean there is no difference in variance between groups of data being compared so it can be concluded that the data is taken from a homogeneous population [11].

3.4 Comparison Test and Correlation Test

Based on the statistical tests that have been carried out, it is found that the research data is normal and homogeneous so that it meets the criteria for parametric statistical tests. Furthermore, the parametric test to be carried out is a comparison test and a correlation test. The parametric comparison test used in this study is the One-Way ANOVA test and the Post-Hoc Test. From the results of the One-Way ANOVA test that has been carried out in this study, the p value was 0.000 (<0.05) which can be concluded that there is a significant difference in the mean between data groups [12]. However, to find out the mean difference between variables more specifically, another comparative test is needed, namely the Post-Hoc Test. From the results of the Post-Hoc Test analysis that has been carried out, it is found that the mean difference is significantly in all data groups (p value <0.05) except for the mean comparison between groups of 40% and 50% concentration variables, there is no significant difference in mean (p value > 0.05) [12].

The next parametric test carried out is the Pearson correlation test. Based on the results of the analysis that has been carried out (Table 6), the Pearson correlation value (r) is 0.888 (> 0.8) which can be concluded that there is a very strong correlation between the concentration of eucalyptus oil and the diameter of the inhibition zone. In addition, (r) value is 0.888 mean the type of correlation that exists is a positive correlation, so it can be concluded that the higher the concentration of eucalyptus oil, the larger the diameter of the inhibition zone formed. In addition, the p value obtained is 0.000 (<0.05), which means that there is a significant correlation between the concentration of eucalyptus oil and the diameter of the inhibition zone [11].

4. Discussion

Eucalyptus oil is an essential oil obtained by distilling Melaleuca leucadendra plants. Eucalyptus oil distilled using the water distillation method (boiled) did not meet Indonesian National Standard, while the water steam distillation method and the steam method met Indonesian National Standard for eucalyptus oil. However, the level of 1,8-Cineol concentration was the highest in water distillation (61.39%) compared to water steam distillation (55.67%) and steam distillation (54.71%) [13]. Different results were found in this study, using the steam distillation method, the eucalyptus oil which was obtained in addition to meeting Indonesian National Standard for eucalyptus oil, it's also contained higher levels of 1,8-Cineol, namely 72.30%, which meant eucalyptus oil used in this study was of super quality.

In another study an analysis of eucalyptus oil (Melaleuca cajuputi) made in Singkawang, West Kalimantan, Indonesia. It was found that 9 bioactive compounds in eucalyptus oil (Melaleuca cajuputi), the highest concentration compound was 1,8-Cineol (71.96%) [14]. In this study 10 compound was found in eucalyptus oil (Melaleuca leucadendra), the highest concentration compound was 1,8-Cineol (72.30%) which is higher. The difference in the concentration of 1,8-Cineol obtained was due to the different types of eucalyptus oil used.

In this study, the inhibition zones are increasing in diameter according to the increasing concentration of eucalyptus oil. This result is in accordance with previous study which found that the inhibitory zone diameter in concentration 10% (7.1 ± 0.3 mm), 15% (8.7 ± 0.3 mm), 20% (9.16 ± 0.2 mm), and 25% (10.5 ± 0.4 mm) [7]. Similar results were also obtained in another study the inhibition zone diameter for concentrations of 100% (14.77±0.68 mm), 75% (14.07±1.08 mm), 50% (13.23±5.30 mm), 25% (7.87±0.23 mm) [15]. In addition, another research also showed that at a concentration of 25 g/l (2.5%) the diameter of the inhibition zone was 3.44±0.34 mm and at a concentration of 50 g/µl (5%) the diameter of the inhibition zone was 4.39±0.48 mm, which further confirm the result in this study [14].

However, in contrast to the previous study, in this study the results of the inhibition diameter at a concentration of 20% (19.00 ± 1.17 mm) were greater than previous study with a concentration of 20% (9.16 ± 0.2 mm) [7]. This happened because the concentration of 1,8-Cineol in this study was higher. Compared to another study, in this study the diameter of the inhibition zone was started to be seen at a concentration of 20% while in the previous study at a concentration of 25% [15]. In addition, in this study the diameter of the inhibitory zone at a concentration of 50% (24.76 ± 1.56 mm) was larger than previous study which at a concentration of 50% (13.23±5.30 mm) [15]. This happens because the concentration of 1,8-Cineol in eucalyptus oil in this study was 72.30% while in the previous study was 17.67% [15]. In another research, although the concentration of 1,8-Cineol was 71.96%, but because the concentration used for the diffusion test was too small, the diameter of the resulting inhibition zone was relatively small.

The results of the One-Way ANOVA statistical test showed that there was a significant difference in the average diameter of the inhibition zone for each test concentration compared to positive control. These results are different from the results of previous research the One-Way ANOVA test that was carried out showed that there was no significant difference in average [15]. This is probably caused by the distribution of data that is not normal and not homogeneous which can be caused by data taken from a population that is not evenly distributed and there are variations between samples. In order for the population to be evenly distributed and homogeneous, sampling can be done by taking random samples and the number of samples is increased [16]. Meanwhile, in this study the results obtained was there was significant average differences because the sample was taken at random with the simple randomization method and the number of samples used was larger because in this study the treatment was repeated four times while in previous study three times [15].

In contrast to the previous research, in this study, in addition to the One-Way ANOVA, other comparative statistical tests were also carried out, namely the Post-Hoc comparison test [15]. Based on the results of the Post-Hoc Test analysis, it can be concluded that all treatment groups were significantly different from the control group (p value < 0.05). All treatment groups had an inhibition zone diameter that was larger than the negative control but still smaller than the positive control, so eucalyptus oil could not replace the control antibiotic but could be an alternative. In addition, there was also an insignificant average difference (p value > 0.05) between the 40% concentration and 50% concentration, so it can be concluded that although the diameter of the 50% concentration inhibition zone is greater than the 40% concentration, 40% concentration is better chosen as the therapeutic concentration.

Another further statistical test conducted in this study was the Pearson correlation test, which is important to made an objective conclusion, compared to previous studies that did not use statistical tests and the study conclusions only subjectively. The results obtained are that there is a very strong, positive, and significant relationship between the concentration of eucalyptus oil and the diameter of the inhibition zone of Escherichia coli bacteria. This means that the higher the concentration of eucalyptus oil, the greater the diameter of the inhibition zone. This is probably due to the presence of various active compounds in eucalyptus oil (Melaleuca leucadendra), but the active compound with the highest concentration was 1,8-Cineol. The higher the concentration of eucalyptus oil, the higher the levels of 1,8-Cineol so that the antibacterial effect was more potent.

According to previous research possible mechanism 1,8-Cineol is able to damage the cell wall of Gram negative bacteria because it reacts with Lipopolysaccharide (LPS) which is a specific molecule on the cell wall of Gram negative bacteria. This reaction with LPS increases the formation of Reactive Oxygen Species (ROS) which causes damage to the bacterial cell wall. This causes an increase in the permeability of the bacterial cell wall, with the increase in the permeability of the cell wall it will cause cytoplasmic leakage so that the nucleotides and proteins of the bacteria are damaged which causes bacterial death [17].

5. Conclusion

Based on this research it can be concluded that there was an inhibition zone on Escherichia coli bacteria treated with eucalyptus oil (Melaleuca leucadendra) made in Lamongan. The inhibition zone began to be seen at a concentration of 20% and the largest diameter at a concentration of 50%. There was also a significant difference between the control group and the treatment group at various concentrations of eucalyptus oil (Melaleuca leucadendra) made in Lamongan. And lastly there was a very strong positive and significant corelation between various concentrations of eucalyptus oil (Melaleuca leucadendra) made in Lamongan and the diameter of the inhibition zone of Escherichia coli bacteria.

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