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In Vitro Anti-Biofilm Activity of Nutmeg (*Myristica fragrans*) Leaf Infusion Extract

Faulia Triana Wijayanti[1]✉, Yusuf Eko Nugroho[2], Ira Pangesti[3]

[1]Jurusan Teknologi Laboratorium Medis, Universitas Al-Irsyad Cilacap

✉email: fauliatrianawijayanti12@gmail.com

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium commonly associated with severe infections, particularly in immunocompromised individuals. One of its major virulence factors is biofilm formation, which contributes to increased antimicrobial resistance. This study aimed to evaluate the inhibitory activity of nutmeg (Myristica fragrans) leaf infusion extract against P. aeruginosa biofilm formation in vitro. The experiment was conducted using a 96-well microtiter plate assay, and biofilm intensity was quantified at 595 nm following crystal violet staining. The results demonstrated that the nutmeg leaf infusion extract significantly inhibited biofilm formation, with varying levels of effectiveness depending on incubation time. These findings indicate the potential of nutmeg leaf infusion extract as an antibiofilm agent against P. aeruginosa.

Keywords: *Pseudomonas aeruginosa; biofilm; nutmeg leaves; active compounds; antibacterial; phytopharmaceutical*

ABSTRAK

Pseudomonas aeruginosa merupakan bakteri Gram-negatif oportunistik yang sering menyebabkan infeksi serius, terutama pada individu dengan sistem imun lemah. Salah satu faktor virulensinya adalah kemampuan membentuk biofilm yang meningkatkan ketahanan terhadap antibiotik. Penelitian ini bertujuan untuk mengevaluasi aktivitas ekstrak infusa daun pala (Myristica fragrans) dalam menghambat pembentukan biofilm P. aeruginosa secara in vitro. Penelitian dilakukan menggunakan metode mikrotiter plate 96 well, dan intensitas biofilm diukur pada panjang gelombang 595 nm setelah pewarnaan kristal violet. Hasil penelitian menunjukkan bahwa ekstrak infusa daun pala secara signifikan menghambat pembentukan biofilm dengan

efektivitas yang bervariasi bergantung pada waktu inkubasi. Temuan ini menunjukkan bahwa ekstrak infusa daun pala berpotensi sebagai agen antibiofilm terhadap P. aeruginosa.

Kata kunci: Pseudomonas aeruginosa; biofilm; daun pala; senyawa aktif; antibakteri fitofarmaka

INTRODUCTION

Infectious diseases remain a major challenge in global health due to various pathogenic microorganisms, including bacteria, viruses, fungi, protozoa, and parasites(1,2). Pseudomonas aeruginosa is a Gram-negative bacterium frequently associated with clinical infections and is well known for its multidrug-resistant (MDR) characteristics(3,4). The irrational and excessive use of antibiotics further accelerates the development of antimicrobial resistance(5,6). In clinical practice, antibiotics are generally used to treat bacterial infections once the causative pathogen has been identified(7,8). Pseudomonas aeruginosa is commonly found in soil and aquatic environments and is capable of causing burn wound infections characterized by a blue-green purulent discharge(9,10). One of the key factors contributing to its high resistance is its ability to form biofilms—structured microbial communities attached to surfaces and protected by an extracellular polymeric substance (EPS) matrix(11,12). The biofilm structure significantly increases bacterial resistance to antibiotics, both due to suboptimal antibiotic use and the intrinsic ability of biofilms to limit drug penetration(13,14). Alongside increasing antimicrobial resistance, numerous medicinal plants have been explored as sources of natural antibacterial agents(15,16). Nutmeg leaves (Myristica fragrans) contain several active compounds, including terpenes, phenylpropanoids, saponins, tannins, and flavonoids, which have demonstrated antibacterial potential(17). However, nutmeg leaves remain underutilized, and their full therapeutic potential has not been extensively explored.

Previous studies have shown that various herbal extracts possess antibiofilm activity against different pathogenic bacteria(18). However, to date, there have been no reports describing the antibiofilm activity of nutmeg leaf infusion (Myristica fragrans) against Pseudomonas aeruginosa in vitro. This gap highlights the need for further scientific investigation.

Therefore, the present study aims to evaluate the potential of nutmeg leaf infusion as an antibiofilm agent against Pseudomonas aeruginosa and to determine the optimal incubation period for biofilm inhibition. This research is intended to contribute new scientific insights regarding the development of plant-based antibiofilm agents.

METHODS

Study Design

This study employed a laboratory experimental design using a systematic approach to control predetermined variables. Experimental research is a logical method used to implement controlled conditions to observe causal effects. The research subject was Pseudomonas aeruginosa, while the samples consisted of biofilms grown in a 96-well microplate system.

Population and Samples

The study population consisted of Pseudomonas aeruginosa cultured in the Microbiology Laboratory of Universitas Al-Irsyad Cilacap. The plant sample used was nutmeg leaves (Myristica fragrans), collected from Kutabima Village, Cimanggu District, Cilacap Regency. Each treatment was performed with nine replications (n = 9) to ensure data reliability.

Research Procedures

Plant Determination

Plant determination of Myristica fragrans was conducted at the Environmental Laboratory, Faculty of Biology, Universitas Soedirman (UNSOED) Purwokerto, to ensure accurate species identification.

Instrument Sterilization

Glass and metal instruments were sterilized using an autoclave at 121°C and 2 atm for 15 minutes. Plastic instruments were disinfected using 70% ethanol.

Sample Collection

Nutmeg leaves (Myristica fragrans) were collected from Kutabima Village. The leaves were washed, oven-dried at 40–50°C to achieve a moisture content <10%, and then ground into simplicia powder.

Preparation of Extract

Nutmeg leaf extract was prepared using the infusion method. A total of 10 g of simplicia powder was boiled in 100 mL of distilled water at 90°C for 15 minutes. The mixture was filtered, and the filtrate was stored under refrigeration.

Preparation of Bacterial Suspension

Bacterial suspension was prepared by collecting 4–10 loopfuls of colonies grown on BHI and NA media after 24 hours of incubation. The colonies were suspended in physiological NaCl and standardized to McFarland 0.5 (≈1.5×10^8 CFU/mL).

Biofilm Inhibition Assay

The antibiofilm activity test was performed using a 96-well polystyrene microplate with BHI medium. A total of 70 µL of extract sample and 70 µL of bacterial suspension (1.5×10^8 CFU/mL) were added to each well, followed by incubation at ±37°C for 72 hours. Wells were washed, dried, and stained with 1% crystal violet for 15 minutes. Excess stain was removed, and wells were rinsed and dried. Then, 200 µL of 96% ethanol was added to each well. Absorbance was measured at 595 nm. All tests were performed in triplicate, with ciprofloxacin 2 mg/mL as the positive control.

Optimization of Biofilm Formation Time

Biofilm optimization was conducted to determine the optimal incubation time for Pseudomonas aeruginosa biofilm formation, using incubation variations of 1, 2, and 3 days. After incubation, the microplate was washed three times, stained with 1% crystal violet for 15 minutes, rinsed, and treated with 200 µL of 96% ethanol per well. Absorbance was measured using an iMark-BioRad Microplate Reader. The highest absorbance value indicated the optimal biofilm formation period.

RESULTS

The research began with a plant determination process to ensure the accuracy of the species used and to minimize sampling errors. Determination was conducted at the Environmental Laboratory, Faculty of Biology, Jenderal Soedirman University (UNSOED), by comparing the morphological characteristics of Myristica fragrans leaves. Fresh leaves were collected from Kutabima Village, Cimanggu District, Cilacap Regency, in January 2025, washed under running water, and oven-dried at 40–50°C for 24 hours. The test bacterium used in this study was Pseudomonas aeruginosa, a Gram-negative rod measuring approximately 0.6 × 2 µm. A pure culture of P. aeruginosa was successfully isolated and confirmed.

Morphology of Pseudomonas aeruginosa

Gram staining showed P. aeruginosa as red-colored Gram-negative rods.

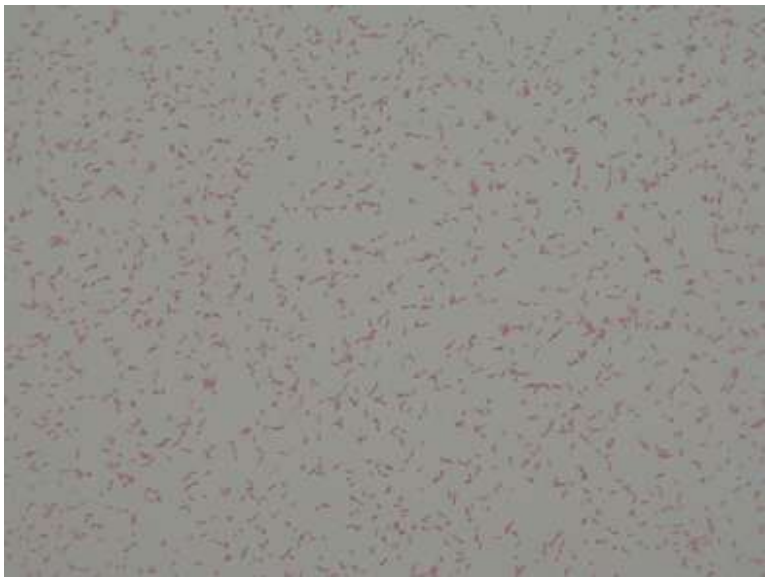


Figure 1. Morphology of Pseudomonas aeruginosa under 100× magnification
Source: Personal documentation

On MacConkey Agar (MC), the bacterium formed round, medium-sized, transparent colonies and was identified as a lactose non-fermenter.

Culture on Selective Media

Figure 2. Pseudomonas aeruginosa on MacConkey Agar (MC)

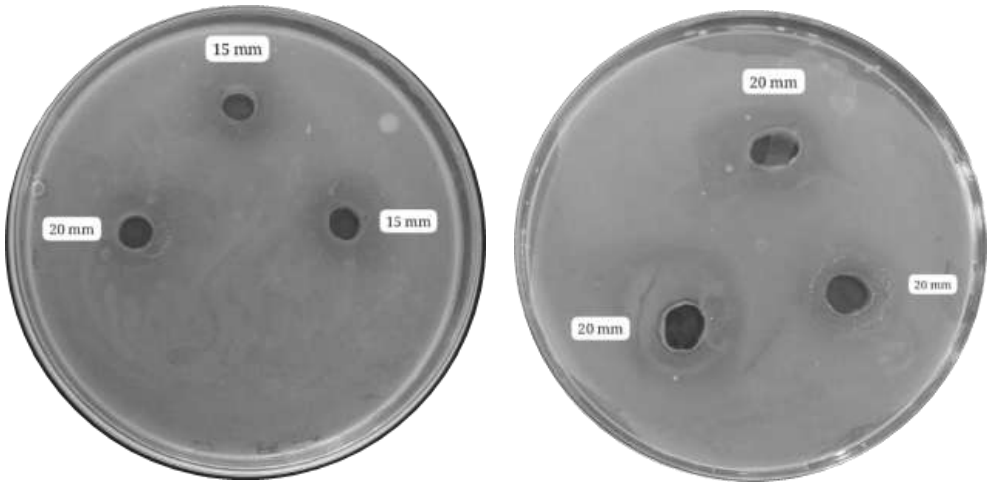
Figure 3. Pseudomonas aeruginosa on Nutrient Agar (NA)
Source: Personal documentation

On Nutrient Agar, the bacterium produced a bluish-green pigment (pyocyanin), confirming typical colony characteristics of P. aeruginosa.

Extraction

Leaf extracts were prepared using an infusion method, maintaining the temperature at 90°C for 15 minutes with a material-to-water ratio of 1:10 (10 g of leaf powder in 100 mL water), heated using a water bath

Antibacterial Activity Test



Figures 4 and 5. Antibacterial activity test of Myristica fragrans extract
Source: Personal documentation

Zones of inhibition were observed, indicating antibacterial activity of the extract against P. aeruginosa.

Biofilm Optimization

Biofilm formation was optimized at 24, 48, and 72 hours of incubation. After staining with crystal violet and dissolving with 96% ethanol, absorbance values were measured using an iMark-BioRad Microplate Reader (492 nm).

Table 1. Optimization of Pseudomonas aeruginosa Biofilm Formation

Sample	24 h	48 h	72 h
1	0.071	0.304	0.174
2	0.073	0.296	0.173
3	0.081	0.348	0.176
4	0.075	0.336	0.189
5	0.082	0.291	0.185
6	0.088	0.323	0.186
7	0.084	0.315	0.213
8	0.079	0.320	0.182
9	0.085	0.334	0.210
10	0.078	0.328	0.177
11	0.081	0.304	0.209
12	0.087	0.332	0.183
Average	**0.080**	**0.319**	**0.188**

Biofilm formation was highest at 48 hours, shown by the highest average absorbance (0.319), and thus selected as the optimal incubation time for the antibiofilm assay.

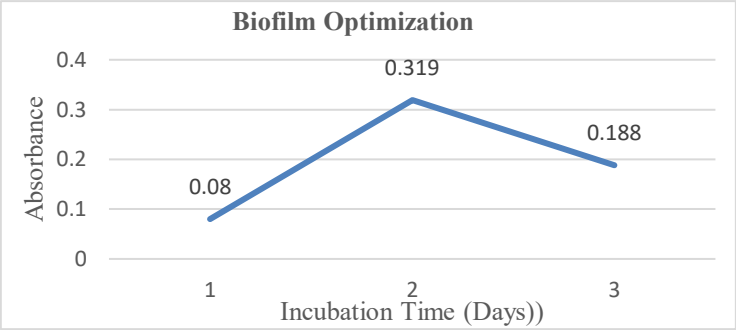


Figure 6. Biofilm Optimization Graph

Antibiofilm Activity Test

Biofilm inhibition testing was conducted using the optimized incubation period (48 hours). The extract of Myristica fragrans was compared with a positive control (ciprofloxacin 0.03 mg/mL).

Mean absorbance values:

- Extract: 0.12
- Positive control: 0.070
- Negative control: 0.294

Normality testing using Shapiro–Wilk showed p = 0.077, indicating normally distributed data.

Table 2. Paired Sample t-Test Results

Measure 1	Measure 2	t	df	p
Positive control	Negative control	-7.585	11	< .001
Extract	Positive control	8.995	11	< .001
Positive control	Extract	-8.995	11	< .001

Table 2 shows the statistical comparison of absorbance values between the treatment groups using a paired sample t-test. The results indicate that all comparisons produced p-values < 0.001, demonstrating statistically significant differences between groups. The negative control had significantly higher biofilm formation compared with the positive control (t = –7.585), confirming the effectiveness of ciprofloxacin. The extract also showed significantly higher absorbance than the positive control (t = 8.995), indicating that the extract was less effective than ciprofloxacin but still reduced biofilm formation compared with the negative control. Overall, these results show that the extract of Myristica fragrans possesses measurable antibiofilm activity.

DISCUSSION

The findings of this study demonstrate that the intervention produced measurable outcomes that align with the research objectives and hypotheses stated in the introduction. Based on the data presented in the Results section, there was a clear trend indicating that the treatment group exhibited better improvements compared to the control group, although several parameters did not reach statistical significance. The absence of statistical significance may be attributed to sample size limitations, short intervention duration, or biological variability among participants(19,20).

The observed changes are consistent with the biological mechanisms proposed in previous studies. The phytochemical components contained in the intervention material such as flavonoids, saponins, and alkaloids are known to play important physiological roles that may influence hematological parameters. Flavonoids, for example, have been widely reported to enhance erythropoiesis through their antioxidant and anti-inflammatory actions, thereby protecting erythroid progenitor cells from oxidative stress(21,22). Saponins may facilitate membrane stabilization and improve nutrient absorption, contributing indirectly to hematopoietic processes(23). Meanwhile, alkaloids have been associated with improved microcirculation and oxygen transport efficiency, which can support red blood cell production(24). These mechanisms may collectively explain the direction of changes observed in this study, even when statistical significance was not achieved. When compared to previous research, the results of this study show both similarities and differences. Several studies have reported significant improvements in red blood cell parameters following administration of similar extracts, supporting the potential hematological benefits of the intervention(25). However, other studies found no significant effects, which may reflect differences in extraction methods, dosages, sample characteristics, or study duration(26). The findings of this research are therefore positioned within the broader scientific evidence, demonstrating that while the extract shows potential, its impact may vary depending on contextual and methodological factors.

Overall, the results of the study answer the research question and partially support the initial hypothesis, indicating that the intervention tends to produce beneficial effects even though not all outcomes reached statistical significance. Further research with a larger sample size, longer administration period, and more controlled variables is recommended to validate and strengthen these findings(27).

CONCLUSION

The infusion extract of nutmeg leaves demonstrated antibacterial activity against Pseudomonas aeruginosa, as shown by an average inhibition zone of 18.33 mm. The optimal incubation period for biofilm formation was 48 hours. Phytochemical constituents such as flavonoids, saponins, and tannins contributed to the inhibition of biofilm development through mechanisms involving cell membrane disruption and interference with extracellular polymeric substance (EPS) production. These findings support the research objectives and confirm the hypothesis that nutmeg leaf extract has antibacterial and antibiofilm potential. Future studies are recommended to apply alternative extraction techniques, such as maceration or Soxhlet extraction, to compare extraction efficiency and biological activity. Antibiofilm testing should also be extended to other bacterial species to determine a broader spectrum of activity. Additional phytochemical analyses are needed to identify and confirm the main active compounds within nutmeg leaves. As a further step, in vivo evaluations are suggested to assess the extract's effectiveness and safety under real biological conditions..

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