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Function of *Moringa oleifera* Lamk Leaf Extract as an Antiseptic for *Pseudomonas aeruginosa* and *Staphylococcus aureus* using Percentage Kill Method

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Abstract
Moringa oleifera Lamk leaf extract has an antimicrobial properties especially against fungal, parasite, Gram positive and negative bacteria by inhibiting DNA synthesis and metabolism an damaging cell walls. The aim of this study was to determine whether *M. oleifera* Lamk leaf extract is effective as an antiseptic against *P. aeruginosa* and *S. aureus* bacteria. Percentage Kill was used in the study to determine the percentage of bacteria death after contact with *M. oleifera* Lamk leaf extract in the 1st, 2nd and 5th minutes against the control and treatment simultaneously. The test is considered to meet the standard if each contact gives a result of ≥90%. For discovered percentage kill to *P. aeruginosa* bacteria in the 1st, 2nd and 5th minutes each test showed a yield of 27.12%. , 47.01%, 57.7%. In the third time of *P. aeruginosa* did not reach the standard. Whereas in *S. aureus* bacteria, each test showed a yield of 92.36%, 95.58% and 96.45%, where it was seen that all results reach the standards criteria of ≥90%. *M. oleifera* Lamk leaf extract was not effective in eliminating *P. aeruginosa* for all contacts because the standard value was below 90%. Whereas for *S. aureus* bacteria, *M. oleifera* Lamk leaf extract was very effective in eliminating bacteria for all contact times ≥90% with the highest value at the 5th minutes (96.45%).

Keywords: *moringa oleifera leaf , pseudomonas aeruginosa, staphylococcus aureus, percentage kill*

Fungsi Ekstrak Daun *Moringa oleifera* Lamk sebagai Antiseptik pada *Pseudomonas aeruginosa* dan *Staphylococcus aureus* Menggunakan Metode Percentage Kill

Abstrak
Tanaman Moringa oleifera Lamk memiliki sifat antimikroba terhadap jamur, parasit, dan bakteri Gram positif maupun Gram negatif melalui penghambatan sintesis dan metabolisme DNA serta merusak dinding sel. Penelitian ini bertujuan untuk mengetahui apakah ekstrak daun M. oleifera Lamk efektif sebagai antiseptik terhadap bakteri P. aeruginosa dan S. aureus. Dalam penilitian ini digunakan metode Percentage Kill untuk mengetahui besarnya persentase kematian terhadap bakteri setelah dilakukan kontak dengan ekstrak daun M. oleifera Lamk pada menit ke-1, ke-2, dan ke-5 terhadap kontrol dan perlakuan secara bersamaan. Uji dinyatakan memenuhi standar jika setiap kontak memberikan hasil ≥90%. Adapun uji terhadap P. aeruginosa pada menit ke-1, ke-2, dan ke-5 masing-masing menunjukkan hasil 27,12%, 47,01%, dan 57,7%. Ketiga perlakukan tersebut tidak memenuhi standar. Sementara itu, pada S. aureus masing-masing menunjukkan hasil 92,36%, 95,58%, dan 96,45%, seluruh perlakuan menunjukkan hasil memenuhi standar. Dari penelitian dapat disimpulkan, ekstrak daun M. oleifera Lamk tidak efektif dalam mengeliminasi bakteri P. aeruginosa untuk semua waktu kontak karena nilai standar di bawah 90%. Adapun pada bakteri S. aureus ekstrak daun M. oleifera Lamk sangat efektif mengeliminasi bakteri untuk semua waktu kontak dengan nilai tertinggi pada menit ke-5 (96,45%).

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Kata Kunci: *Ekstrak daun Moringa oleifera, Pseudomonas aeruginosa, Staphylococcus aureus, Percentage kill.*

Introduction

M. oleifera belongs to the family Moringaceae, can be easily found around Africa and Asia. *M. oleifera* has a variety of active components which include phenolic acids, vitamins, flavonoids, saponins, isothiocyanates, and tannins. *M. oleifera* leaves contain high amounts of vitamin C, beta-carotene, polyphenols, and vitamin E and are also a source of antioxidants. *M. oleifera* is known to have pharmacological value and is useful as an antibacterial. WHO states that in developing countries 80% of the population choose to use herbs as the main therapy that underlies modern medicine. *M. oleifera* was chosen because it has antibacterial or antiseptic properties and is safe to use for humans and animals. Meanwhile, *M.oleifera* leaves have a high active content for chronic diseases such as diabetes, high blood pressure, anti-cancer, hypercholesterolemia, anti-inflammatory, insulin resistance, neuroprotective functions, and many others.[1]

WHO defines an antiseptic as a disinfectant that can destroys or inhibits the development of microorganisms present in tissues, does not have the potential to cause harm and has an effect on the body. Antiseptics played a significant role in inhibiting or slowing down the growth of microorganisms in the midst of the COVID-19 pandemic and even being able to kill them. In order to lower the risk of infection, antiseptics are generally used when dealing with wounds, as well as during surgery, certain procedures or washing hands.[2]

P. aeruginosa is a Gram-negative pathogen commonly found in the environment that has become an important cause of infection in humans and can be associated with significant morbidity and mortality. This microorganism is one of the most frequent and severe causes of hospital-acquired infections, particularly affecting immunocompromised (especially neutropenic) and intensive care unit (ICU) patients.[3,4]

S. aureus is a Gram-positive bacteria a crucial pathogen for humans which causes diverse kinds of clinical manifestations. *S. aureus* not only originated from the environment but it also can be found in normal human flora. Infections that are originate from *S. aureus* are infective endocarditis, bacteremia, septic arthritis, soft tissue infections, skin infections, prosthetic device infections, osteomyelitis, gastroenteritis, pulmonary infections, toxic shock syndrome, meningitis, and infections of the urinary tract.[5]

Based on the high rate of infection caused by *P. aeruginosa* and *S. aureus* both in the community and in hospitals, the use of antiseptics is important to prevent these bacteria from manifesting more severely. The high burden felt in developing countries coupled with an increase in disease infections has caused people to seek alternative treatments using herbal plants. *M. oleifera* which is easy and can be found in developing countries is the right choice. To prove the efficacy of *M. oleifera* as an antiseptic against *P. aeruginosa* and *S. aureus* would evaluated using the percentage kill test. This study aims to find out how the *M. oleifera* leaf extract used functions as an antiseptic and can treat infections caused by *P. aeruginosa* and *S. aureus* bacteria.

Methodology

Extract preparation. Preparation of M. *oleifera* Lamk leaf carried out at The Department of Medical Pharmacy, Medical Faculty Universitas Indonesia. With the total weight of 120.8495 g Extract of *M. oleifera*, it consists of 15.30% level of water. To find out about the pure percentage of the extract, the water percentage is by substracting 100% with the water percentage, which equals to 84.70%. Furthermore, to get the consentration of the pure extract, the pure percentage should be divided with 100 mL with the result of 847 mg/mL. From this result, it can be interpreted that in 1 mL of the extract equals to 847 mg of pure *M. oleifera* extract. This study will be using a concentration of 800 mg/mL and volume 50 mL before dilution by using CMC (Carboxymethylcelllulose) Na 1%. Prepare by weighed 1 g of CMC and prepare 100 mL aquadest and heat it, insert the CMC into the beaker glass and add 50 mL of warm water, then mixed it until it changed into clear. After that, add another 50 mL of warm water. Then to know about how much volume of pure extract is there in 50 mL of *M. oleifera* extract calculation is needed where the result of the volume is 47.2 mL. Which means the extract that is used is 47.2 mL + CMC 1% 2.8 mL, yet to change the volume into gram (g), the result of the volume will be calculated again with the result of 40 mg. In conclusion the extract 40 mg will be diluted with CMC 1% 2.8 mL.

Bacterial preparation. *P. aeruginosa* and *S. aureus* bacteria from the Microbiology Laboratory, Department Microbiology Clinic, FKUI should be re-cultured on nutrient and blood agar in 18-24 hours. Following that, re-cultured bacteria should be equalized for turbidity up to 0.5 Mc-Farland. These bacteria that has been through this process which will be used for percentage kill test.

Sampling technique. The procedure is add one loop of inoculating microorganism to 10 mL Tryptic Soy Broth (TSB) and incubate at 35°C for about 18-24 hours. A ten-fold serial dilution of the suspension in TSB up to 9 tubes is performed aseptically using the micropipette, For each dilution, transfer 1 ml to two sets of Petri dishes. To each of the two plates, add a 15 mL aliquot of molten and cooled agar. To obtain a uniform distribution and allow the plates to be solid, swirl the plates gently. Incubate all plates at 35°C for 24-48 hours in an inverted position, Count the amount of colonies on each plate by using the electronic colony counter. Count only plates that are statistically valid and contain between 200 and 300 colonies. This colonies will be continued to percentage kill test

Percentage kills. Add 0.5 mL of microorganism to 4.5 mL of sterile water, Transfer 1 mL to 9 mL of sterile water after 1st minute, 2nd and 5th minutes (thrice repetition), respectively. Add 15 mL of molten plate Count Agar (PCA) to each of the two dishes, allow to uniform distribution and to be solid, swirl the plates gently then incubate in an inverted position at 35°C for 24-48 hours, Counted the amount of colonies on each plate using the electronic colony counter.[7,8] This method is being use to see the percentage of the leave extract in inhibiting *P. aeruginosa* and *S. aureus* growth with contact time of 1st, 2nd, and 5th minutes. The result of the research will show the effect of different contact time with the extract to the growth of *P. aeruginosa* and *S. aureus*.[9,10,11]

Percentage Kill = (C–X)/C x 100%
C : Total colony of control
X :Total colony of *M.oleifera* mixture
Significant percentage kill is achieved with ≥90% .

Result

Based on experiments that have been carried out using the percentage kill method with three repetitions at the 1st, 2nd and 5th minutes, the optimal concentration of the second bacteria was obtained on *P. aeruginosa* and *S. aureus* was 10^5. The results of the dilution of *P. aeruginosa* with *M. oleifera* leaf extract in control with three repetitions and contact time. Result of Percentage kill procedure with contacts time control for *P. aeruginosa* in the 1st minute were 155, 145, and 137. For the 2nd minute were 139, 135, and 129. Whereas for contact in the 5th minute were 130, 122, and 115. In the treatment, the number of colonies *P. aeruginosa* with *M. oleifera* leaf extract (three repetition) for contact time in the 1st minute were 155, 83, and 68. for the 2nd minute contact were 108, 71, and 53. while the 5th minute contact was 86, 59, and 40 can be seen in table.1. the chart bar represents the average growth of *P. aeruginosa* colonies in the 1st, 2nd, and 5th minutes for control and treatment. A significant difference can be observed every minute, so that it can be stated that *M. oleifera* leaf extract can inhibit the growth of P. *aeruginosa* with a yield of more than 90%. (Figure 1).

Table 1. The average number of P. *aeruginosa* bacterial colony growth every minute as well as experimental repetitions for both control and treatment with *M. oliefera* leaf extract

Time	Colony Count (Control/C)			Average Colony Growth (Control/C)	Colony Count (Treatment/T)			Average Colony Growth (Treatment/T)
	I	II	III		I	II	III	
1 Minute	155	139	130	141.33	155	108	86	103
2 Minutes	145	135	122	134	83	71	59	71
5 Minutes	137	129	115	127	68	53	40	53.67

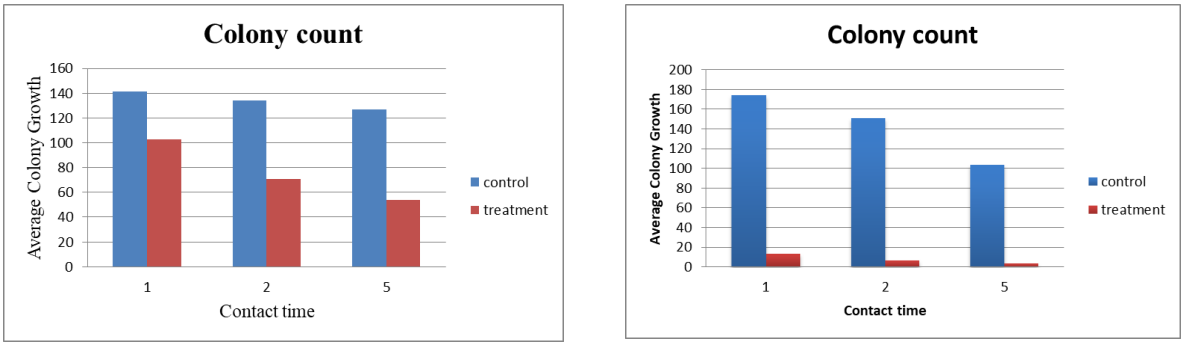
Colony count (C and T)= I: First repetion; II: second repetion and III: Thrid repitition
C= Total average colony growth control
T= Total average colony growth treatment

Table 2. The average number of *S. aureus* bacterial colony growth every minute as well as experimental repetitions for both control and treatment with *M. oliefera* leaf extract

Time	Colony Count (Control/C)			Average Colony Growth (Control/C)	Colony Count (Treatment/T)			Average Colony Growth (Treatment/T)
	I	II	III		I	II	III	
1 Minute	194	180	150	174.67	17	13	10	13.33
2 Minutes	165	148	100	151	8	7	5	6.67
5 Minutes	115	100	95	103.33	6	3	2	3.67

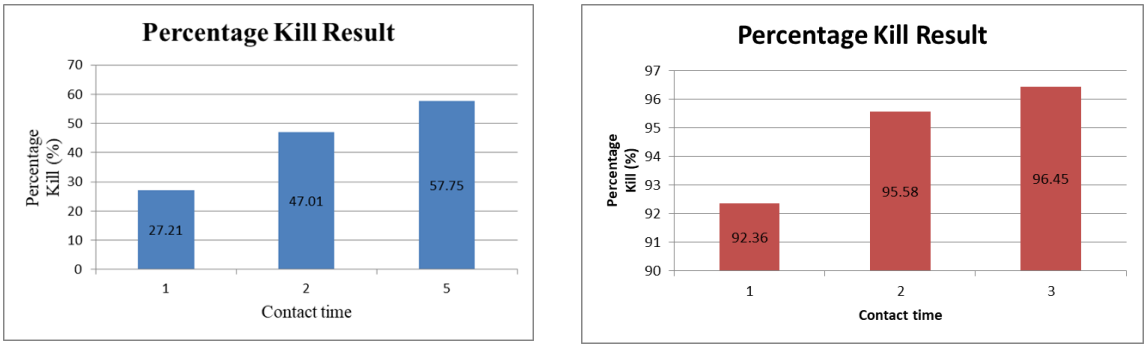
Colony count (C and T)= I: First repetion; II: second repetion and III: Thrid repitition
C= Total average colony growth control
T= Total average colony growth treatment

Result of Percentage kill procedure with contacts time for control with *S. aureus* in the 1st minute were 194, 165, and 115. For the 2nd minute were 180, 148, and 100. Whereas for contact in the 5th minute were 150, 100 and 95. In the treatment, the number of colonies *S. aureus* with *M. oleifera* leaf extract (three repetition) for contact time in the 1st minute were 17, 8, and 6. for the 2nd minute contact were 13, 7, and 3. while the 5th minute contact was 10, 5 and 2 can be seen in Table 2, the chart bar represents the average growth of *S. aureus* colonies in the 1st, 2nd, and 5th minutes for control and treatment. A significant difference can be observed every minute, so that it can be stated that *M. oleifera* leaf extract cannot inhibit the growth of *S. aureus* with a yield of less than 90% (Figure 1).



(A) (B)

Figure 1. Diagram average number of *P. aeruginosa* (A) and *S. aureus* (B) bacterial colony growth every minute as well as experimental repetitions for both control and treatment with *M. oliefera* leaf extract



(A) (B)

Figure 2. Diagram Percentage Kill results from of *P. aeruginosa* (A) and *S. aureus* (B) with *M. oliefera* leaf extract

Based on calculations using Percentage Kill with contact time in the 1st, 2nd, and 5th minutes respectively for the results of the Percentage Kill from *Pseudomonas aeruginosa* bacteria are in the following 27.21%, 47.01%, 57.75%. and the results of the Percentage Kill from *Staphylococcus aureus*

bacteria are in the following 92.36%, 95.58%, 96.45%. Therefore, the outcome of the Percentage Kill can be considered as good due to the numbers exceeding 90%. Based on the contact results, it can be stated that the longer contact time, will be given the higher value of the Percentage Kill. This value was found in *S. aureus* bacteria and has shown a higher percentage level increase (above 90%), so it can be said that *M. oliefera* leaf extract has provided benefits in terms of inhibiting the growth of S. *aureus* by increasing level 96.45% in the 5th minute. Percentage Kill results can be interpreted as good because it exceeds ≥ 90%. Whereas for *P. aeruginosa* from the 1st until 5th minutes does not reach the minimum requirement of 90%. This means that *M. oleifera* leaf extract does not have an eradication level against *P. aeruginosa*

Discussion

This research proves the existence of antimicrobial properties of *M. oleifera* leaf extract to inhibit the growth of *P. aeruginosa* and *S. aureus*. This can be seen from the growth of bacterial colonies on each agar plate and the replicates in the control and treatment. Based on the average colony growth in each replicate, it can be stated that *M. oleifera* leaf extract has the ability to inhibit bacterial growth. The hypothesis based on these results states that *M. oleifera* leaf extract has antiseptic properties that can slow down the growth rate *S. aureus* bacteria based on several possible mechanisms of action.

For *P. aeruginosa* from the first minute was never reached 90%, the percentage since 1st, 2nd and 5th minutes were the smallest with an average of 73.3 It means that the bacteria has been killed below 90% of the bacteria colonies or that *M. oleifera* is not effective enough to be used as a disinfectant. Though it's a small number, *M. oleifera* still gives an effect of decreasing the number of *P. aeruginosa* in every contact time. This might be the result of the compound that is contained by *M. oleifera* such as tripernoid, saponin, and tanin, that is able to disrupts the bacteria membrane. Tanin especially, has the ability to inactivate the adhesive substances that is produced by enzymes, microbes, and transport protein in cell membranes. Antioxidants such as cytosterol and glucopyranoside are also found in *M. oleifera*.[12] Another kind of antioxidant that is found in *M. oleifera*, 300 Flavonoid, is able to damage the permeability of bacterial cell walls, they are also considered as lipophilic, meaning that there are more chances of them damaging the cell walls of *P. aeruginosa*.[13,14]

The percentage kill test result shows that *M. oleifera* is able to hinder the growth of *P.aeruginosa* most effectively on the 5th minute is 57.74% effective. This finding is proven just the same as research done by Widowati *et al*. (2014) where they also concluded that 50% of *M. oleifera* extract concentration is the most effective as the antibacterial solution for *P. aeruginosa*.[13] Dewangan *et al*. (2010) improvised their trial by using another part of *M. oleifera* which is their root bark. Their trial used disc diffusion method and included several gram-negative and gram-positive bacteria. They also separate the extracts to different parts which are Methanol, acetone, chloroform, Ethyl acetate, and aqueous. They also included Ciprofloxacin, to estimate the sensitivity of the bacteria. In this trial, ethyl acetate and acetone of *M. oleifera* is found to show the most antibacterial activities against the bacteria including *P. aeruginosa*. [15] Abdalla *et al*. (2016) mentioned that clinically isolated *P. aeruginosa* can't be effectively eradicated using *M. oleifera* as it shows no inhibition zone in their test.[16]

Despite the ineffectiveness of *M. oleifera* antibacterial effect on *P. aeruginosa*, we can still maximize the antibacterial capability of *M. oleifera* by mixing it with another material. Proven by Wajdi *et al*. (2017), they found out through their research that mixing *M. oleifera* with *M. calabura* could significantly increase the diameter of their *P. Aeruginosa*, trial inhibition zone compared to a single *M. oleifera* treatment.[17]

For *S. aureus* bacteria, *M. oleifera* is also proven by the Percentage Kill results where from the first minute it already reached above 90%, the percentage of minute 1,2 and 5 are 92.36 %, 95.58 % and 96.45 % (Figure 2). This means that the bacteria has been killed for more than 90% of the bacteria colonies. Furthermore, we can also compare it with study conducted by Subhan *et al*., in 2019 about effectivity of handrub according to *World Health Organization* (WHO) in 2010 that formula to kill *Staphylococcus epidermidis, Escherichia coli*, Methicillin resistance *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*. They stated that the usage of handrub can kill the microbacterias that has been mentioned above by 99.9% eradication level in the 1st, 2nd, and 5th minutes. Therefore, we can say our research is significant due to the previous result that stated that a good antiseptic has >90% of Percentage Kill time, in which in our result is kind of similar with

the Percentage Kill time of the usage of handrub.[18,19]

Another breaktrough of herbal antiseptic for *S. aureus* is the study done by Yagnik *et al.* (2021), about the function of apple cider vinegar (ACV) as antibacterial for MRSA and resistant *E. coli*. In their study, they stated that apple cider vinegar have a direct effects of anti-microbial on MRSA and *E. coli* using microbe growth inhibition zone measurement using mueller hinton agar plates and co-cultured with different concentrations of ACV at 37°C for twenty four hours. Their research found the inhibition zone of resistanf *E. coli* + ACV is 8±1mm, while in MRSA + ACV is 8.4±0.7. Moreover, study of *Melaleuca armillaris (M. amillaris)* esstential oil as an antibacterial for *S. aureus* also being tested by Buldain *et al* .(2021). The MIC of *M. armillaris* essential oils (EO) necessary to inhibit *S. aureus* was 25 µL/mL at pH 7.4 and 6.5 but decreased to 12.5 at pH 5.0. Their research using time-kill method proven that the higher concentration and acidity will result in better antibacterial effect with the optimal decrease of and 3.9 Log10 (CFU/mL) for 8 MIC.[20,21]

Reduced growth of *S. aureus* bacteria can be caused by *M. oleifera* containing aqueous and ethanolic extract which has antibacterial properties with inhibitory effect of a few bacteria including *S. aureus*. In addition, antibacterial effect in *M. oleifera* is also because it has secondary metabolites compound namely alkaloids, fenol, and flavonoid, which can inhibit the growth of bacterias. Flavonoid compound effected the growth by inhibition of energy metabolism, synthesis of nucleic acid, function of cell membrane, bacteria movement and prevent the energy formation; furthermore, it can cause the energy metabolism inhibition by inhibit the oxygen use of the bacteria.[22,23] Moreover, Wigunarti *et al.* (2019), also has a similar result where where *M. oleifera* decrease the growth of *S. aureus*.[24] According to research done by Ginarana *et al.* (2020), where the extract of *M. oleifera* is use in gel formation by adding *M. oleifera* extract to dispertion of carbomere mixed with paraben and gliserin, with sump method on Mueller Hinton Agar media. The antebacterial activities which effected the growth of *S. aureus* can be seen by the formation of inhibition zone.[23]

Conclusion

We concluded that *M. oleifera* leaf extract can inhibit the growth of S. *aureus*. This can be seen from the Percentage Kill which was > 90% in the 1st minute of 92.36% and continued to increase in the 2nd and 5th minutes (95.58% and 96.45%). Meanwhile for *P. aeruginosa*, *M. oliefera* leaf extract also can inhibit growth from the 1st, 2nd and 5th (27.12%, 47.01%, and 57.7%) minutes, but the results are still below < 90%. These results prove that *M. oleifera* leaf extract has the ability as an antiseptic against *S. aureus* compared to *P. aeruginosa* bacteria.

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