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Antibacterial effects of *Melaleuca leucadendra* Ecoenzyme on *Pseudomonas aeruginosa*

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Abstract

Introduction: *Pseudomonas aeruginosa*, a Gram-negative opportunistic pathogen, poses a significant threat in hospital settings, causing nosocomial infections with severe consequences. The bacterium's high antibiotic resistance, particularly through biofilm formation via quorum sensing (QS), complicates treatment strategies. This study explores the potential of eucalyptus (*Melaleuca leucadendra*), known for its antimicrobial properties especially due to 1,8-cineole, ecoenzyme in inhibiting *Pseudomonas aeruginosa*.

Materials and Methods: This study is a posttest-only control group design. *Pseudomonas aeruginosa* bacteria were samped using simple random sampling. The study utilized the agar-well diffusion method on Mueller Hinton agar to assess the antibacterial effect of eucalyptus (*Melaleuca leucadendra*) ecoenzyme. Concentrations tested ranged from 10% to 100% with three repetitions and incubation at 37 °C for 24 hours. Data were obtained by measuring the inhibition zone using calipers.

Results: Contrary to expectations, the study found no antibacterial effect of eucalyptus (*Melaleuca leucadendra*) ecoenzyme on *Pseudomonas aeruginosa* at all concentrations tested (100%, 90%, 70%, 60%, 50%, 40%, 30%, 20%, and 10%).

Conclusion: The investigation into eucalyptus (*Melaleuca leucadendra*) ecoenzyme revealed no discernible antibacterial activity against *Pseudomonas aeruginosa*. These findings challenge the initial hypothesis and underscore the importance of thorough experimentation in assessing the potential of natural agents for combating nosocomial infections. Further research is needed to elucidate the complex interactions between *Pseudomonas aeruginosa* and eucalyputs (*Melaleuca leucadendra*).

Keywords: Antibacaterial effects; *Melaleuca leucadendra*; Ecoenzyme/Garbage enzyme; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and facultative anaerobic which could thrive in hospital environments [1]. Nosocomial infection caused by *P. aeruginosa* have serious implications for public health, including treatment cost, clinical complications, lower quality of live, and mortality rate [2]. *P. aeruginosa* infection could lead to various health issues, including ventilator-associated pneumonia, urinary tract infection, burn wound infection, bacteremia, otitis externa, and meningitis [3].

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P. aeruginosa exhibits a high level of antibiotic resistance, thus complicating the treatment of infections [4]. One significant resistance mechanism of *P. aeruginosa* is the formation of biofilms, making infections challenging to control and prone to recurrence [5]. The formation of *P. aeruginosa* biofilm depends on the quorum sensing (QS) system, where bacteria communicate one another to regulate gene expression in response to population density [5].

Tea tree (*Melaleuca leucadendra*) oil emerges as a potential agent to address *P. aeruginosa* infections [6,7]. Tea tree oil is known to possess antimicrobial properties that can inhibit the growth of various Gram-positive and Gram-negative bacteria [7]. The most prominent molecule in tea tree oil, 1,8-Cineole, is known to inhibit the formation of *P. aeruginosa* biofilm by interfering with the QS system [8].

This research involves the use of ecoenzyme from tea tree (*M. leucadendra*) leaves, a product of fermentation from raw materials, sugar, and water [9]. Besides their cleaning and insect-repelling functions, ecoenzyme are reported to have antimicrobial, antioxidant, and biocatalytic properties [10,11]. Through the relatively simple production method of ecoenzyme, this research aims to uncover the potential of tea tree leaf (*M. leucadendra*) ecoenzyme in addressing nosocomial infections cause by *P. aeruginosa*. Therefore, this study aims to investigate the potential of tea tree leaf (*M. leucadendra*) ecoenzyme in inhibiting *P. aeruginosa* growth. The study's results are expected to provide new insights into the use of tea tree leaf ecoenzyme as potential material for the development of effective disinfectant products to prevent the growth and biofilm formation of *P. aeruginosa*.

2. Material and methods

The method used in this study was agar-well diffusion assay with post-test only control group design. The research took place at Microbiology Laboratory of Faculty of Medicine Universitas Airlangga since March-August 2023.

This study utilized the following materials: tea tree (M. leucadendra) ecoenzyme (tea tree leaf, water, brown sugar), Mueller Hinton agar (OXOID CM0337), Nutrient Broth (OXOID CM0001), ciprofloxacin disc (OXOID CT0425), distilled water, 0.5 McFarland reference standard, cotton swab. The instruments used in this study were: Erlenmeyer, test tube, petri dish, autoclave (TOMY SX-500), inoculation loop, micropipette, digital caliper.

2.1. Controls

In this study, the positive control group received known antibacterial agent. The antibiotic used was ciprofloxacin disc (OXOID CT0425). Negative control was included to account for any potential contamination and unintended effects or confounders. The negative control used was distilled water.

2.2. Procedure

2.2.1. Production of Melaleuca leucadendra Ecoenzyme

The tea tree (*M. leucadendra*) ecoenzyme was made by combining 3 (300g) parts of tea tree leaves, 1 (100g) part of brown sugar, and 10 (1000g) parts of water in a bottle and left for 3 months.

2.2.2. Preparation of Melaleuca leucadendra Ecoenzyme

The tea tree (*M. leucadendra*) ecoenzyme made after 3 months of fermentation period was then filtered and made into 10 different concentrations (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) by adding distilled water. Sterility test was carried out to the tea tree (*M. leucadendra*) ecoenzyme using direct inoculation method to ensure the tea tree (*M. leucadendra*) ecoenzyme is not contaminated by bacteria. Direct inoculation is done by streaking the tea tree (*M. leucadendra*) ecoenzyme on Mueller Hinton agar then incubating it at 37°C for 24 hours. The result after direct inoculation test showed that tea tree (*M. leucadendra*) ecoenzyme samples were sterile.

2.2.3. Preparation of Pseudomonas aeruginosa bacteria

Pseudomonas aeruginosa bacteria were cultured on Mueller Hinton agar and incubated at 37°C for 24 hours. Inoculated bacteria were then taken from *the P. aeruginosa culture* by random sampling and put into Mueller Hinton broth. *P. aeruginosa* suspension was made to match 0.5 McFarland turbidity, the inoculum standard of 108 CFU ml-1 used for disk diffusion method antibiotic susceptibility testing.

2.2.4. Assessment of Antibacterial Effects

The agar well-diffusion array used in this study showed result in diameter of inhibition zones against *P. aeruginosa*. This study consists of two controls and 10 treatments. The positive control used was ciprofloxacin disc (OXOID TC0425), which is known for its use in treating *P. aeruginosa* infections such as bacteremia, ostheochondritis, and eye and ear infections [12]. The negative control used was distilled water. Distilled water is able to dissolve tea tree econezyme and have no known antibacterial activity prior to testing. The ten treatments used tea tree (*M. leucadendra*) ecoenzyme in concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. All experiments were done in three replications.

The *Pseudomonas aeruginosa* bacteria suspension was taken using a swab and streaked on a petri dish with Mueller Hinton agar. The wells were then made and the treatments were inserted into each well with different concentrations and incubated at 37˚C for 24 hours.

The inhibition zone results are area around the wells with no bacteria colony formed. The inhibition zones were measured with digital caliper in mm.

3. Results and discussion

The result of tea tree (*M. leucadendra*) ecoenzyme inhibition against *P. aeruginosa* are displayed in table 1. The result indicated that tea tree (*M. leucadendra*) ecoenzyme fermented for 3 months does not have antibacterial effects against *P. aeruginosa* as could be seen that all treatments yield the same result as the negative control.

Table 1 Result of agar-well diffusion array of tea tree (*M. leucadendra*) ecoenzyme against *P. aeruginosa*

Treatment	Diameter (mm)			
	Replication I	Replication II	Replication III	Mean
(+) Control	25.35	29.10	30.35	28.27
(-) 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 lack of antibacterial activity at all concentrations of tea tree (*M. leucadendra*) ecoenzyme contradicts previous research that reports tea tree (*M. leucadendra*) has antibacterial effects [7]. Possible explanations include the effectiveness of the molecules present in the tea tree (*M. leucadendra*) in inhibiting bacteria, the content of tea tree (*M. leucadendra*) ecoenzyme, and the resistance of *P. aeruginosa* to the antibacterial effects of tea tree (*M. leucadendra*).

The tea tree (*M. leucadendra*) ecoenzyme were then tested using thin-layer chromatography (TLC) screening method to detect terpenoid contents and the result were positive terpenoid contents. Terpenoid, including 1,8-Cineole, are the main components of tea tree (*M. leucadendra*) [13]. Terpenoid from tea tree (*M. leucadendra*) have been reported to have antibacterial effects against various Gram-positive bacteria (*Bacillus cereus, Bacillus subtilis, Corynebacterium diphtheriae, Corynebacterium minutissimus, Enterococcus faecium, Listeria monocytogenes, Micrococcus luteus,*

Staphylococcus aureus, S. capitis, S. epidermidis, S. faecais, Klebsiella spp., and Staphylococcus aureus) and Gram-negative bacteria (*Alcaligenes faecalis, Enterobacter cloacae, Escherichia coli, and Proteus vulgaris*) [7]. The antibacterial mechanism of 1,8-Cineole involves causing protein and nucleic acid leakage due to increased permeability of the bacterial cell membrane [14]. Another molecule of terpenoid, α-Terpineole, also increases the permeability of the bacterial cell membrane [15]. tea tree (*M. leucadendra*) ecoenzyme contains the same molecules as tea tree (*M. leucadendra*) essential oil, but no inhibitory effect was observed.

This is in contrast to some previous studies that reported antibacterial effects of ecoenzyme. It is reported that lemon (*Citrus lemon L*.) ecoenzyme has the antibacterial effects against five pathogenic bacteria, namely *E. Coli, Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhi*, and *Pseudomonas aeruginosa* [16]. Antibacterial testing of lemon essential oil against *P. aeruginosa* using the diffusion method resulted in an inhibition zone with a diameter of 13 mm at a concentration of 15%. These results indicate that lemon essential oil (*C. lemon L*.) has a strong antibacterial effect [16,17].

Another study tested the antibacterial effects of ecoenzyme extracts from papaya, pineapple, and kaffir lime fruits using the diffusion method and found that eoenzymes from these fruits had strong antibacterial effects against *E. coli*. Ecoenzymes from papaya, pineapple, and kaffir lime produced inhibition zones with a diameter of 18 mm at a concentration of 100% [17,18]. Another study created and tested the antibacterial effects of ecoenzyme extracts with yeast (*Saccharomyces cerevisiae*) and sweet lime (*Citrus limetta*), pomegranate (*Punica granatum*), pineapple (*Ananas comosus*), or papaya (*Carica papaya*) fruits, as well as a mixture of fruits or vegetables [19]. Antibacterial effects against *P. aeruginosa* were tested using the diffusion method, and the results showed inhibition zone diameters as follows: sweet lime enzyme (*C. limetta*) 17 mm, pomegranate enzyme (*P. granatum*) 13 mm, pineapple enzyme (*Ananas comosus*) 25 mm, papaya enzyme (*Carica papaya*) 21 mm, mixed fruit enzyme 18 mm, and vegetable enzyme 0 mm [19].

The tea tree (*M. leucadendra*) ecoenzyme were also tested for asetic acid components using high-performance liquid chromatography (HPLC) and the result was negative, indicating no detectable acetic acid in the tea tree (*M. leucadendra*) ecoenzyme. Previous research on the antibacterial effects of acetic acid on *P. aeruginosa* bacteria found that acetic acid can cause the death of *P. aeruginosa* bacteria present in biofilms [20]. Acetic acid in the enzyme is formed from glucose that is hydrolyzed into ethyl alcohol (ethanol) and then forms acetic acid [21]. At pH 3.5, acetic acid is known to cause the death of *P. aeruginosa* bacteria, while at pH >4.2, acetic acid does not significantly affect bacterial growth [20]. The absence of acetic acid in tea tree (*M. leucadendra*) ecoenzyme is consistent with the lack of inhibition zones in the diffusion test.

Another possible explanation for this is that the concentration of terpenoid molecules present in tea tree (*M. leucadendra*) ecoenzyme is different from that in tea tree (*M. leucadendra*) essential oil. The concentration of antibacterial substances is related to their ability to inhibit bacterial growth; the greater the concentration of antibacterial substances, the larger the inhibition zone formed [22]. TLC testing of tea tree (*M. leucadendra*) ecoenzyme did not measure the total concentration of terpenoids and individual molecules such as 1,8-Cineole and α-Terpineole, as well as other molecules found in tea tree (*M. leucadendra*) ecoenzyme. Therefore, further research on the concentration of molecules in tea tree (*M. leucadendra*) ecoenzyme has the potential to reveal additional scientific knowledge.

The absence of an inhibition zone in the testing of tea tree (*M. leucadendra*) ecoenzyme against *P. aeruginosa* bacteria using the diffusion method aligns with a previous study. Based on previous research, screening for the antibacterial effects of tea tree (*M. leucadendra*) essential oil on Gram-negative bacteria such as *P. aeruginosa* and *Escherichia coli* did not show inhibition of the growth of these bacteria [23]. This indicates that *P. aeruginosa* bacteria are not sensitive to the antibacterial effects of tea tree (*M. leucadendra*) essential oil. *P. aeruginosa bacteria*, Gram-negative bacteria, have a phospholipid bilayer and lipopolysaccharide layer that selectively hinders the penetration of antibiotics. Within the phospholipid bilayer and lipopolysaccharide layer, there are porins that form protein channels and release hydrophilic molecules, allowing them to diffuse slowly [5]. tea tree (*M. leucadendra*) ecoenzyme is a product of fermentation with a water-based solvent. Based on its characteristics, tea tree (*M. leucadendra*) ecoenzyme can diffuse and enter *P. aeruginosa* bacteria. The resistance of *P. aeruginosa* bacteria to tea tree (*M. leucadendra*) ecoenzyme may be explained by the presence of an efflux pump in its cell wall. Previous studies have reported that *P. aeruginosa* bacteria have the MexAB-OprM efflux pump [24]. Terpenoid molecules, such as 1,8-Cineole and α-Terpineole, present in tea tree (*M. leucadendra*) ecoenzyme are substrates for the MexAB-OprM efflux pump, making *P. aeruginosa* bacteria resistant to tea tree (*M. leucadendra*) ecoenzyme [25]

4. Conclusion

The tea tree (*M. leucadendra*) ecoenzyme does not have antibacterial effects against *P. aeruginosa* bacteria. This finding was obtained by testing the antibacterial effects of tea tree (*M. leucadendra*) ecoenzyme on *P. aeruginosa* bacteria using the agar-well diffusion method. Future research could contribute to clarify the antibcaterial effects of tea tree (*M. leucadendra*) ecoenzyme by further testing on each compound and its concentration inside tea tree (*M. leucadendra*) ecoenzyme and using different method of antimicrobial assay.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declare no conflict of interest.

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