World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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Antibacterial effect of eucalyptus (*Melaleuca leucadendra*) ecoenzyme produced by Lamongan's MSMEs against *Salmonella* Typhi

Bilal Burhanuddin Baihaqi [1], Wiwin Retnowati [2,*], Danti Nur Indiastuti [3] and Eko Budi Koendhori [2]

[1] *Medical Study Program, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, 60131.*
[2] *Department of Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, 601313.*
[3] *Department of Anatomy, Histology, and Pharmacology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, 60131.*

World Journal of Advanced Research and Reviews, 2024, 21(03), 634–640

Publication history: Received on 27 November 2023; revised on 06 January 2024; accepted on 08 January 2024

Article DOI: https://doi.org/10.30574/wjarr.2024.21.1.0051

Abstract

Typhoid fever is a disease with extensive epidemiological distribution, causing high morbidity and mortality. The lack of effectiveness in prevention and treatment contributes to its continued mass occurrence. *Salmonella* Typhi bacteria are the main etiological cause of typhoid fever and have demonstrated resistance to various typhoidal antibiotics. This study aims to explore alternative possibilities for these issues, both curatively and preventively. Eucalyptus (*Melaleuca leucadendra*) is known to contain numerous antibacterial compounds, with 1.8 cineole as the main antibacterial compound. This compound exerts antibacterial effects by disrupting bacterial cell membranes and walls. Ecoenzyme are also recognized for their antibacterial effects, containing antibacterial compounds such as acetic acid, citric acid, lipase enzyme, trypsin enzyme, and amylase enzymes. This research is a laboratory experimental study conducted using the agar-well diffusion method on Mueller Hinton agar media, with the diameter of the inhibition zone as the test result. The findings indicate that all tested concentrations of eucalyptus ecoenzyme (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) show no inhibitory zone in all three test replications. This result suggests that eucalyptus (*Melaleuca leucadendra*) ecoenzyme produced by Lamongan's MSMEs does not exhibit antibacterial effects on *Salmonella* Typhi.

Keywords: Ecoenzyme; Eucalyptus (*Melaleuca leucadendra*); *Salmonella typhi*; Antibacterial; Typhoid fever

1. Introduction

Ecoenzyme are products made from raw materials through a three-month fermentation process. Producing ecoenzyme does not require special skills or a significant amount of money because it only uses raw materials as the main ingredients, such as used plastic bottles, brown sugar, and simple tools [1]. Therefore, ecoenzyme are products that anyone can make at an inexpensive cost. Ecoenzyme have been proven to exhibit antibacterial, antifungal, and anti-insect capabilities [2].

Eucalyptus oil (*Melaleuca leucadendra*) is known as a traditional Indonesian medicine widely used by the public. Eucalyptus is known to contain various compounds with antibacterial properties, including 1,8-cineole, linalool, 4-terpineol, and α-terpineol. 1,8-cineole is a compound with the most potent antibacterial and antifungal capabilities, constituting the highest percentage in *Melaleuca leucadendra* [3]. It damages bacterial cell walls and membranes through the down-regulation of carbohydrate metabolism and protein genes [4]. Essential oils, including *Melaleuca leucadendra*, can also be a solution to increasing antibiotic resistance through the synergistic use of essential oils with antibiotics [5].

* Corresponding author: Wiwin Retnowati

Salmonella enterica serotype Typhi is a rod-shaped Gram-negative bacterium with a flagellum and spreads via the oral-fecal route, primarily through undercooked food, contaminated drinking water, and the vomit of infected patients [6]. This bacterium generally spreads massively in densely populated slum areas with poor sanitation because humans are the only hosts that can transmit it. *Salmonella* Typhi is the main bacterium that causes typhoid fever [7].

Typhoid fever is an enteric fever that can spread systemically to various organs, with the main symptoms being abdominal pain and fluctuating fever. Typhoid fever has no specific symptoms, but patients usually experience enterocolitis with nausea, diarrhea, and abdominal pain, as well as flu-like symptoms [7]. Other symptoms that can occur in typhoid fever are weakness, headache, constipation, cough, jaundice, and loss of appetite. Typhoid fever usually reaches temperatures of 39 – 40°C [8]. According to Stanaway et al. [9], it is estimated that typhoid fever, together with paratyphoid, occurs in 14.3 million cases and results in the deaths of around 76.9 – 218.9 thousand people worldwide in a year. This fever also results in a disability-adjusted life year (DALYs) of 5.6 – 15.8 million in 2017. The incidence of typhoid fever based on age is 15% in children aged less than two years, and around 75% of cases occur in children under 10 years of age [10].

The main therapy for typhoid fever is antibiotics. The most potent antibiotic that can be used in typhoid fever is Ciprofloxacin from the Fluoroquinolone group, with Amoxicillin, Chloramphenicol, and Trimethoprim-sulfamethoxazole as alternatives [7]. However, resistance of *Salmonella* Typhi to various antibiotics has occurred worldwide. Globally, *Salmonella* Typhi has shown resistance to Ampicillin, Chloramphenicol, Trimethoprim-sulfamethoxazole, Nalixylic Acid, Ciprofloxacin, Ceftriaxone, and Azithromycin [11]. This bacterium has even shown multi-drug resistance (MDR) in South Asian countries, namely India, Pakistan, and Bangladesh [12].

The high rate of *Salmonella* Typhi antibiotic resistance makes research related to alternative solutions to the problem important. One possible approach is to study the potential of eucalyptus oil (*Melaleuca leucadendra*). However, the process of making eucalyptus oil requires tools and abilities that not everyone has, especially in the distillation process, which can take up to 24 hours and involves drying with anhydrous sodium sulfate [3].

This research uses eucalyptus ecoenzyme, considering that not everyone can make eucalyptus oil. The conversion of eucalyptus into ecoenzyme also has the potential to increase the antibacterial ability of eucalyptus because ecoenzyme are known to have antibacterial abilities [2]. It is hoped that the eucalyptus ecoenzyme will show its potential use as a preventive or curative agent. Eucalyptus ecoenzyme products can be preventive agents as disinfectants that can be easily made and do not cause irritation, considering that many currently available disinfectant products can cause skin irritation [13]. It is also hoped that this product can become a curative agent through a synergistic mechanism with antibiotics or as a medicine, considering the large number of resistance that has occurred in society.

One of the producers of eucalyptus, providing ecoenzyme material, is in Lamongan Regency, East Java. Eucalyptus produced in this area is quite ideal because around 90% of the Lamongan area is 0-100 meters above sea level, making it suitable for eucalyptus, which has an ideal elevation habitat of up to 400 meters [14]. The use of eucalyptus from these producers in this research also provides a positive economic contribution and supports the development of Micro, Small, and Medium Enterprises (MSMEs). This contribution will have an even greater impact if, in the future, eucalyptus ecoenzyme can be mass-produced as a health solution.

This research aims to understand the antibacterial effect of eucalyptus ecoenzyme on *Salmonella* Typhi considering the antibacterial compounds contained therein. It is hoped that this research can contribute to providing information in solving problems related to typhoid fever, both in preventive and curative measures, as well as making a positive economic contribution to eucalyptus-producing MSMEs in Lamongan or Indonesia in the future.

2. Materials and Methods

This research is a laboratory experimental study that aims to test the antibacterial effect of eucalyptus (*Melaleuca leucadendra*) ecoenzyme on *Salmonella* Typhi using the agar-well diffusion assay with a post-test only control group design. The concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% of eucalyptus (*Melaleuca leucadendra*) ecoenzyme act as the independent variable, with the diameter of the inhibition zone on *Salmonella* Typhi media as the dependent variable. The research was conducted from May to September 2023 and carried out at the Microbiology Laboratory of the Faculty of Medicine, Universitas Airlangga, Surabaya.

2.1. Materials and Instruments

The materials used in this research were eucalyptus (*Melaleuca leucadendra*) ecoenzyme (composed of eucalyptus leaf, brown sugar, and water fermented for three months), *Salmonella* Typhi bacteria, Selenite Broth, Mueller Hinton Agar, Chloramphenicol antibiotic disc, distilled water, H2SO4 1%, and BaCl2 1%. The instruments used in this research included petri dishes, Erlenmeyer tubes, test tubes, measuring cups, micropipettes, incubators, tubes, vortexes, tweezers, calipers, autoclaves, aluminum foil, stirring rods, hot plates, paper discs, knives, cotton wool, sterile sticks, and plastic bottles.

2.2. Sampling Technique

The samples of *Salmonella* Typhi bacteria used in this study were collected through a simple random sampling method. The bacterial samples utilized were those meeting the inclusion criteria, specifically samples with homogeneous species bacterial colonies that had been identified and then randomly selected. The eucalyptus (*Melaleuca leucadendra*) ecoenzyme sample material was obtained from MSMEs Sendang Arum, District Sambeng, Lamongan Regency. The eucalyptus raw material would then be transformed into ecoenzyme and diluted to 10 different concentrations (100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10%) with distilled water.

2.3. Procedures

2.3.1. Production of Ecoenzyme

Eucalyptus leaves (*Melaleuca leucadendra*) were cleaned with running water and cut into small pieces. Subsequently, 300g of eucalyptus leaves were placed into a plastic bottle along with 1 liter of clean water and 100g of brown sugar. The mixture was then left for 3 months for fermentation.

2.3.2. Media Preparation

Mueller Hinton Agar was prepared by placing 28 grams of Mueller Hinton Agar powder into an Erlenmeyer tube, dissolving it in 1 liter of distilled water, and then heating it on a hot plate. The Erlenmeyer tube was covered with aluminum foil and sterilized by autoclaving at 121°C for 15 minutes. The resulting mixture was then poured evenly into a petri dish.

Selenite Broth was also prepared by adding 23 grams of selenite sodium powder into an Erlenmeyer tube, dissolving it in 1 liter of distilled water, and heating it until it boiled. The solution was then sterilized in a sterile test tube to a minimum depth of 5 cm and boiled for 10 minutes at 100°C.

2.3.3. Preparation of Salmonella Typhi suspension

Salmonella Typhi bacteria from the culture stock were grown on selective Selenite Broth media for 24 hours at 37°C. The bacterial suspension was then homogenized using a shaker with a speed of 120 rpm at room temperature. Subsequently, the suspension was incubated at 37°C for 24 hours. A 5 ml portion of the bacterial suspension was transferred into a centrifuge tube and centrifuged for 3 minutes at a speed of 1500 rpm. The formed supernatant was discarded, and a phosphate buffer saline solution was added to the precipitate up to 5 ml. The turbidity of the bacterial suspension was then compared with a standard solution of 0.5 McFarland (1.5 x 10^8 CFU/ml).

2.3.4. Preparation of Eucalyptus (Melaleuca leucadendra) Ecoenzyme

Eucalyptus (*Melaleuca leucadendra*) ecoenzyme, fermented for 3 months, were filtered and subjected to a sterility test using the direct inoculation method for 24 hours at a temperature of 37°C. The test results indicated that the eucalyptus ecoenzyme were sterile. Subsequently, the filtered ecoenzyme were diluted to concentrations of 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10% by adding distilled water.

2.3.5. Antibacterial Activity Test using the Diffusion Method

The antibacterial effect test of eucalyptus (*Melaleuca leucadendra*) ecoenzyme against *Salmonella* Typhi bacteria was conducted using the agar-well diffusion method, with the inhibition zone diameter as the result parameter. Eucalyptus ecoenzyme were prepared at concentrations of 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10%. The positive control for this test was the chloramphenicol antibiotic disc, and the negative control was distilled water. Each test group was replicated three times.

A standardized *Salmonella* Typhi bacterial suspension with a 0.5 McFarland standard solution (1.5 × 10^8 CFU/mL) was prepared, and sterilized cotton swabs were dipped in the liquid bacterial culture. The cotton swab was then rubbed over the entire surface of the Mueller Hinton Agar, repeated twice, while rotating the plate 60°. Wells of 6 mm diameter were prepared in two Mueller Hinton Agar media (each media containing 6 wells). A volume of 100 µL of distilled water was dropped into one of the wells as the negative control. The chloramphenicol disc was taken with tweezers and placed in one of the wells. Eucalyptus ecoenzyme with concentrations of 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10% were each dripped as much as 100 µL on 10 different wells. The media were then incubated for 24 hours at 37°C. After incubation, the diameter of the inhibition zone formed was measured using a caliper, and the data were analyzed.

3. Results and Discussion

The antibacterial effect test of eucalyptus (*Melaleuca leucadendra*) ecoenzyme produced by Lamongan's MSMEs against *Salmonella* Typhi bacteria was conducted using the agar-well diffusion method. Eucalyptus ecoenzyme were prepared at concentrations of 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10%. The positive control for this test was chloramphenicol antibiotic, while the negative control used was distilled water. The test was replicated three times, and the results include the diameter of the inhibition zone measured in millimeters (mm) using a digital caliper. The table below presents the measurements of the inhibition zone diameter for eucalyptus ecoenzyme (*Melaleuca leucadendra*) produced by Lamongan's MSMEs against *Salmonella* Typhi bacteria.

Table 1 Inhibition zone diameter of eucalyptus (*Melaleuca leucadendra*) ecoenzyme produced by Lamongan's MSMEs against *Salmonella* Typhi bacteria

Treatment	**Diameter (mm)**			
	Replication I	**Replication II**	**Replication III**	**Mean**
(+) Control	31.59	29.85	30.95	30.8
(-) 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
10%	0.00	0.00	0.00	0.00

The test yielded negative results, with no inhibition zone observed at any concentration of eucalyptus ecoenzyme. The absence of an inhibition zone in the eucalyptus ecoenzyme test mirrors the absence of an inhibition zone in the negative control of distilled water employed in this study. This outcome contradicts the research conducted by Sun et al. [4], which indicates that the primary content of 1,8-cineole in eucalyptus can harm the cell walls and membranes of *Salmonella* bacteria. Several potential causes may explain these negative results. The lack of antibacterial effect in this study could be attributed to low levels of terpenoid compounds and the absence of other compounds with antibacterial properties in the eucalyptus ecoenzyme content. Additionally, *Salmonella* Typhi bacteria resistance to the eucalyptus ecoenzyme content might also contribute to these negative results.

The primary antibacterial components in *Melaleuca leucadendra* (eucalyptus) are terpenoid compounds, with 1,8-cineole or eucalyptol being the major one, constituting 61% and serving as its chemotype [15]. Known for its antibacterial effects, 1,8-cineole targets various Gram-positive bacteria such as *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *S. epidermidis*, as well as Gram-negative bacteria like *Klebsiella pneumonia*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa* [16]. It also exhibits antibacterial effects against *Salmonella* bacteria by disrupting their cell membranes through the downregulation of mRNA metabolism for carbohydrates and membrane proteins [4]. Other active antibacterial compounds present in eucalyptus include linalool, 4-terpineol, and α-terpineol [3].

Ecoenzyme are products derived from raw materials through fermentation processes [1]. Recognized for their antibacterial effects, ecoenzyme contain various chemical compounds, including acetic acid, citric acid, lipase enzymes, trypsin enzymes, amylase enzymes, and a pH around 3.5 [2]. Acetic acid, an antibacterial compound in ecoenzyme, forms through anaerobic hydrolysis of complex organic compounds like ethanol, increasing in concentration during fermentation. This acid disrupts cellular metabolism by passing through cell membranes, elevating osmotic pressure within cells and leading to osmolysis [17]. Research by Qadir et al. [18] confirms the antibacterial effects of a derivative of acetic acid, 2,4-dihydroxybenzyliminodiacetate, against *Salmonella* Typhi by inhibiting peptidoglycan synthesis.

Neupane and Khadka [19] assert that the antibacterial effects of ecoenzyme also stem from the enzymes they contain, such as amylase, protease, caseinase, cellulase, and lipase. Their study demonstrates the antibacterial properties of ecoenzyme derived from sweet lime (*Citrus limetta*), pomegranate (*Punica granatum*), pineapple (*Ananas comosus*), and papaya (*Carica papaya*) against *Salmonella* Typhi. The diffusion method shows inhibition zones at a 100% concentration, with result of *Citrus limetta* at a 20 mm diameter, *Punica granatum* at 18 mm, *Ananas comosus* at 20 mm, and *Carica papaya* at 15 mm [19]. Abdullah et al.'s [20] research further validates the antibacterial ability of ecoenzyme produced from a mixture of dragon fruit skin, orange, papaya, and pineapple core against *Salmonella* Typhi at concentrations of 50% (1.483 mm inhibition zone), 75% (4.733 mm), and 100% (6.083 mm).

Laboratory testing of eucalyptus ecoenzyme produced by MSMEs in Lamongan at Airlangga University's Life Sciences, Engineering, and Engineering Research Institute reveals the presence of terpenoid compounds but the absence of acetic acid in the ecoenzyme. The absence of these crucial antibacterial compounds is noteworthy, particularly considering negative results from diffusion method antibacterial tests. The lack of acetic acid may diminish or eliminate the antibacterial potential of eucalyptus ecoenzyme, as acetic acid is known for its potent antibacterial effects [21].

Testing results also indicate the presence of terpenoid compounds in the ecoenzyme. However, the lack of antibacterial effects against *Salmonella* Typhi may be attributed to the low levels of terpenoid content. Sun et al. [4] suggest that a concentration of 0.25 mg/mL of 1,8-cineole is needed to initiate antibacterial mechanisms against *Salmonella* over a 3-hour period. Another possible reason is the absence of specific active antibacterial terpenoid derivatives in the eucalyptus ecoenzyme, such as 1,8-cineole, linalool, 4-terpineol, and α-terpineol. Therefore, further research is necessary to investigate these possibilities and confirm the reasons behind the lack of antibacterial effects of eucalyptus ecoenzyme produced by MSMEs in Lamongan against *Salmonella* Typhi.

The primary mechanism of *Salmonella* Typhi's resistance to antibiotics involves the destruction of the chemical structures of antibacterial agents, rendering them inactive. This can occur through the production of extended-spectrum β-lactamases (ESBLs), which cut the chemical ring structure of beta-lactam antibiotics (penicillin and cephalosporin), making them ineffective in disrupting bacterial cell wall formation [22]. Chemical structure alterations also contribute to resistance to chloramphenicol antibiotics through the action of the enzyme chloramphenicol acetyltransferase. *Salmonella* Typhi's resistance to tetracycline antibiotics results from the activation of efflux pumps, expelling tetracycline from inside the cell. For quinolone antibiotics, resistance occurs through the protection of targeted enzymes, such as DNA gyrase, by pentapeptide proteins, preventing their inhibition [23].

Various resistance mechanisms make many *Salmonella* Typhi bacteria fall into the category of Multi-Drug Resistant (MDR), resistant to antibiotics like ampicillin, chloramphenicol, and cotrimoxazole. Some even reach the Extensively Drug-Resistant (XDR) category, showing resistance to fluoroquinolone and third-generation cephalosporin antibiotics [12]. These findings highlight the highly complex and potent resistance capabilities of *Salmonella* Typhi against various antibacterial agents.

The outer lipopolysaccharide (LPS) membrane of Gram-negative MDR bacteria, including *Salmonella* Typhi, may contribute to resistance against lipophilic compounds like the terpenes found in eucalyptus ecoenzyme, acting as a physical barrier to these antibacterial substances [24]. Hence, further research on *Salmonella* Typhi's potential

resistance to antibacterial agents present in eucalyptus ecoenzyme produced by MSMEs in Lamongan, as well as its mechanisms, is warranted.

4. Conclusion

The eucalyptus (*Melaleuca leucadendra*) ecoenzyme produced by Lamongan's MSMEs, tested for antibacterial effects against *Salmonella* Typhi using the agar-well diffusion method, did not exhibit any antibacterial effects. Despite the negative research findings and the limitations encountered during the study, the researcher provides recommendations. In future tests, the antibacterial effects of eucalyptus ecoenzyme produced by Lamongan's MSMEs against *Salmonella* Typhi could be evaluated using methods other than agar-well diffusion. To enhance the accuracy of test results, standardizing the thickness of Mueller Hinton Agar media and ensuring the production of standardized ecoenzyme is recommended. Additionally, conducting qualitative and quantitative analyses of the chemical compounds present in the eucalyptus ecoenzyme produced by Lamongan's MSMEs can offer a more comprehensive understanding of the research outcomes.

Compliance with ethical standards

Acknowledgements

The authors express their gratitude to the Microbiology Department, Faculty of Medicine, Universitas Airlangga, for the support and assistance, enabling the timely completion of this paper.

Disclosure of Conflict of Interest

All authors declare no conflict of interest.

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