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Antimicrobial Activity and Time-kill Kinetics of *Moringa oleifera* Lam. Leaf Extracts Against Human Pathogenic Bacteria

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ABSTRACT

Moringa oleifera Lam. exhibits various pharmacological activities, including antibacterial, antioxidant, and anti-inflammatory effects. This study aimed to investigate the antimicrobial properties of *M. oleifera* leaf extracts and their time-kill kinetics against human pathogenic bacteria. Fresh *M. oleifera* leaves were collected, cleaned, dried, and extracted with ethanol. Phytochemical screening and total flavonoid content (TFC) were determined. Antimicrobial activity against *Staphylococcus aureus, S. epidermidis, Escherichia coli, and Pseudomonas aeruginosa* was assessed via disk diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill kinetics. Phytochemical analysis revealed the presence of flavonoids, alkaloids, terpenoids, tannins, and saponins, with a TFC of 8.95 mgEQ/g. Antibacterial testing demonstrated that higher concentrations of moringa leaf extract (2%, 4%, 6%, and 8%) produced larger inhibition zones, with the 8% concentration showing the most significant ($p \leq 0.005$) effect, particularly against *Staphylococcus aureus* (9.89 ± 0.28 mm), *Staphylococcus epidermidis* (9.59 ± 0.17 mm), *Pseudomonas aeruginosa* (9.05 ± 0.12 mm), and *Escherichia coli* (9.00 ± 0.27 mm). The MIC for all tested bacteria was 1.25 mg/mL, and the MBC ranged from 1.25 to 5 mg/mL. Time-kill assays showed that the extract was bactericidal to *S. aureus* and *S. epidermidis* at 4 x MIC, with complete bacterial elimination after 4 hours. However, *P. aeruginosa* and *E. coli* exhibited a reduction of more than 3 $\log_{10}$ CFU/mL, indicating bactericidal activity at higher concentrations. These findings support moringa leaf extract as a potential natural alternative for combating bacterial infections, particularly against Gram-positive bacteria.

Keywords: *Moringa oleifera*, Antimicrobial activity, Time-kill kinetics, Pathogenic bacteria, Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC)

Introduction

In developing countries, infectious diseases pose the biggest threat to public health. Bacterial infections are the most common kind. Natural materials have continuously encouraged the development of novel therapeutic medications intended to cure microbial illnesses in people while also reducing the incidence of microbial resistance. *Moringa oleifera* Lam. is one of many plants that have been investigated and shown to have pharmacological qualities that allow them to treat a variety of illnesses.[1] The medical plant species *Moringa oleifera*, which is widely grown in subtropical and tropical countries, originated in India.[2,3] In addition to being a vegetable that people can frequently eat, *Moringa oleifera* also possesses several medicinal uses.

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The plant is referred to as the miracle tree because of its capacity to treat several diseases and certain chronic illnesses.

To separate the beneficial components from *Moringa oleifera*, numerous studies have been conducted. Moringa has comparable nutritional values to spirulina.[4] It is reported that *Moringa oleifera* contains several nutrients, such as beta-carotene, protein, amino acids, minerals, and lipid.[5] *Moringa oleifera* also possesses many bioactive compounds, such as flavonoids, alkaloids, glucosinate, isothiocyanates, phenolic, saponins, and tannins.[6-9] Almost all parts of the moringa plant, such as the fruit, leaves, seeds, and roots, have been used to treat several types of diseases.[10] Previous studies have reported that *Moringa oleifera* exhibits antibacterial, antifungal, anti-inflammatory, antidiabetic, antioxidant, antihyperglycemic, antihyperlipidemic, anticancer, antipyretic, gastroprotective, antidote, and antidiarrhea properties.[11-17]

Pathogenic bacteria are those that can cause infection in humans. They can spread through a variety of routes, including contaminated food or drink, direct contact, and the air. *Staphylococcus aureus, S. epidermidis, Escherichia coli, and Pseudomonas aeruginosa* are some of the harmful bacteria. Skin infections ranging from mild to severe can be caused by *S. aureus* bacteria.[18] *Staphylococcus epidermidis* is a frequent cause of hospital-acquired infections, which frequently causes infections associated with implanted medical devices, such as catheters and prosthetic joints.[19] Furthermore, *P. aeruginosa* can infect the urinary system in addition to the lungs.[20] The most common cause of diarrhea is *E. coli*.[21] The technique of the time-kill kinetics test was used to determine the interaction between an antimicrobial agent and a microbial strain. This test examines the effects of the concentration and

time of an antimicrobial agent on a microbial strain. The test determines whether an antimicrobial agent is bacteriostatic or bactericidal. Bactericidal activity is defined as a reduction ≥3 $\log_{10}$ colony-forming units (CFU)/mL, which is equivalent to 99.9% killing of the inoculum. Based on this value, it shows the ability of a substance or agent to kill bacteria significantly, which is indicated by a 3-fold decrease in the number of live bacteria (CFU) on a logarithmic scale per milliliter. While a reduction of <3 $\log_{10}$ CFU/mL indicates a bacteriostatic activity of the antimicrobial agent.[22,23] Thus, the time-kill kinetics test can determine whether the moringa leaf extract has bactericidal or bacteriostatic activity.

The present study aimed to evaluate the antimicrobial activity and time-kill kinetics of *Moringa oleifera* leaf extracts against human pathogenic bacteria. This research provides novel insights into the time-dependent antimicrobial effects of *Moringa oleifera* leaf extract, detailing its bactericidal activity against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *E. coli*, and linking bioactive compounds to antimicrobial efficacy. The research methods, including phytochemical screening, antimicrobial testing (disk diffusion, minimum inhibitory concentration [MIC], minimum bactericidal concentration [MBC], and time-kill assays), are crucial for evaluating *Moringa oleifera* leaf extract's antibacterial potency, providing comprehensive insights into its efficacy against human pathogens.

Materials and Methods

Sample collection

Fresh moringa leaves were collected from Pekanbaru City and identified at the Botany laboratory in the Department of Biology, Riau University. The plant materials were assigned a specimen number: 058/UN19.5.1.1.3-4.1/EP/2022. Before being chopped to accelerate the desiccating process, the specimens were purified with running tap water to get rid of any debris. The materials were ground into a powder and stored in a tightly sealed container after being dried at room temperature until a constant weight was achieved.

Sources of materials

Ethanol, nutrient agar, Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), and dimethyl sulfoxide were all purchased from Merck, Germany. The bacterial strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli* were provided by PT. Multi Medika Laboratory, Indonesia. Mayer, Wagner, Dragendorff, and Bouchardat reagents, concentrated HCl, magnesium, and $FeCl_3$ powders were purchased from PT. Brataco Chemika, Indonesia. The apparatus employed in the research included a rotary evaporator (Model RE-1000HN, LabTech, China), analytical scale, pH meter, laminar airflow (LabTech, China), UV-Vis spectrophotometry T60 (PG-Inst, United Kingdom), incubator (IN55, Memmert), autoclave (Model GEA YX-18LDJ, China), and oven (UN110, Memmert).

Preparation of simplicia and extract of moringa leaves

To remove any adhering dirt, the prepared moringa leaves were rinsed under running water. The leaves were also cut into tiny pieces to increase the surface area and accelerate the desiccation process. The simplicia was ground into a fine powder after the specimens were sorted and allowed to dry at room temperature. Following three days of maceration with 96% ethanol, the resultant powder was filtered to separate the filtrate from the residue. The same type and quantity of solvent were used in the maceration process up to three times. Additionally, a rotary evaporator was used to concentrate the collected extracts by removing the solvent.[24,25]

Phytochemical screening of moringa leaf extract

The phytochemical analysis was carried out using standard qualitative procedures. For alkaloid analysis, 0.5 mL of the extract was pipetted into three separate test tubes, and a few drops of Mayer's, Wagner's, and Dragendorff's reagents were added to each tube, respectively, with the formation of a precipitate indicating the presence of alkaloids. To test for flavonoids, 0.5 mL of the extract was treated with three drops of concentrated hydrochloric acid and a small quantity of magnesium powder, then heated for five minutes. The development of a reddish or pink colouration confirmed the presence of flavonoids. In the saponin test, 10 mL of hot water was added to the extract, and the mixture was shaken vigorously for about 10 seconds, after which the formation of stable froth indicated the presence of saponins. For tannin detection, three drops of a 1% ferric chloride ($FeCl_3$) solution were added to 0.5 mL of the extract, and the appearance of a blue-black or greenish precipitate confirmed the presence of tannins. To examine terpenoids, the extract was dissolved in chloroform, followed by the addition of 0.5 mL of anhydrous acetic acid, after which 2 mL of concentrated sulfuric acid was carefully added down the wall of the test tube. The appearance of a reddish-brown coloration at the interface indicated the presence of terpenoids.[26]

Determination of total flavonoid content

The total flavonoid content (TFC) of the extract was carried out using ultraviolet-visible (UV-Vis) spectrophotometry. The calibration curve was established using quercetin concentrations of 50, 70, 90, 110, and 130 parts per million (ppm). From each concentration of the quercetin standard solution, 1 mL was pipetted, followed by the addition of 8 mL of 5% CH_3COOH and 1 mL of 10% $AlCl_3$. The mixture was allowed to stand at 25 °C for 30 minutes. Then, UV-Vis spectrophotometry was then used to measure the absorbance of the standard solution at a maximum wavelength of 412 nm. To measure the TFC of the extract, the procedure described above was repeated using 1 mL of the extract solution instead of quercetin. The TFC was expressed as mg quercetin equivalents per gram (mg QE/g), and the test was performed in triplicate.[27]

Inoculum preparation

The growing bacterial culture was inoculated into 25 mL of sterilized nutrient broth (NB) medium and incubated on a shaker incubator at 37°C, 120 rpm for 15-18 hours. Next, the absorbance value was measured with a UV-Vis spectrophotometer at a wavelength of 625 nm. An absorbance value of 0.08-0.1 indicates the equivalent of a bacterial count of 1.5 x 10^8 CFU/mL.[28]

Antimicrobial activity test

The antimicrobial activity test was conducted employing Muller Hilton Agar (MHA) media and the disk diffusion method. The McFarland turbidity standard of 0.5, or 10^8 CFU/mL, was used to calibrate bacterial suspensions. The extracts were then concentrated to four different concentrations: 2%, 4%, 6%, and 8%. Each Petri dish was filled with 15 ml of sterile MHA medium. Using a swab approach, test microorganisms were inoculated into agar media. Then, at regular intervals, 5 empty discs and 1 chloramphenicol disc were placed on the surface of the medium. Subsequently, 10 µL of the extract was applied to four discs with concentrations of 2%, 4%, 6%, and 8%, along with one disc serving as a negative control. After that, the samples were placed in an incubator for 24-48 hours at 37°C. The diameter of the resulting inhibitory zone after making observations was calculated. Each treatment was carried out three times to minimize errors.[29]

Determination of MIC and MBC of moringa leaf extract

A modified liquid dilution method was utilized to determine the minimum inhibitory concentration (MIC). The liquid medium used was MHB. Each extract was prepared at five different concentrations: 0.313 mg/mL, 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL. Each test tube was filled with 5 mL of sterilized MHB medium, followed by the addition of 0.5 mL of the extract. Then 0.5 mL of bacterial suspension was added to the medium at 10^6 CFU/mL, which had been adjusted to a standard of 0.5 McFarland standard. Using a UV-Vis spectrophotometer (λ = 480 nm), the experiment was carried out to measure the absorbance values before and after incubation to assess the growth of the test bacteria. The absorbance value measured before treatment was subtracted from the absorbance value recorded after treatment in order to determine the MIC. The MIC is the lowest concentration that inhibits bacterial growth, indicated by the absence of turbidity in the test tube (with a bacterial OD of ≤ 0). After obtaining the MIC value, the minimum bacteriocidal concentration (MBC) was computed. The absorbance values of all the concentrations and control

groups were recorded, and then the samples were streaked onto NA media. A colony counter was used to count the colonies after they had been incubated at 37˚C for 24-48 hours. The MBC value is determined by identifying the lowest concentration that results in a colony count of 0 CFU/mL.[30]

Time-kill curve assay
The test microbial culture was inoculated with a final concentration of 10^6 CFU/mL into the Erlenmeyer flask, along with the extract at a specified concentration and 10 mL of MHB. In this experiment, treatment variations were established by adding the extract to each Erlenmeyer flask at concentrations of 0 x MIC, 0.5 x MIC, 1 x MIC, 2 x MIC, and 4 x MIC. Chloramphenicol was used as the control. A 0.1 mL sample was taken at 0, 0.5, 1, 2, and 4-hour intervals. After that, the samples were inoculated using MHA in the pour plate method and incubated at 37ºC for 24 hours. The bacterial colonies that grew were then counted, and the count was expressed in logarithmic values ($\log_{10}$ CFU/mL). This experiment was carried out in triplicate. Bactericidal action was determined if the log kill value reached >3 $\log_{10}$ CFU/mL.[31]

Statistical analysis
The data were presented as the mean of three replicates, and the results are presented as the mean ± standard deviation. The study's results were assessed at a 95% confidence level with a significance of $p \leq 0.05$, using a one-way analysis of variance (ANOVA) for statistical analysis.

Results and Discussion

Phytochemical screening and extract yield
The objective of this study was to assess the antibacterial activity of moringa leaves. Ethanol was used as a solvent to extract the moringa leaves, and ethanol is classified as a polar solvent. As a result, most of the ionic compounds present in moringa leaves were extracted. Furthermore, the ethanol solvent is non-toxic and evaporates easily. Due to its slightly lower dipole moment and dielectric constant, which contribute to its moderate polarity, ethanol dissolves compounds more effectively than water.[32] A phytochemical screening is then conducted to determine whether a plant contains secondary metabolite compounds. The same plant may contain varying secondary metabolite compounds, as factors such as climate, soil, temperature, humidity, and other variables can influence these variations. Phytochemical screening was performed on the obtained moringa leaf extract to identify the secondary metabolite compounds present. The extract from moringa leaves contained terpenoids, alkaloids, flavonoids, saponins, and tannins. Five hundred grams of moringa leaf *simplicia* were extracted using the maceration technique with 96% ethanol. A rotary evaporator was then used to concentrate the filtrate. The extract yield was 11.97%, obtained from an extract weight of 58.97 grams.

Total flavonoid content of the Moringa oleifera leaf extracts
To determine the TFC, quantitative examination was carried out using UV-Vis spectrophotometry. Conjugated aromatic structures in flavonoid compounds enable them to absorb light in both the visible and ultraviolet spectrums. Quercetin, a flavonoid belonging to the flavonol group, was used as the standard solution for this analysis.[33] The absorbance of an ethanol extract made from moringa leaves was evaluated using quercetin's maximum wavelength, determined to be 412 nm. $AlCl_3$ was added to the sample solution to determine the TFC. This reaction forms a compound that shifts the wavelength towards the visible spectrum, causing the solution to turn yellow. The addition of acetic acid helps maintain the wavelength in the visible range. A 30-minute incubation period was allowed to ensure the reaction proceeded optimally, maximizing the final colour intensity.[34] The TFC of the extract was 8.9546 mgQE/g, as shown in Table 1. According to research by Utami *et al*. (2024), the extract of *Etlingera elatior* (Jack) R.M. Smith from North Luwu, Indonesia, has a TFC of 4.6 mgQE/g.[35] This result suggests that the TFC of the leaf extract of moringa exceeds that of the extract of *Etlingera elatior*. According to the study, flavonoids offer several benefits, including antibacterial, antitumor, anticancer, antiproliferative, and antioxidant properties.[36]

Table 1: Total flavonoid content of moringa leaf ethanol extract.

Replication	Absorbance	Flavonoid Content (mgEQ/g)	Average Flavonoid Content (mgEQ/g)
1	0.594	9.0875	
2	0.582	8.9281	8.9546 ± 0.1217
3	0.576	8.8484	

Antimicrobial activity of the Moringa oleifera leaf extracts
Antimicrobial activity was tested against four different extract concentrations: 2%, 4%, 6%, and 8%. The test results are displayed in Table 2. According to the results, the size of the inhibitory zone increased with higher extract concentrations. Therefore, a larger inhibition zone is correlated with stronger antibacterial action of the extract against the tested microbial species. Moringa leaf extract was then tested for antibacterial efficacy against pathogenic bacteria. Chloramphenicol is a broad-spectrum bacteriostatic antibiotic used as a positive control.[37] According to the results of the antibacterial tests, the highest inhibition zone for each species of bacteria was obtained at a concentration of 8%. Therefore, a greater inhibitory zone is observed with increasing extract concentration.
Based on the results in Table 2, *S. aureus* exhibited the largest inhibitory zone, while *E. coli* showed the smallest. This difference is attributed to the simpler cell wall structure of *S. aureus* (Gram-positive) compared with *E. coli* (Gram-negative), which explains the variation in inhibitory zone formation between the two bacteria.[38] Gram-negative bacteria have three layers in their cell wall: the outer layer, the middle layer, and the innermost layer. On the other hand, Gram-positive bacteria have a single layer inside their cell walls. As a result, the extract can penetrate the cell walls of Gram-positive bacteria more easily than those of Gram-negative bacteria. To determine whether there were significant differences in inhibitory zone sizes among the bacteria, an ANOVA was performed. The results yielded a p-value of 0.000 ($p < 0.05$), indicating that the inhibitory zones of each bacterium are significantly affected by the concentration of moringa leaf extract.

MIC and MBC of the Moringa oleifera leaf extracts
The MIC test identifies the lowest concentration of an antimicrobial agent that significantly inhibits bacterial growth, whereas the MBC determines the lowest concentration that causes bacterial death. Minimum inhibitory concentration tests were conducted using five different concentrations. Absorbance measurements were taken before and after incubation to determine the extract's MIC. The observed differences are summarized in Table 3. A decrease in absorbance after incubation indicates that the extract inhibits bacterial growth. The MIC of the extract against all tested bacteria was 1.25 mg/mL. Furthermore, the MBC for *S. aureus* was 1.25 mg/mL, *S. epidermidis* was 2.5 mg/mL, *P. aeruginosa* and *E. coli* were 5 mg/mL, as presented in Table 4.

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Table 2: Antibacterial activity of moringa leaf ethanol extract.

Concentration of Extract (%)	Zone of inhibition (mm)			
	Escheriacia coli	***Pseudomonas aeroginosa***	***Staphylococus aureus***	***Staphylococus epidermidis***
2	7.34 ± 0.25	7.35 ± 0.27	7.66 ± 0.12	8.55 ± 0.17
4	8.47 ± 0.12	8.62 ± 0.11	8.80 ± 0.22	9.07 ± 0.47
6	8.72 ± 0.30	8.70± 0.08	9.15 ± 0.13	9.20 ± 0.46
8	9.00 ± 0.27	9.05± 0.12	9.59 ± 0.17	9.89 ± 0.28
Positive Control	20.5 ± 0.10	22.6 ± 0.03	25.3 ± 0.38	24 + 0.29
Negative Control	6 ± 0.00	6 ± 0.00	6 ± 0.00	6 ± 0.00

Table 3: The minimum inhibitory concentration of moringa leaf ethanol extract

Bacteria	Conc. (mg/mL)	Absorbance		Mean difference	Remark	Sig.
		Before incubation	After Incubation			
Staphylococcus	5.00	0.301 ± 0.03	0.246 ± 0.05	-0.055	Decrease	
aureus	2.50	0.285 ± 0.04	0.269 ± 0.02	-0.016	Decrease	
	1.25	0.269 ± 0.02	0.211 ± 0.02	-0.058	Decrease	
	0.63	0.305 ± 0.03	0.312 ± 0.06	+0.007	Increase	0.001
	0.31	0.296 ± 0.01	0.345 ± 0.03	+0.049	Increase	
	K+	0.392 ± 0.01	0.257 ± 0.10	-0.135	Decrease	
	K-	0.277± 0.03	0.412 ± 0.03	+0.135	Increase	
Staphylococcus	5.00	0.388 ± 0.04	0.266 ± 0.04	-0.122	Decrease	
epidermidis	2.50	0.268 ± 0.05	0.196 ± 0.02	-0.072	Decrease	
	1.25	0.373 ± 0.05	0.299 ± 0.05	-0.074	Decrease	
	0.63	0.412 ± 0.06	0.420 ± 0.009	+0.008	Increase	0.008
	0.31	0.409 ± 0.10	0.473 ± 0.05	+0.064	Increase	
	K+	0.530 ± 0.07	0.331 ± 0.10	-0.199	Decrease	
	K-	0.468 ± 0.08	0.585 ± 0.06	+0.117	Increase	
Eschericia coli	5.00	0.468 ± 0.01	0.242 ± 0.15	-0.226	Decrease	
	2.50	0.372 ± 0.11	0.275 ± 0.07	-0.097	Decrease	
	1.25	0.294 ± 0.03	0.210 ± 0.04	-0.084	Decrease	
	0.63	0.273 ± 0.03	0.298 ± 0.04	+0.025	Increase	0.002
	0.31	0.410 ± 0.07	0.487 ± 0.18	+0.077	Increase	
	K+	0.495 ± 0.09	0.273 ± 0.02	-0.222	Decrease	
	K-	0.335 ± 0.05	0.477 ± 0.09	+0.142	Increase	
	5.00	0.512 ± 0.13	0.385 ± 0.08	-0.127	Decrease	
Pseudomonas	2.50	0.400 ± 0.06	0.312 ± 0.02	-0.088	Decrease	
aeruginosa	1.25	0.366 ± 0.06	0.284 ± 0.01	-0.082	Decrease	
	0.63	0.246 ± 0.01	0.295±0.03	+0.049	Increase	0.007
	0.31	0.388 ± 0.04	0.452 ± 0.002	+0.064	Increase	
	K+	0.371 ± 0.003	0.242 ± 0.04	-0.129	Decrease	
	K-	0.246 ± 0.07	0.411 ± 0.07	+0.165	Increase	

The mean difference is significant at the 0.05 level

Figure 1 compares the absorbance values of moringa leaf ethanol extract against the bacteria before and after incubation. At a concentration of 1.25 mg/mL, a decrease in absorbance was observed for all four bacterial species tested. These results indicate that the secondary metabolites present in moringa leaf extract can inhibit bacterial growth. The decrease in absorbance after incubation suggests that the extract may inhibit bacterial growth. Meanwhile, an increase in absorbance after incubation indicates that the extract does not inhibit bacterial growth. Consequently, the lowest concentration at which a decrease in absorbance is observed during incubation is considered the MIC value.

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Table 4: The minimum bactericidal concentration of moringa leaf ethanol extract.

Bacteria	Concentration (mg/mL)	Bacterial growth		
		First replicate	Second replicate	Third replicate
Staphylococcus aureus	5.00	-	-	-
	2.50	-	-	-
	1.25	-	-	-
	0.63	+	+	+
	0.31	+	+	+
	control	-	-	-
Staphylococcus epidermidis	5.00	-	-	-
	2.50	-	-	-
	1.25	+	+	+
	0.63	+	+	+
	0.31	+	+	+
	control	-	-	-
Eschericia coli	5.00	-	-	-
	2.50	+	+	+
	1.25	+	+	+
	0.63	+	+	+
	0.31	+	+	+
	control	-	-	-
Pseudomonas aeruginosa	5.00	-	-	-
	2.50	+	+	+
	1.25	+	+	+
	0.63	+	+	+
	0.31	+	+	+
	control	-	-	-

-: no bacterial growth; +: bacterial growth

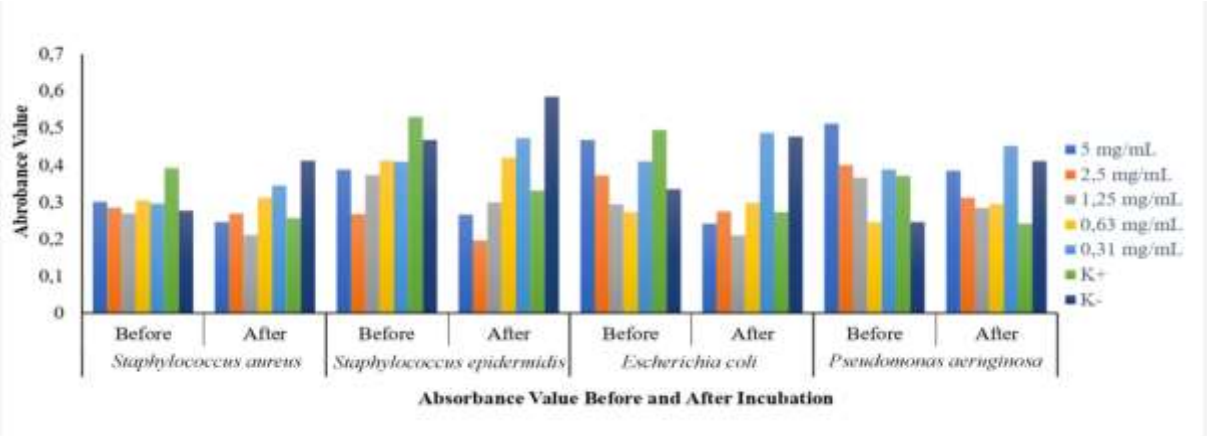

Figure 1: The minimum inhibitory concentration of moringa leaf ethanol extract before and after incubation.

Determination of MIC was also conducted against positive control and negative control. The purpose of positive control is to compare and validate the antibacterial efficacy of the test samples with a known active agent. Meanwhile, the negative control confirmed that the solvent exhibited no antibacterial activity, ensuring that the observed effects were solely due to the tested sample. The positive control used in the MIC test was the antibiotic chloramphenicol, while the negative control was the solvent DMSO. The absorbance value of the positive control against *S. aureus, S. epidermidis, P. aeruginosa*, and *E. coli* bacteria showed a decrease after incubation. This indicates that the positive control is effective in inhibiting bacterial growth. Meanwhile, the negative control showed that the absorbance value still increased after incubation for all test bacteria, which indicates that the negative control is ineffective in inhibiting bacterial growth. Therefore, the

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concentration of the extract showing nearly similar effectiveness to the positive control is at a concentration of 1.25 mg/mL for all tested bacteria.

Then, the MBC test was also compared to a negative control, which serves to ensure the validity and accuracy of the results by detecting contamination, bias, or false positives. A negative control must not contain any active ingredients so that the test results obtained are purely from the sample being tested. In this study, the control used was nutrient agar medium. Based on the test results, no bacteria grew in the negative control. This indicates that the experimental conditions (such as the growth media, equipment, and solvents used) were not contaminated by unwanted bacteria. Thus, the MBC value obtained reflects the activity of the compounds present in the extract and not any contamination.

Although the extract exhibits antibacterial activity, testing showed that its effectiveness is not exactly the same compared to antibiotics or positive controls. This is evident from the greater decrease in absorbance observed with the controls after incubation compared to the extract. A greater decrease in absorbance indicates higher effectiveness in inhibiting bacterial growth. This variation in activity may be attributed to differences in the chemical composition of the extract, which influence its antibacterial potency.[39] The MIC test results, analyzed by ANOVA, showed a significant value ($p < 0.05$), indicating that the effectiveness of moringa leaf extract in inhibiting bacterial growth varied significantly with concentration.

Plant extracts have been widely evaluated using MIC and MBC tests. For example, Siramon *et al*. (2022) assessed the MIC and MBC of coconut coir dust extract against *S. aureus* and *S. epidermidis*. The MBC values ranged from 3.2 to 102.4 mg/mL, while the MIC values were between 0.8 and 25.6 mg/mL.[40] Extracts from *M. oleifera* L. seeds exhibited an MIC of 12.5 mg/mL and an MBC of 25 mg/mL against *P. gingivalis*, according to Madhloom *et al*. (2022).[41] In this study, the MBC ranged from 1.25 to 5 mg/mL against *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *E. coli*, while the MIC was 1.25 mg/mL for moringa leaf extract. These results indicated that moringa leaf extract exhibits lower MIC and MBC values compared to moringa seed extract. Since it requires a lower concentration to inhibit and eliminate bacterial growth compared to the higher MIC and MBC values of the seed extract, moringa leaf extract demonstrates greater antimicrobial effectiveness than moringa seed extract.

Antibacterial activity of the Moringa oleifera leaf extracts based on the time-kill assay

The antibacterial activity exhibited by plant extracts can vary, being either bacteriostatic or bactericidal in nature.[42] Therefore, a time-kill assay is required to analyze the characteristics of moringa leaf extract's antibacterial activity. Figure 2 shows the bacteriostatic or bactericidal potential of the ethanol extract of moringa leaves against bacteria. Based on the time-kill curve, the extracts exhibit varying mechanisms of action that depend on extract concentration, bacterial species, and incubation period. Each bacterium responds differently, with the rate of killing varying accordingly. As shown in Figures 2a and 2b, the ethanol extract of moringa leaves eliminated *S. aureus* and *S. epidermidis* within 4 hours at a concentration of 4 × MIC in the time-kill assay. Thus, the ethanol extract of moringa leaves exhibits a bactericidal effect against *S. aureus* and *S. epidermidis*. Furthermore, as shown in Figures 2c and 2d, the extract did not completely eliminate *P. aeruginosa* and *E. coli* at a concentration of 4 × MIC; however, it still demonstrated bactericidal activity, achieving a log reduction greater than 3 $\log_{10}$ CFU/mL. A reduction of more than 3 $\log_{10}$ CFU/mL is generally considered indicative of bactericidal action.[43]

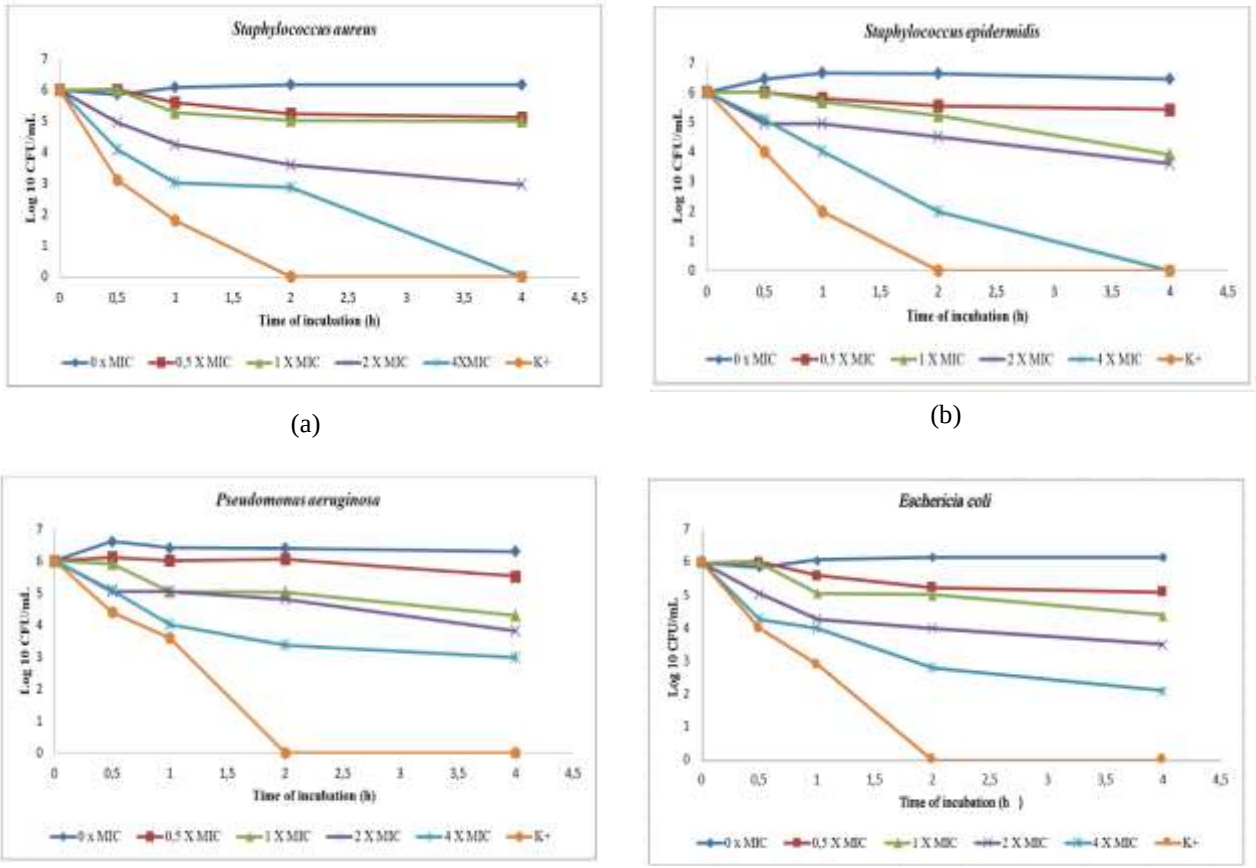

Figure 2: Time-kill kinetics curve of moringa leaf ethanol extract against test microorganisms.
a: *S. aureus;* b: *S. epidermidis;* c: *P. aeruginosa*; d: *E. coli;* MIC: minimum inhibitory concentration

In *S. aureus* and *S. epidermidis*, the extract at 2 × MIC was able to kill the bacteria, as indicated by a log reduction greater than 3 $\log_{10}$ CFU/mL. Therefore, at this concentration, the extract exhibits bactericidal activity, since such activity is defined by a log reduction exceeding 3 $\log_{10}$ CFU/mL. At a concentration of 4 × MIC, *S. aureus* and *S. epidermidis* were completely eradicated after 4 hours of exposure to the extract. Bacterial eradication is defined by the complete absence of viable colonies in the culture medium. According to research by Witkowska *et al*. (2013), sage extract had a bactericidal effect on *S. aureus* when applied at a concentration more than 40 mg/mL for a duration of 24 hours.[43] Furthermore, at doses of 5 and 10 mg/mL for durations of 4 and 6 hours, respectively, extracts of clove and rosemary can eradicate *S. aureus*. This study demonstrated that *Moringa oleifera* extract can completely eliminate *S. aureus* at a concentration of 5 mg/mL within 4 hours of incubation. *Moringa oleifera* extract exhibits antibacterial activity comparable to sage extract and shows similar efficacy to clove and rosemary extracts. Moreover, *S. aureus*, being a Gram-positive bacterium, is generally more susceptible to antimicrobial agents due to its relatively less complex and more easily disrupted cell membrane compared to Gram-negative bacteria.

For *P. aeruginosa* and *E. coli*, concentrations of 0.5 × MIC, 1 × MIC, 2 × MIC, and 4 × MIC did not completely eradicate the bacteria after 4 hours of incubation. However, at 4 × MIC, the log reduction exceeded 3 $\log_{10}$ CFU/mL, indicating a bactericidal effect. In contrast, at 0.5 × MIC, 1 × MIC, and 2 × MIC, the log reduction was less than 3 $\log_{10}$ CFU/mL, suggesting a bacteriostatic effect at these concentrations. Consequently, the extract exhibits a stronger bactericidal effect against *S. aureus* and *S. epidermidis* than against *E. coli* and *P. aeruginosa*. Therefore, moringa leaf extract is a more effective antibacterial agent against Gram-positive bacteria, such as *S. aureus* and *S. epidermidis*. In another example, *Melastoma malabathricum* extract has been shown to successfully eliminate *P. aeruginosa* at a concentration of 1.56 mg/mL over the course of 8 hours.[44] A study by Mamman *et al*. (2013) reported the bactericidal activity of *Azadirachta indica* extract against *E. coli* at a concentration of 250 mg/mL. Compared to these findings, *Moringa oleifera* extract at 4 × MIC did not completely eradicate *E. coli* or *P. aeruginosa*. Therefore, while *Melastoma malabathricum* and *Azadirachta indica* exhibit bactericidal activity against Gram-negative bacteria, *M. oleifera* demonstrates a primarily bacteriostatic effect in these cases.[45] In general, plant extracts exhibit varying abilities to inhibit or eradicate different bacterial species. This variability may be attributed to the presence of essential oils and polyphenolic compounds, which possess distinct bacteriostatic and bactericidal properties against diverse bacterial strains. The MIC is an important parameter for evaluating antimicrobial activity, as it reflects both the kinetics of bacterial inhibition and the effective concentration of the extract required for antibacterial action.[46]

Increasing the concentration of an extract can enhance its diffusion into the bacterial cell membrane, leading to membrane disruption. Consequently, higher extract concentrations correspond to a greater bactericidal potential. According to Miksusanti (2009), higher extract concentrations can cause leakage of the cytoplasmic membrane, ultimately leading to bacterial cell death.[47] For an extract to effectively kill bacteria, it must remain at the target site long enough to disrupt metabolic activities and interfere with essential bacterial biochemical processes. Increasing the extract concentration can enhance its bactericidal effect.[48] The bioactive constituents of the extract, including flavonoids, saponins, and tannins, play a crucial role in mediating its antibacterial activity.[49]

Flavonoids exhibit antibacterial activity by forming complexes with extracellular proteins, compromising bacterial cell membrane integrity, disrupting the microbial life cycle, and interfering with cellular functions. Alkaloids primarily act by intercalating into bacterial DNA and cell walls. Tannins inhibit peptidoglycan synthesis, leading to defective cell wall formation and bacterial inactivation. Saponins are another class of bioactive compounds that contribute to antibacterial activity. By reducing the surface tension of bacterial cells, saponins make it easier for the germs to leak and lyse.[50]

Conclusion

The present study demonstrated that moringa leaf extract exhibits moderate antimicrobial activity, with greater efficacy against Gram-positive bacteria than Gram-negative bacteria. The MIC for all tested bacteria was 1.25 mg/mL, while the MBC ranged from 1.25 to 5 mg/mL. Time-kill kinetics assays revealed that the extract is bactericidal against *S. aureus* and *S. epidermidis* but bacteriostatic against *P. aeruginosa* and *E. coli*. These findings suggest that *Moringa oleifera* leaf extract holds potential as a natural antimicrobial agent for the treatment of bacterial infections in humans. Future studies should explore the isolation and identification of specific bioactive compounds responsible for *Moringa oleifera*'s antimicrobial activity, evaluate its potential synergistic effects with other natural antibiotics, and assess its clinical efficacy and safety in treating bacterial infections *in vivo*.

Conflict of Interests

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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