Journal of Indonesian Medical Laboratory and Science
pISSN 2775-0108 || eISSN 2774-2504

Inhibitory Effect of Nutmeg (*Myristica fragrans*) Leaf Extract on Biofilm Formation by Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Fahri Ramadhan[1*], Yusuf Eko Nugroho[1], Imam Agus Faizal[1]

[1] Bachelor of Applied Medical Laboratory Technology Study Program, Universitas Al-Irsyad, Cilacap, Indonesia

Correspondence
Fahri Ramadhan
JL. Cerme No.24 Cilacap,Central Java 53223
Email: ramadanfahri120@gmail.com

Received: 2025-07-21
Revised: 2025-10-09
Accepted: 2025-10-04
Available online: 2025-10-10

DOI:
https://doi.org/10.53699/joimedlabs.v5i2.235

Citation
Ramadhan Faahri, Nugroho E.Y., Faizal A.I., (2025). Inhibitory Effect of Nutmeg (*Myristica fragrans*) Leaf Extract on Bioflm Formation by Methicillin-Resistant *Staphylococcus aureus* (MRSA). Journal of Indonesian Medical Laboratory and Science,6(2),122-131.
https://doi.org/10.53699/joimedlabs.v5i2.25

Abstract

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is an opportunistic pathogen with a strong capacity for biofilm formation, which enhances resistance to antibiotics. Although nutmeg (*Myristica fragrans*) seeds and mace have been extensively studied, research on nutmeg leaves is limited despite their content of flavonoids, tannins, saponins, and triterpenoids with antimicrobial potential. *Objective:* This study evaluated the antibiofilm activity of nutmeg leaf extract against MRSA biofilm formation in vitro. Materials and Methods: Biofilm assays were conducted using MRSA isolates. The optimal incubation time for biofilm formation was first determined, followed by treatment with nutmeg leaf extract. *Results:* MRSA formed optimal biofilms at 48 h (OD = 0.101 ± 0.012). Nutmeg leaf extract significantly reduced biofilm formation (OD = 0.083 ± 0.010) compared with the negative control (OD = 0.118 ± 0.009) and the positive control, tetracycline (OD = 0.096 ± 0.011) ($p = 0.001$). While the reduction was statistically significant, the difference from tetracycline was modest. *Conclusion:* Nutmeg leaf extract demonstrated significant antibiofilm activity against MRSA in vitro. These findings support its potential as a complementary natural agent for managing biofilm-associated infections, warranting further studies to isolate active compounds and assess synergistic effects with standard antibiotics.

Keywords

Biofilm, Flavonoids, MRSA, *Myristica fragrans*

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen responsible for a wide spectrum of infections, from superficial skin diseases to life-threatening conditions such as pneumonia, septicemia, and endocarditis. Its strong biofilm-forming capacity exacerbates resistance to antibiotics and immune defenses, contributing to high morbidity and mortality worldwide(Mishra et al., 2024; Wu et al., 2024). Biofilm-associated infections are estimated to account for up to 80% of human infections, with increased tolerance to antimicrobial agents and frequent treatment failure (Cangui-Panchi et al., 2022; Silva et al., 2021).

Given these challenges, natural products have attracted attention as potential antibiofilm agents.

Several plant extracts, including *Pongamia pinnata*, *Coriandrum sativum*, and *Aloe vera*, have demonstrated inhibitory effects on biofilm formation by pathogenic bacteria(Peele et al., 2017). However, studies focusing specifically on antibiofilm activity remain limited.

Nutmeg (*Myristica fragrans*) is traditionally recognized for its anti-inflammatory, antioxidant, and antimicrobial properties. While most studies have investigated nutmeg seeds and mace, the leaves remain underutilized despite containing bioactive compounds such as flavonoids, tannins, saponins, and triterpenoids with antibacterial potential (Ummah, 2019; Wisdyafanny & Silviani, 2023). These phytochemicals may interfere with biofilm formation through mechanisms including membrane disruption, inhibition of nucleic acid synthesis, and modulation of biofilm-associated gene expression.

Thus, the novelty of this study lies in evaluating the antibiofilm activity of nutmeg leaf extract against MRSA. By focusing on the leaves—an underexplored plant part—this research aims to provide new insights into their potential as a complementary strategy for managing MRSA-related infections.

2. Materials and Methods

2.1. Methods

The study was conducted over two months, covering permit acquisition, sampling, laboratory testing, and report preparation. The research took place at the Microbiology Laboratory, Faculty of Pharmacy, Science, and Technology, Al-Irsyad University, Cilacap.

2.2. Materials and Equipment

The tools included an autoclave (B-One Medical Equipment Co., Ltd., China; Model: B-One 23L) and a microplate reader (AMR-100, Allsheng Instrument). The materials used were *Myristica fragrans* (nutmeg) leaves obtained from Cilacap, Central Java, Indonesia; distilled water, MRSA isolates, Mannitol Salt Agar (MSA), Himedia Laboratories, India; Cat. No. M118, Lot No. [0000613432], Blood Agar Plate Himedia Laboratories, India; Cat. No. M073, Lot No. [0000652449]., 1% crystal violet, and 96% ethanol. Tetracycline hydrochloride (Sigma-Aldrich, USA; Cat. No. T7660 was prepared at 0.03 mg/mL as positive control, while physiological NaCl served as a negative control. Nutmeg leaves were processed using the aqueous infusion method. This method was selected to reflect traditional usage, ensure safety, and effectively extract polar compounds such as flavonoids, tannins, and saponins, which are reported to contribute to antibiofilm activity. Experimental Design: A true experimental design with a post-test only control group design

was applied. Three groups were tested: (1) treatment group (nutmeg leaf extract), (2) positive control (tetracycline), and (3) negative control (NaCl physiological solution). Each experiment was conducted with twelve independent replicates (n = 12). Quantitative data (OD values) were expressed as mean ± standard deviation (SD). Data were analyzed using JASP v18. The Shapiro–Wilk test was performed to assess normality. If the data were normally distributed, comparisons between groups were made using a paired-sample t-test; otherwise, the Wilcoxon signed-rank test was applied. A p-value < 0.05 was considered statistically significant

2.3 Nutmeg leaf extract

The extract is made by drying nutmeg leaves and then grinding them using a blender. Once smooth, 10 grams of nutmeg leaf powder is weighed and added to 100 mL of distilled water. The solution is then heated in a water bath at 95°C for 15 minutes. Finally, the residue is filtered using gauze (Winarsih et al., 2019). The infusion method was selected because it represents a simple and widely used traditional technique that is practical, safe, and suitable for extracting polar phytochemicals such as flavonoids, tannins, and saponins. These compounds have been reported to contribute to antibacterial and antibiofilm activities. In addition, the use of hot water as a solvent reflects traditional medicinal practices and avoids toxic organic solvents, making the extract safer for potential therapeutic applications (Bitwell et al., 2023; Verep et al., 2023).

2.4 Optimization of MRSA bacterial biofilm formation

This optimization aims to determine the optimal incubation time for MRSA bacteria in forming a biofilm. The incubation time variations used were 1, 2, and 3 days. After incubation, the microplate was rinsed using running water three times, then 200 µL of 1% crystal violet solution was added to each well and incubated at room temperature for 15 minutes. The microplate was rinsed again with running water three times. Then, 200 µL of 96% ethanol solution was added to each well and incubated at room temperature for 15 minutes. Next, readings were taken using a Microplate Reader. The results of the highest absorbance value were declared as the optimal MRSA biofilm formation (Besan et al., 2023).

2.5 MRSA Bacterial Biofilm Inhibition Test

Antibiofilm activity assays was conducted using a 96-well polystyrene round-bottom microplate with liquid BHI media. A total of 70 µL of sample in media was added to each well, then 70 µL of bacterial suspension in media equivalent to 1.5 x 108 CFU/mL was added to the well containing the sample, incubation was carried out at a temperature of ± 37°C for the optimal time for biofilm formation.

After incubation, the contents of the wells were discarded and the plate was washed with running water and then dried for 15 minutes by inverting the microplate at room temperature. A total of 200 μL of 1% crystal violet solution was added to each well with a staining time of 15 minutes. The contents of the wells were discarded and the wells were rinsed again with running water. The microplate was dried by inverting it at room temperature for one hour. Then, 200 μL of 96% ethanol solution was added to each well on the plate and the optical density was read at a wavelength of 492 nm (Besan et al., 2023). Positive control using Tetracycline with a concentration of 0.03 mg/mL (Rivani et al., 2022)

2.6 Data Analysis

Data analysis was performed by first assessing data normality using the Shapiro–Wilk test. If the data were normally distributed, a parametric paired-sample t-test was applied; otherwise, a non-parametric Wilcoxon signed-rank test was used. Statistical analyses were conducted with JASP v18 software, comparing each treatment group with the respective controls in the bacterial sensitivity assay (Kim, 2014; Talikan & Ajan, 2025).

3 Results and Discussion

3.4 Optimization Assay for Biofilm Formation of MRSA

This optimization aims to determine the optimal incubation time for MRSA bacteria to form a biofilm. The incubation time variations used were 1, 2, and 3 days. After incubation, the microplate was rinsed with running water three times, then 200 μL of 1% crystal violet solution was added to each well and incubated at room temperature for 15 minutes. The microplate was rinsed again with running water three times. Then, 200 μL of 96% ethanol solution was added to each well and incubated at room temperature for 15 minutes. Next, readings were carried out using a Microplate Reader. The highest absorbance value was declared as optimal MRSA biofilm formation (Hernández-Cuellar et al., 2023).

Table 1. Optimization biofilm of MRSA

Sample	Time of Absorbance		
	24 Hours	48 Hours	72 Hours
1	0,091	0,098	0,086
2	0,093	0,091	0,105
3	0,087	0,096	0,09
4	0,086	0,096	0,084
5	0,099	0,101	0,086
6	0,091	0,104	0,085

7	0,088	0,107	0,093
8	0,085	0,115	0,105
9	0,072	0,117	0,097
10	0,072	0,108	0,09
11	0,078	0,102	0,104
12	0,079	0,084	0,086
Average	**0,085**	**0,101**	**0,092**

OD (Optical Density) readings were carried out using an ALLSHENG microplate reader with a wavelength of 492 nm, this refers to the journal (Tobi et al., 2022) which optimized the wavelength using three variations, namely 492 nm, 595 nm, and 655 nm to read biofilm growth which is indicated by the largest OD value (Kaneko et al., 2021).

The table shows the average results of biofilm optimization for various treatments. The average absorbance value for the 24-hour incubation period was 0.085; for the 48-hour incubation period, it was 0.101; and for the 72-hour incubation period, it was 0.092. The magnitude of the values is directly proportional to the biofilm growth rate. Therefore, it can be concluded that MRSA bacteria can form optimal biofilms with a 48-hour incubation period.

3.5 Assessment of Antibiofilm Activity Against MRSA Biofilm Formation

Based on the results of the MRSA antibiofilm activity test conducted with a 48-hour incubation period with the addition of Myristica fragrans extract, the results of the activation test were compared with the positive control using 0.03 mg/ml tetracycline (Rivani et al., 2022). The average absorbance results in the activity test showed absorbance data for the extract test of 0.083; for the positive control of 0.095; and for the negative control of 0.117.

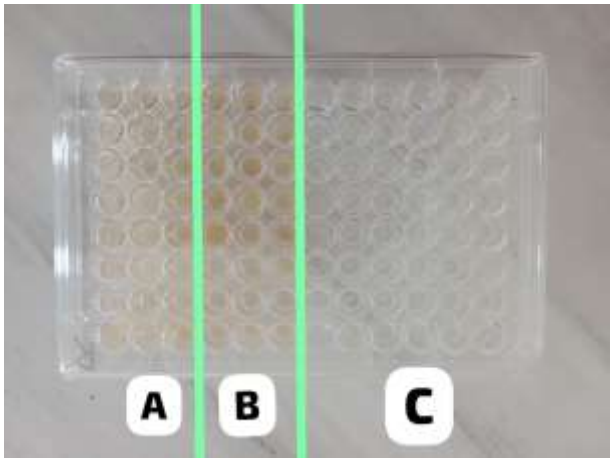

Figure 1. Antibiofilm activity assay of MRSA using the microtiter plate crystal violet method. Three treatment groups are shown: (A) positive control with tetracycline (0.03

mg/mL), (B) negative control with distilled water, and (C) test group with nutmeg (*Myristica fragrans*) leaf extract..

A paired-sample t-test comparing the extract with the positive control yielded a statistically significant difference ($p = 0.001$). The effect size was large (Cohen's $d = 0.85$), with a 95% confidence interval for the mean difference ranging from −0.018 to −0.005 OD units, indicating that the observed reduction in biofilm formation was both statistically and biologically relevant. This result demonstrates that nutmeg leaf extract has significant antibiofilm activity against MRSA. However, given the marginal difference in OD values, further studies are required before drawing conclusions about its comparative efficacy with standard antibiotics.

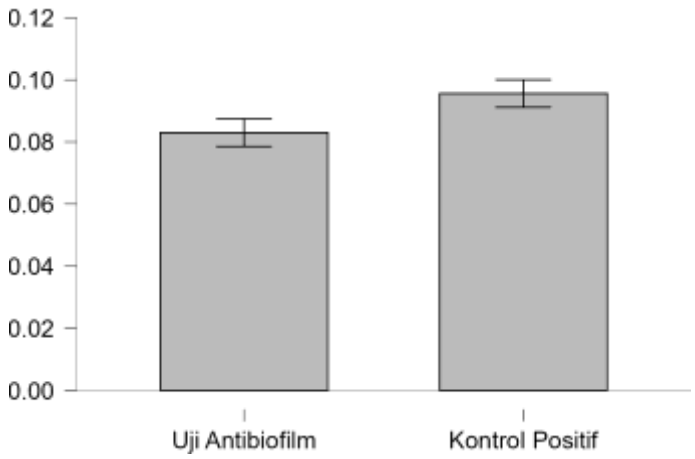

Figure 2. Bar plot paired sample T-test

Flavonoid compounds have been shown to physically disrupt the binding process of BAP (biofilm associated protein) and BAP polymerase, thereby inhibiting the formation of the MRSA bacteria biofilm (Fawwaz et al., 2019). The effects of tannins include reducing biofilm slime production, reducing the expression of the icaA and icaD genes, thus disrupting polysaccharide production, and inhibiting the quorum-sensing regulator RNAIII (Aboelnaga et al., 2024).

Saponin compounds can form complex compounds with cell membranes through hydrogen bonds, resulting in damage to the protein structure, thus unbalancing the permeability of the cell membrane and causing cell lysis (Santajit et al., 2024). Triterpenoid compounds have been shown to degrade biofilms, thereby reducing their formation and killing bacteria within the formed biofilm (Rahmadeni et al., 2019).

Our findings are in line with recent reports on plant-derived antibiofilm activity. For

example, Gallic acid (GA) has been shown to disrupt MRSA biofilms both in formation and established stages via structural degradation (Santajit et al., 2024). Isolated flavonoids have also demonstrated antibiofilm effects on *Staphylococcus* spp (Majnooni et al., 2023), reinforcing the mechanistic plausibility of the flavonoid content in nutmeg leaves. Moreover, polifenol-rich wild edible plants displayed potent antibiofilm activity against MRSA in Mediterranean studies (Donati et al., 2025). In addition, *Paederia foetida* leaf extract was reported to inhibit MRSA biofilm and downregulate quorum sensing genes (Sang et al., 2024). Compared with these, our extract showed a modest yet significant effect; this suggests that while nutmeg leaf extract is promising, future work should include mechanistic evaluations (e.g., gene expression, EPS quantification) and isolate active compounds to enhance potency.

4 Conclusions

This study demonstrated that nutmeg (*Myristica fragrans*) leaf extract significantly inhibited biofilm formation by MRSA in vitro. The extract reduced optical density values compared with both negative and positive controls, indicating that the phytochemicals present in the leaves—such as flavonoids, tannins, saponins, and triterpenoids may have a main role in antibiofilm activity. These findings highlight the potential of nutmeg leaves, an underutilized part of the plant, as a source of bioactive compounds with relevance for managing biofilm-associated infections.

While these findings support the potential of nutmeg leaf extract as a natural source of antibiofilm compounds, the observed differences with standard antibiotics such as tetracycline were modest. Therefore, the extract should be considered a promising complementary therapeutic candidate rather than a substitute for established antibiotics. Further studies are recommended to isolate active compounds, assess their mechanisms of action, and evaluate safety and efficacy in *in vivo* models.

The strengths of this study include the use of twelve replicates to improve statistical reliability and the application of standardized biofilm assays to assess activity. Nevertheless, several limitations should be acknowledged. First, the extraction method employed was limited to aqueous infusion, which may not fully capture the activity of non-polar metabolites. Second, the study was restricted to in vitro conditions, which do not fully represent the complexity of host environments. Finally, the OD differences compared to tetracycline, although statistically significant, were relatively modest and may not reflect strong clinical.

Future studies should aim to isolate and characterize the specific active compounds responsible for antibiofilm activity in nutmeg leaves. Further investigations should also explore different extraction methods to maximize yield of bioactive compounds, evaluate potential synergistic effects with conventional antibiotics, and assess toxicity and efficacy in in vivo models. Such studies would help to establish nutmeg leaf extract not only as a complementary therapeutic candidate but also as a scientifically validated option for managing MRSA biofilm-related infections.

Acknowledgments: Thanks are addressed to the supervisor and D4 TLM Al-Irsyad University Cilacap, both directly and indirectly, who have provided ideas and support to the author so that this article can be completed

Funding: None

Conflicts of Interest: There is no conflict of interest in this research

Author Contributions: FR : Conceptualize and implement research and data analysis. YEN and IAF: Evaluate and review research until it becomes a draft manuscript.

5 References

Aboelnaga, N., Elsayed, S. W., Abdelsalam, N. A., Salem, S., Saif, N. A., Elsayed, M., Ayman, S., Nasr, M., & Elhadidy, M. (2024). Deciphering the dynamics of methicillin-resistant *Staphylococcus aureus* biofilm formation: From molecular signaling to nanotherapeutic advances. *Cell Communication and Signaling*, 22(1). BioMed Central Ltd. https://doi.org/10.1186/s12964-024-01511-2

Besan, E. J., Rahmawati, I., & Saptarini, O. (2023). Antibiofilm Activity of Extracts and Fractions of Butterfly Pea Flowers (Clitoria ternatea L.) against *Staphylococcus aureus*. *PHARMACY: Indonesian Pharmaceutical Journal*, 20(1), 1. https://doi.org/10.30595/pharmacy.v0i0.14437

Bitwell, C., Sen, S., & Luke, C. (2023). A review of modern and conventional extraction techniques and their applications for extracting phytochemicals from plants. Scientific African, 19, e01585. https://doi.org/10.1016/j.sciaf.2023.e01585

Cangui-Panchi, S. P., Ñacato-Toapanta, A. L., Enríquez-Martínez, L. J., Reyes, J., Garzon-Chavez, D., & Machado, A. (2022). Biofilm-forming microorganisms causing hospital-acquired infections from intravenous catheters: A systematic review. *Current Research in Microbial Sciences*, 3(November). https://doi.org/10.1016/j.crmicr.2022.100175

Donati, E., Ramundi, V., Nicoletti, I., Righetti, L., Cimini, S., De Gara, L., & Mariani, F. (2025). *Bioprospecting of six polyphenol-rich Mediterranean wild edible plants reveals antioxidant, antibiofilm, and bactericidal properties against methicillin-resistant Staphylococcus aureus.*, 1–18.

Fawwaz, M., Nurdiansyah, S., & Baits, M. (2019). Potential of Nutmeg Leaves (Myristica fragrans Houtt) as a Source of Phenolic Compounds. *Indonesian Phytopharmacy Journal*, 4(1).

Hernández-Cuellar, E., Tsuchiya, K., Valle-Ríos, R., & Medina-Contreras, O. (2023). Differences in biofilm formation by methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. *Diseases*, 11(4). MDPI. https://doi.org/10.3390/diseases11040160

Kaneko, H., Nakaminami, H., Ozawa, K., Wajima, T., & Noguchi, N. (2021). In vitro anti-biofilm effect of anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) agents against the USA300 clone. *Journal of Global Antimicrobial Resistance*, 24, 63–71. https://doi.org/10.1016/j.jgar.2020.11.026

Kim, H. (2014). *Statistical notes for clinical researchers: Nonparametric statistical methods: 1. Nonparametric methods for comparing two groups.*, 7658, 235–239.

Majnooni, M. B., Ghanadian, S. M., Mojarrab, M., Bahrami, G., Mansouri, K., Mirzaei, A., & Farzaei, M. H. (2023). Activities of flavonoids isolated from Allium colchicifolium leaves. 2023. https://doi.org/10.1155/2023/5521661

Mishra, A., Aggarwal, A., & Khan, F. (2024). Medical device-associated infections caused by biofilm-forming microbial pathogens and controlling strategies. *Antibiotics*, 13(7), 1–16. https://doi.org/10.3390/antibiotics13070623

Peele, A., Jagarlapoodi, S., & Vekateswarulu, T. C. (2017). *Investigation of the potential antibiofilm activities of plant extracts*. September 2017.

Rahmadeni, Y., Febria, F. A., & Bakhtiar, D. A. (2019). Potential of Pakih Sipasan (Blechnum orientale) as an antibacterial agent against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. *Journal of Biological Sciences*, 6(2), 224–229. https://doi.org/10.24843/metamorfosa.v06.i02.p12

Rivani, E., Arfijanto, M. V., & Widodo, A. D. W. (2022). Vancomycin for methicillin-resistant *Staphylococcus aureus* biofilm eradication is associated with the emergence of heterogeneous vancomycin-intermediate *Staphylococcus aureus*. *International Journal of Health Sciences*, 811–818. https://doi.org/10.53730/ijhs.v6ns9.12536

Sang, H., Jin, H., Song, P., Xu, W., & Wang, F. (2024). Gallic acid exerts antibiofilm activity by inhibiting methicillin-resistant *Staphylococcus aureus* adhesion. *Scientific Reports*, 1–11. https://doi.org/10.1038/s41598-024-68279-w

Santajit, S., Tunyong, W., Horpet, D., Binmut, A., Kong-Ngoen, T., Wisessaowapak, C., Thavorasak, T., Pumirat, P., & Indrawattana, N. (2024). Unveiling the antimicrobial, anti-biofilm, and anti-quorum-sensing potential of Paederia foetida Linn. leaf extract against *Staphylococcus aureus:* An integrated in vitro–in silico investigation. *Antibiotics*, 13(7). https://doi.org/10.3390/antibiotics13070613

Silva, V., Almeida, L., Gaio, V., Cerca, N., Manageiro, V., Caniça, M., Capelo, J. L., Igrejas, G., & Poeta, P. (2021). Biofilm formation of multidrug-resistant MRSA strains isolated from different types of human infections. *Pathogens*, 10(8). https://doi.org/10.3390/pathogens10080970

Talikan, A., & Ajan, R. (2025). On paired samples t-test: Applications, examples and limitations. March. https://doi.org/10.5281/zenodo.10987546

Tobi, C. H. B., Saptarini, O., & Rahmawati, I. (2022*)*. Antibiofilm activity of extracts and fractions of areca nut seeds (Areca catechu L.) against *Staphylococcus aureus* ATCC 25923. *Journal of Pharmaceutical Science and Clinical Research (JPSCR)*, 7(1), 56. https://doi.org/10.20961/jpscr.v7i1.43698

Ummah, M. S. (2019). Potential of nutmeg leaf extract (*Myristica fragrans Houtt*) as a natural preservative on the quality of broiler chicken meat. *Fillia Cendekia Journal*, 11(1), 1–14. https://doi.org/10.32503/fillia.v5i2.1170

Verep, D., Ateş, S., & Karaoğul, E. (2023). A review of extraction methods for obtaining bioactive compounds in plant-based raw materials. *Bulletin of Advanced Research*, 25(3), 492–513. https://doi.org/10.24011/barofd.1303285

Winarsih, S., Khasanah, U., & Alfatah, A. H. (2019). Antibiofilm activity of ethyl acetate fraction of Mimosa pudica leaf extract against methicillin-resistant *Staphylococcus aureus (MRSA*) in vitro. *Health Journal*, 6(2), 76–85. https://doi.org/10.21776/ub.majalahkesehatan.006.02.1

Wisdyafanny, M. W., & Silviani, Y. (2023). Antibacterial effectiveness test of nutmeg leaf ethanol extract (Myristica fragrans Houtt) against *Staphylococcus epidermidis*. *Journal of Pharmacy*, 12(1). Morch.

Wu, X., Wang, H., Xiong, J., Yang, G. X., Hu, J. F., Zhu, Q., & Chen, Z. (2024). *Staphylococcus aureus* biofilm: Formulation, regulatory, and emerging natural product-derived therapeutics. *Biofilm*, *7*. *Elsevier B.V*. https://doi.org/10.1016/j.bioflm.2023.100175