ORIGINAL RESEARCH REPORT

In Vitro Antibacterial Activity of Eco Enzyme of Eucalyptus (*Melaleuca leucadendra*) against *Escherichia coli*

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ABSTRACT

Background: Eucalyptus (*Melaleuca leucadendra*) is known to have antimicrobial potential due to its bioactive terpenoid compounds, including 1,8-cineole. This compound has the potential to inhibit the growth of *Escherichia coli*, a bacterium responsible for various infectious diseases. The eco-enzyme method, which utilizes fermentation, is simple to perform and does not require complex materials. **Objective**: This study aimed to assess the antibacterial activity of eucalyptus eco-enzyme against *Escherichia coli*. **Material and Method**: The *M. leucadendra* used in this study was sourced from Candisari Village, Lamongan, Indonesia and *E. coli* was obtained from laboratory isolates. Antibacterial activity was measured by observing the zone of inhibition in the well diffusion test on Muller-Hinton agar, with chloramphenicol as the positive control and distilled water as the negative control. The incubation period was 24 hours at 36°C. **Result**: The inhibition zone around the positive control was 25.94±1.1 mm. No inhibition zone (0 mm) was observed around the negative control or the eucalyptus eco-enzyme solution at concentrations ranging from 10% to 100%. However, a clearer zone was observed around the eco-enzyme well. The inability of the eco-enzyme to inhibit the growth of *E. coli* may be attributed to several factors, including the ingredients, processing method, acidity level, and bacterial resistance. **Conclusion**: The eucalyptus eco-enzyme did not exhibit sufficient antibacterial activity against *E. coli* at any of the tested concentrations.

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Highlights

1. It has been proven that the eucalyptus eco-enzyme from Lamongan does not exhibit strong enough antibacterial activity against *E. coli* at any concentration.

2. The eucalyptus eco-enzyme method is less effective at extracting active compounds compared to distillation processing.

BACKGROUND

Escherichia coli (*E. coli*) is a beneficial bacterium in the human digestive tract but can also cause disease under certain circumstances. When *E. coli* spreads to a supposedly sterile part of the body, such as the urinary tract, an infection can occur in that area (urinary tract infection). Data from 2022 showed that 67.3% of urinary tract infections in Indonesia were caused by *E. coli* (Putra, 2022). Hand sanitizer is a liquid often used to clean hands and prevent *E. coli* infection. However, repeated and frequent use of alcohol-based sanitizers can cause the skin to become dehydrated (Hakimah, 2021). The level of antibiotic resistance in the treatment of *E. coli* infections is also increasing; tetracycline sensitivity is only 20%, and gentamicin sensitivity is only 60% (Shrestha, et al., 2022).

M. leucadendra is a natural material that is widely grown and utilized in Indonesia (Wibowo, et al., 2023). This plant contains terpenoid compounds in the form of 1,8-cineole. Scientists have found that it can stop the growth of *E. coli* by blocking the LuxS gene. This gene is involved in biofilm formation, motility, structure, and pathogenicity of bacteria. By blocking this gene, the growth of *E. coli* will be inhibited (Wang, et al., 2022). When *M. leucadendra* is processed using the distillation method, it can produce an inhibition zone of 24.76 mm (Wilujeng, et al., 2022). However, processing using the distillation method is challenging and requires materials that are difficult to obtain on a small scale. Therefore, it is necessary to implement a simpler processing method, such as Eco enzyme. This method processes organic materials by utilizing fermentation to extract compounds from these materials. Water, sugar, and organic materials are combined to allow the growth of bacteria that can decompose and ferment active compounds of the organic material (Rukmini & Herawati, 2023).

Lamongan is one of the districts in Indonesia that produces eucalyptus, which has been shown to contain 72% of the compound 1,8-cineole in its distillation product. However, to date, there have been no antibacterial test results using eucalyptus processed by the eco-enzyme method (Targanski, et al., 2023). Therefore, it is important to conduct research to determine and verify the antibacterial activity of this eucalyptus ecoenzyme. The results of this research are expected to provide additional data on the antibacterial ability of *M. leucadendra*, which can then serve as a basis for the community to produce useful and easy-to-make eucalyptus-processed products.

OBJECTIVE

This research aimed to study the antibacterial activity of the eucalyptus (*M. leucadendra*) eco enzyme against *E. coli* bacteria in vitro.

MATERIAL AND METHOD

This study employed a posttest-only control group design, a type of true experimental research. The antibacterial activity was tested using the agar well diffusion method. Positive and negative controls were used in the form of chloramphenicol and distilled water, respectively. The diffusion test was conducted using Mueller-Hinton agar media (OXOID CM0405, Basingstoke, Hampshire, UK) at the Microbiology Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. *E. coli* bacteria as research samples in this study were obtained from existing stock stored in the Laboratory of the Department of Microbiology, Faculty of Medicine, Universitas Airlangga. The required sample size was calculated using the Federer formula (Trisia, et al., 2018; Gaspersz & Fitrihidajati, 2022), which indicated a minimum of 3 repetitions and a total of 30 samples. *E. coli* was then inoculated into nutrient broth according to the 0.5 McFarland standard (standard inoculum 108 CFU/mL).

The eco-enzyme production involved mixing water, eucalyptus, and brown sugar in a 10:3:1 ratio, respectively, and fermenting the mixture in a closed container, which was periodically opened to release the fermentation gas. The eucalyptus leaves (*M. leucadendra*) from Candisari Village, Lamongan

Regency, Indonesia, were confirmed through a species identification process at the UPT Herbal Materia Medika Laboratory, Batu, Indonesia. After six months of fermentation, the eco-enzyme was harvested and filtered through a Sartorius filter with a pore size of 0.22 µm. The inoculation method confirmed that the eucalyptus eco-enzyme was free from microbiological contaminants. The eco-enzyme was then diluted with distilled water to achieve concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, which were then homogenized using a vortex.

For the diffusion test, four wells of 6 mm diameter were made on each petri dish containing Mueller-Hinton agar that had been streaked with *E. coli*. Each well was filled with 100 µL of one of the ten different concentrations of Lamongan eucalyptus eco-enzyme, along with the positive and negative controls, using a micropipette. The petri dishes were then incubated at 37°C for 24 hours, and the zone of inhibition was measured.

RESULT

Eucalyptus fermentation was carried out for six months, from August 2023 to February 2024, resulting in an aqueous, clear brown eco-enzyme liquid with a distinctive eucalyptus aroma and a slightly sour taste. The eucalyptus eco-enzyme was then tested for sterility, content, and acidity, and the results are shown in Table 1.

Table 1. Results of several eucalyptus eco enzyme tests.

Test	Parameters	Method	Result	Test site
Sterility	Contaminant microorganisms	Inoculation	Sterile, no colonies of contaminant microorganisms were found on the test medium	Laboratory of the Department of Microbiology, Faculty of Medicine, Universitas Airlangga
Compound content	Acetic acid	HPLC	No acetic acid was detected in the sample	Institute of Life Sciences and Technology, Universitas Airlangga (LIHTR)
	Phytochemical screening (terpenoids)	TLC	Samples detected to contain terpenoid compounds	Institute of Life Sciences and Technology, Universitas Airlangga (LIHTR)
Acidity	pH	pH Meter	pH value = 4.95	Laboratory of the Department of Microbiology, Faculty of Medicine, Universitas Airlangga

After several content tests, an agar well diffusion antibacterial test was performed, with 24 hours of incubation at 37°C. The results of the inhibition zone measurements for the positive and negative controls, as well as the ten eco-enzyme concentrations, are presented in Table 2.

Table 2. Results of inhibition zone diameters.

Treatment	Diameter (mm)			
	Repetition I	Repetition II	Repetition III	Mean
Positive Control	24.73	27.28	26.78	25.94
Negative Control	0.00	0.00	0.00	0.00
100%	0.00	0.00	0.00	0.00
90%	0.00	0.00	0.00	0.00
80%	0.00	0.00	0.00	0.00
70%	0.00	0.00	0.00	0.00
60%	0.00	0.00	0.00	0.00
50%	0.00	0.00	0.00	0.00
40%	0.00	0.00	0.00	0.00
30%	0.00	0.00	0.00	0.00
20%	0.00	0.00	0.00	0.00

The inhibition zone test on the positive control (chloramphenicol) resulted in an average inhibition zone of 25.95±1.1 mm. This value indicates that the positive control used in this study has a very strong inhibitory effect, based on the David and Stout classification, and that the *E. coli* bacteria used are sensitive to the positive control according to the Clinical and Laboratory Standards Institute (CLSI) classification (Ouchari, et al., 2018). In contrast, the negative control (distilled water) and all concentrations of Lamongan eucalyptus eco-enzyme did not produce inhibition zones (0 mm). However, the growth of *E. coli* bacteria was reduced around the eco-enzyme wells, and the colonies appeared less dense.

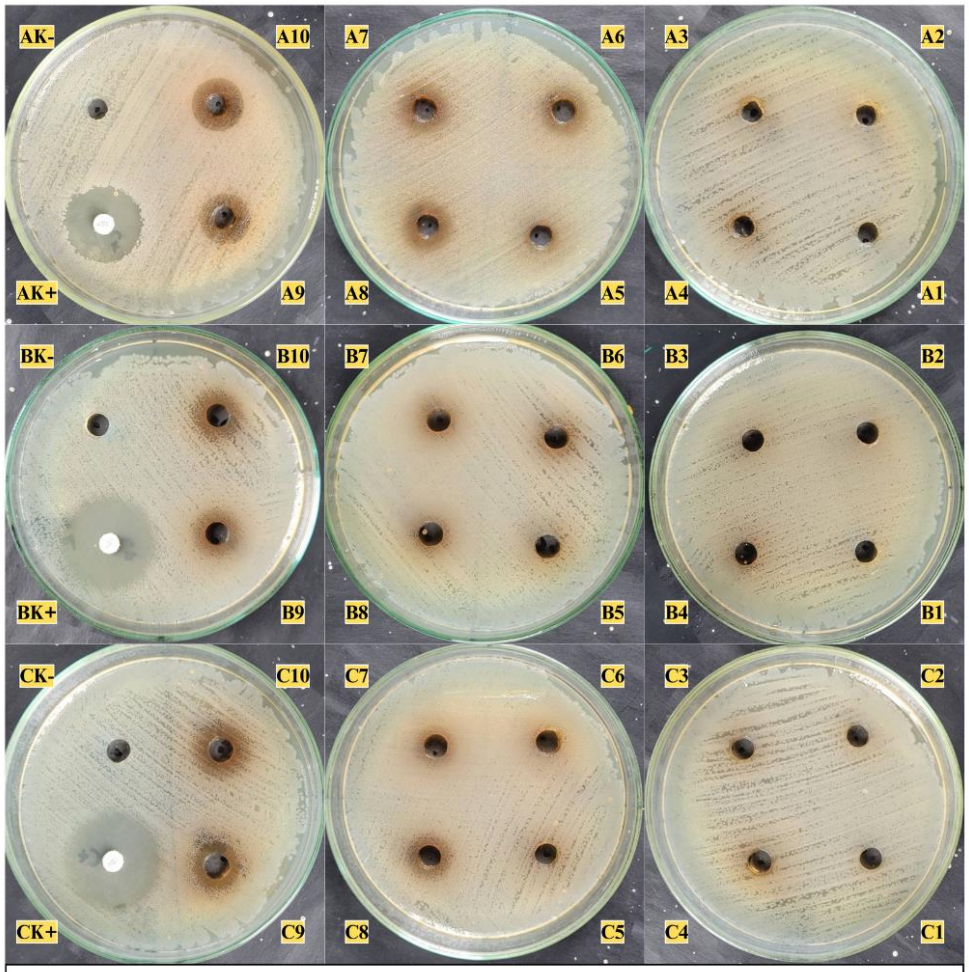

Figure 1. Agar well diffusion test after 24 hours incubation.

This study employed descriptive statistics and a simple comparative analysis to compare the antibacterial activity of the eco-enzyme with that of a positive control. Chloramphenicol, used as the positive control, produced an average inhibition zone of 25.95 ± 1.1 mm. The standard deviation of 1.1

mm for the positive control indicated the consistency of the measurements across repetitions, supporting the validity of the agar well diffusion method used. The average inhibition zone of the positive control formed a significant and very strong inhibition zone. In contrast, the eucalyptus eco-enzyme at various concentrations did not produce any inhibition zones (0 mm). This striking difference suggested that the eco-enzyme was not effective enough to inhibit the growth of *E. coli*.

DISCUSSION

Content and characteristics of eucalyptus eco enzymes

The characteristics of the eco-enzyme identified in this study aligned with those found in previous research, including its brown colour and acidic aroma (Dewi, et al., 2015; Rijal, et al., 2021). The similarity in these characteristics suggests that the stages involved in producing the Lamongan eucalyptus eco-enzyme are generally appropriate and functioning effectively. However, the content test conducted on the eco-enzyme showed some differences. For example, acetic acid compounds were not detected, which contrasted with the findings of previous studies that demonstrated positive results for acetic acid content. Factors contributing to these differences may include the selection of raw materials as a carbon source during the fermentation process, such as the geographical origin of the eucalyptus plants, the variety of organic materials added, and the type of sugar used.

In this study, eucalyptus leaves from Lamongan were used, which may contain fewer volatile compounds, such as 1,8-cineole. The results were different from eucalyptus leaves from Maluku used in Rijal's study (Rijal, et al., 2021). The geographical conditions in Maluku, including higher humidity and rainfall, may contribute to the higher concentration of volatile compounds in their commodities. This is likely due to the influence of water availability and plant adaptation to environmental stress (Anwar, 2022). The eco-enzyme produced in this study also had the lowest pH compared to those in Rijal's and Andam's previous studies. This could be attributed to the more limited variety of organic carbon sources used in the Lamongan eco-enzyme fermentation. In contrast, Rijal, et al., (2021) incorporated salak, tomato, orange, and mango fruit waste in his eucalyptus eco-enzyme, whereas only eucalyptus leaves were used in this study. Another research has shown that eucalyptus leaves contain less carbon than other fruits and plants (Mulyana & Purwanto, 2020).

Sugar plays a crucial role in the fermentation process of eco-enzymes, serving as a carbon source for microbial metabolism and facilitating enzyme production. Therefore, the use of different types of sugar as raw materials for eco-enzymes can influence the production of the resulting eco-enzymes (Supriyani, et al., 2020; Phanama, 2023). Molasses, a by-product of sugar production, contains a variety of complex sugars and higher mineral content than brown sugar. As a result, using molasses as a sugar source can impact microbial activity and enzyme formation during the fermentation process (Supriyani, et al., 2020). The differences in raw materials and other research aspects between this study and others are summarized in Table 3.

Table 3. Mapping of raw materials and important aspects of some related research.

Researcher (year)	Extraction method	Source of organic matter	Source of organic matter	Acidity (pH)	Bacteria	Antibacterial Test Method	Result
Rijal, et al., (2021)	Eco enzymes	Maluku eucalyptus (*M. leucadendra*) and fruit waste	Molasses	4.2	*E. coli*	Diffusion	Zone of inhibition 9.17 (1x24 hours) 14.42 (2x24 hours) mm (positive)
Wilujeng, et al., (2022)	Oil refining	Lamongan eucalyptus (*M. leucadendra*)	No sugar involved	No acidity test was performed	*E. coli*	Diffusion	Zone of inhibition 24.76 mm (positive)
Dewi, et al., (2015)	Eco enzymes	Fruit waste	Brown sugar	3.81	*E. coli*	Diffusion and Dilution	Zone of Inhibition 0 mm (negative) and MIC 60% (positive)
This research (2024)	Eco enzymes	Lamongan eucalyptus (*M. leucadendra*)	Brown sugar	4.95	*E. coli*	Diffusion	Zone of inhibition 0 mm (negative)

Zone of inhibition in eucalyptus eco enzyme antibacterial effect test against *E. coli*

All the eco-enzyme concentrations tested did not kill *E. coli* bacteria, as evidenced by the absence of inhibition zones around the eco-enzyme wells. This result aligned with Dewi, et al., (2015) eco-enzyme diffusion test results, where no inhibition zones were produced, and a clearer area was observed surrounding the wells. However, Dewi, et al., (2015) also tested the eco-enzyme using the dilution method, which resulted in the eco-enzyme successfully inhibiting *E. coli* at a minimum inhibitory concentration of 60%. This suggests that the diffusion test method may be less suitable for testing the antibacterial activity of eco-enzymes. The diffusion ability of the active compounds in eucalyptus, particularly terpenoids, on agar media is limited, as terpenoids and agar have different polarities. In contrast, the dilution method uses liquid media, which allows the active compounds to interact directly with the test bacteria, leading to results that are not influenced by the diffusion ability (Dewi, et al., 2015; Prayoga, 2015).

The Lamongan eucalyptus eco-enzyme tested positive for terpenoids, specifically 1,8-cineole, which has been shown to exhibit antibacterial effects against various bacteria, including *Staphylococcus aureus*, *E. coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella enterica*, and *Pseudomonas aeruginosa* (Allung, 2019; Hakim, et al., 2019; Joen, 2020; Monzote, et al., 2020). However, the absence of an inhibition zone in this study suggests that the presence of terpenoids does not necessarily guarantee the ability of a substance to inhibit bacterial growth. This may be due to the low concentration of terpenoids, particularly 1,8-cineole. According to Wang, et al., (2022), the minimum concentration of terpenoids required to inhibit the growth of *E. coli* is 6.2 µg/ml, and to kill the bacteria, a concentration of at least 12.4 µg/ml is needed. It is possible that the terpenoid content in the Lamongan eucalyptus eco-enzyme did not reach these levels. Therefore, further testing is required to determine the levels and concentrations of terpenoids in the Lamongan eucalyptus eco-enzyme produced in this study, which was not feasible due to several limitations.

In the eco-enzyme method, water serves as a medium to capture the active compounds extracted during the fermentation process. However, water has high polarity, making it ineffective at capturing terpenoids, which have low polarity (Rijal, et al., 2021). Terpenoids are hydrocarbon compounds derived from lipids or fats, and as such, they are not easily soluble in water (Musman, 2017). In contrast, the distillation method uses oil as an extraction medium, which is more effective at capturing terpenoid compounds. For example, a study on eucalyptus oil from Lamongan by Wilujeng, et al., (2022) produced a positive inhibition zone and contained 61% terpenoids in its product. It is also important to note that terpenoids inhibit bacterial growth primarily by interfering with the formation of peptidoglycan in bacterial cell walls. *E. coli*, a Gram-negative bacterium, has a cell wall structure characterized by a thin peptidoglycan layer, which is surrounded by thick lipopolysaccharides (Rohde, 2019). This structural difference means that the potential inhibition effect of eucalyptus eco-enzyme may be more pronounced when tested on Gram-positive bacteria, which have a thicker peptidoglycan layer that plays a crucial role in their defense. Therefore, for future research on Lamongan eucalyptus eco-enzyme, it may be beneficial to test it on Gram-positive bacteria.

Strength and limitations

Despite the negative results of this study, it has provided several valuable insights and benefits. These include the expansion of eco-enzyme research, particularly in relation to eucalyptus, and the examination of the antibacterial potential of eucalyptus treated with the eco-enzyme method against *E. coli* bacteria. Moreover, this study has highlighted the limitations of using the agar diffusion method as a test for eco-enzyme antibacterial activity, as well as the effectiveness of the eco-enzyme method in extracting active compounds from plants. However, this study also has several limitations, including the untested concentrations and exact levels of eco-enzyme content, as well as the use of only one test method. The absence of alternative test methods for comparison or validation further limits the robustness of the findings.

CONCLUSION

It was concluded that the 10%-100% concentrations of Lamongan eucalyptus eco enzyme did not exhibit sufficient antibacterial activity against *E. coli*.

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Conflict of Interest
All authors have no conflict of interest.

Ethic Consideration
The research protocol was declared ethically feasible by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga (No. 43/EC/KEPK/FKUA/2023) issued on 19-02-2023 in accordance with the 7 (seven) WHO 2011 standard.

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Author Contribution
MABA contributed to the conception and design, analysis and interpretation of data, and drafting of the article. WR contributed to the conception & design of the study and critical revision of the article. UM contributed to the critical revision of the article and final approval of the article. EBK contributed to the critical revision of the article and final approval of the article.

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