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RESEARCH ARTICLE

Total Phenolic and Flavonoid Contents of Ethanol Extract of *Manilkara kauki* Leaves and Antibiofilm Activity Test Against Biofilms of *Pseudomonas aeruginosa*

Lilik FARKHIYAH[*], Tukiran TUKIRAN[**°]

Total Phenolic and Flavonoid Contents of Ethanol Extract of Manilkara kauki Leaves and Antibiofilm Activity Test Against Biofilms of Pseudomonas aeruginosa

SUMMARY

One of the infectious bacteria that can cause high morbidity and mortality is Pseudomonas aeruginosa. P. aeruginosa is a difficult pathogen to treat and often causes resistance to antibiotics. One of the causes of antibiotic resistance is the formation of biofilm. Manilkara kauki is one of the plants that has potential as an antibiofilm. The absence of publications related to information on total phenolic and flavonoid contents and the use of M. kauki leaves as antibiofilm makes this research important and necessary. This study aims to determine the total phenolic and flavonoid contents in the ethanol extract of M. kauki leaves and antibiofilm activity tests, which include optimization of biofilm formation time, inhibition activity test, and biofilm destruction using the Microtiter Plate Assay method with positive control of ciprofloxacin. According to the LC-MS results, the flavonoid content was relatively high at 46.15%, while the non-flavonoid phenolic content in the ethanol extract of small sapodilla leaves was 23.08%. Meanwhile, the total phenolic and flavonoid content tests yielded similar results, namely 48.9830 mg/g GAE and 21.3270 mg/g QE, respectively. The results of the antibiofilm activity test showed that P. aeruginosa is a strong biofilm-forming bacterium with an optimum formation time of 48 hours and an OD value of 0.5298. Meanwhile, the highest percentage of biofilm inhibition and destruction was observed at a concentration of 1000 ppm, with IC50 values of 134 and 108.4 µg/mL, respectively.

Keywords: *Antibiofilm, flavonoid, phenolic, Pseudomonas aeruginosa, Manilkara kauki.*

Manilkara kauki Yapraklarının Etanol Ekstraktının Toplam Fenolik ve Flavonoid İçeriği ve Pseudomonas aeruginosa Biyofilmlerine Karşı Antibiyofilm Etkinlik Testi

ÖZ

Yüksek morbidite ve mortaliteye neden olabilen enfeksiyöz bakterilerden biri Pseudomonas aeruginosa'dır. P. aeruginosa tedavisi zor bir patojendir ve sıklıkla antibiyotiklere karşı direnç geliştirir. Antibiyotik direncinin nedenlerinden biri biyofilm oluşumudur. Manilkara kauki, antibiyofilm potansiyeline sahip bitkilerden biridir. M. kauki yapraklarının toplam fenolik ve flavonoid içerikleri ile antibiyofilm olarak kullanımıyla ilgili yayınların bulunmaması, bu araştırmayı önemli ve gerekli kılmaktadır. Bu çalışmanın amacı, M. kauki yapraklarının etanol ekstraktındaki toplam fenolik ve flavonoid içeriklerini belirlemek ve antibiyofilm aktivitesini incelemektir. Antibiyofilm testleri, biyofilm oluşum süresinin optimizasyonu, inhibisyon aktivitesi testi ve biyofilm yıkımı testlerini kapsamakta olup, pozitif kontrol olarak siprofloksasin kullanılmıştır. Tüm testler Microtiter Plate Assay yöntemi ile gerçekleştirilmiştir. LC-MS sonuçlarına göre, flavonoid içeriği %46,15 ile nispeten yüksek bulunurken, küçük sapodilla (M. kauki) yapraklarının etanol ekstraktındaki flavonoid olmayan fenolik bileşiklerin oranı %23,08 olarak belirlenmiştir. Buna karşılık, toplam fenolik ve flavonoid içerik analizleri sırasıyla 48,9830 mg/g GAE ve 21,3270 mg/g QE değerlerini vermiştir. Antibiyofilm aktivite testi sonuçları, P. aeruginosa'nın güçlü bir biyofilm oluşturan bakteri olduğunu, optimum biyofilm oluşum süresinin 48 saat olduğunu ve OD değerinin 0,5298'e ulaştığını göstermiştir. Bu arada, biyofilm inhibisyonu ve yıkımının en yüksek yüzdesi 1000 ppm konsantrasyonda gözlemlenmiş olup, IC50 değerleri sırasıyla 134 ve 108,4 µg/mL'dir.

Anahtar Kelimeler: *Antibiyofilm, fenolik, flavonoid, Pseudomonas aeruginosa, Manilkara kauki.*

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INTRODUCTION

Infectious diseases are often experienced by developed and developing countries, including Indonesia (Santoso et al., 2020). Infection is a disease caused by pathogenic microbes such as bacteria (Konoralma, 2019). According to the National Institute of Health, microbial biofilms are responsible for about 65% of nosocomial infections and 80% of all microbial infections and chronic diseases (Singh et al., 2024). The effectiveness of antibiotics as antimicrobial agents is decreasing as the prevalence of drug resistance among microorganisms increases (Muteeb et al., 2023).

One of the bacteria that causes serious infections is *Pseudomonas aeruginosa* (Chen et al., 2024). This bacterium is capable of causing infections in the urinary tract and bloodstream (Qin et al., 2022) and is the dominant pathogen causing pulmonary infections in cystic fibrosis patients (Pang et al., 2019). This bacterium can cause a mortality rate as high as 50%, depending on the nature of the infection (Purnama et al., 2024). *P. aeruginosa* is a bacterium resistant to multiple drugs, making treatment challenging and frequently linked to high mortality rates (Hirsch & Tam, 2010).

The development of biofilms is one of the factors contributing to antibiotic resistance (Pamudi et al., 2024). The Centers for Disease Control and Prevention (CDC) in 2007 reported that approximately 1.7 million infections in hospitals were caused by biofilm formation (Goel et al., 2021). Biofilms consist of a community of bacterial cells that can produce a polymer matrix composed of exopolysaccharides, thereby inhibiting antibiotic penetration and resulting in chronic infection (Jayadi et al., 2023; Maghfirah et al., 2017). Until now, no drug specifically functions as an antibiofilm, so the development of antibiofilm agents is very necessary (Purnama et al., 2024).

Bacterial resistance to many drugs directs the search for new strategies, one of which is by utilizing natural materials (Prianggawe & Hajrin, 2024). The content of natural materials such as phenolics and flavonoids is considered to have potential as a natural antibiofilm that has the ability to damage bacterial polysaccharides (Rosyada et al., 2023). One of the plants that is predicted to be potentially useful as an antibiofilm is *Manilkara kauki.* (L.) Dubard. *M. kauki* (Indonesian people called as sawo kecik) is a fruit-producing tree from the Sapotaceae family and the Manilkara genus (Susilawati et al., 2023). The genus Manilkara comprises approximately 70–80 species widely distributed in tropical and subtropical regions, particularly in Southeast Asia, South Asia, Africa, and Central America. In Indonesia itself, *M. Kauki* can be found on the islands of Karimun, Kangean, Bali, Nusa Tenggara, Buton, Sulawesi, Maluku, and Papua (Pratiwi et al., 2021). *M. Kauki* is widely used in traditional medicine. Several parts of the plant have reported ethnomedical uses, where the bark and roots are used as astringents and to treat diarrhea in children, the seeds are used as antipyretics, anthelmintics, and antileprosy agents, while the leaves are used as compresses for tumors (Hegde et al., 2025). Although *M. kauki* leaves have long been used in traditional medicine, research on their antibiofilm activity has not yet been reported.

Previous studies have shown that *M. kauki* has various biological activities, including antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, as well as antidiabetic activity through lowering blood glucose levels in diabetic rats (Pratiwi et al., 2021; Prayudhani et al., 2013; Purba et al., 2023). These findings show similar activity to that reported in other Manilkara species, such as *Manilkara zapota* (L.) van Royen, reported by Kaneria et al. (2009), which showed that *M. zapota* methanol extract has antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Enterobacter aerogenes* bacteria, with an average activity value of 12.4.

According to research by Srisupap and Chaicharoenpong (2021), water extracts from small *M. kauki* leaves have the highest phenolic content compared to extracts from the fruit, seeds, stems, and roots. In addition, the flavonoid content in small *M. kauki* leaves for methanol extracts is also higher than that in extracts from the fruit, seeds, and stems. Therefore, leaves were used in this study. Referring to the background previously described, the researcher is interested in researching the ethanol extract of *M. kauki* leaves, which contain total phenolic and flavonoid compounds, as determined using UV-Vis Spectrophotometry. While the previous study focused on antibacterial activity against planktonic cells, this study specifically evaluated antibiofilm activity, which better represents chronic infection conditions. Then it is necessary to carry out a series of activities, including optimization of biofilm formation time, inhibitory activity test, and biofilm destruction to determine the antibiofilm potential of *M. kauki* leaf ethanol extract against *P. aeruginosa* bacterial biofilm. This approach allows for quantitative assessment of biofilm inhibition and destruction activities. The results of this study provide preliminary information on the potential antibiofilm activity of *M. Kauki* leaves. The absence of publications related to the information on *M. kauki* leaves as antibiofilm makes this research important to do.

MATERIALS AND METHODS

Materials

The materials used in this study included *M. kauki* leaves, ethanol 96%, quercetin (Merck), 10% (b/v) aluminum chloride ($AlCl_3$), filter paper, sodium acetate, distilled water, sodium carbonate (Na_2CO_3), acetic acid, barium chloride ($BaCl_2$), Folin–Ciocalteu reagent (Merck), and a pure culture of the bacterium *P. aeruginosa* (ATCC 27853), nutrient agar (NA) medium (Merck), physiological saline, peptone (Oxoid), dipotassium hydrogen phosphate (K_2HPO_4), glucose (Merck), sodium chloride (NaCl), tryptone (Merck), and crystal violet (Merck).

Instrumental

Equipment and instruments used in this study are incubator (Memmert IN55), volumetric flask (Herma), beakers (Iwaki), laminar air flow (Thermo Scientific 1300-Series A2), UV-Vis spectrophotometer (Shimadzu UV-1800), micropipettes, test tubes (Iwaki), inoculating loop, autoclave (HICLAVE HVE-50), 96-well plates, blue tips, ELISA microplate reader (BK-EL10A), and rotary evaporator (R-215, Buchi).

Characterization methods

M. kauki leaf collection and extraction process

The *M. Kauki* leaf plant used in this study was identified and verified botanically by Heri Santoso, Department of Botan on November 19th, 2024. The specimen is stored under the number BT-112495 at the Herbarium of Yayasan Generasi Biologi Indonesia. The plant material was collected from Tropodo, Sidoarjo Regency, East Java Province, Indonesia, on September 4th, 2024, with coordinates -7.360310, 112.764195. A total of 1.3 kg of the collected leaves was dried under sunlight for three days with occasional flipping. After drying, the leaves were pulverized with a grinder until they became powder and were weighed. 750 g of dry powder was obtained. The *M. kauki* leaf powder was extracted using ethanol as the solvent at a ratio of 1:2 within 24 hours. Maceration was repeated 3 times with occasional stirring. After that, filtering was carried out with a Buchner funnel assisted by a vacuum pump, and an ethanol filtrate was obtained. The ethanol filtrate was concentrated through evaporation to yield a thick extract, and weighed to obtain a thick extract of 169 grams. As much as approximately 0.5 mg of the extract was tested for phytochemicals using the LC-MS test. In this study, *M. kauki* leaf extract was prepared using ethanol, which is a polar solvent, because the test target was *Pseudomonas aeruginosa*, a Gram-negative bacterium. Previous research on the species *M. zapota* showed that extraction with polar solvents, such as acetone, produced broader antimicrobial activity compared to non-polar solvents, with effects on all

test bacteria (Kaneria & Chanda, 2012). Maceration is used because it is a simple method that does not involve heating, allowing for the extraction of compounds without damaging heat-sensitive natural ingredients (Riasari et al., 2022).

Total phenolic content

The procedure for this test follows the method described by Saputri et al. (2021). The total phenolic content in the ethanol extract of *M. kauki* leaves was measured using the Folin-Ciocalteu method. Gallic acid was used as the standard at concentrations of 10, 20, 30, 40, and 50 ppm. Each extract and standard solution was taken 0.4 mL, and then 2 mL of Folin-Ciocalteu reagent was added. Then, 1.6 mL of 1M Na_2CO_3 was homogenized and incubated at room temperature for 30 minutes in the absence of light, and then measured using a UV-Vis spectrophotometer at a wavelength of 743 nm.

Total flavonoid content

Total flavonoid content using quercetin as a control with concentrations of 20, 40, 60, 80, and 100 ppm. Extract and standard solutions were each taken 0.5 mL and then 0.1 mL of 10% (b/v) $AlCl_3$ solution, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, then shaken until homogeneous. The solution was subsequently incubated at 37°C for 30 minutes and measured using a UV-Vis spectrophotometer at a wavelength of 424.6 nm.

Identification of the chemical content of the ethanol extract of *M. kauki* leaves using LC-MS

The LC-MS testing was performed according to the method described by Martha et al. (2023) with modifications. The ethanol extract of *M. kauki* leaves was analyzed to identify its secondary metabolites using a Shimadzu LC-MS instrument, model 8040. Before analysis, the crude ethanol extract was filtered through a 0.22 µm membrane filter to remove particulate matter. The concentrated extract was prepared as a stock solution by dissolving it in 95% ethanol with a concentration of 1 mg/mL. The stock solution was sonicated for 10 minutes to ensure complete dissolution and homogeneity. A 1 µL sample was injected into the system with a capillary voltage of 3.0 kV. Compound separation was carried out using a Shimadzu Shim-Pack FC-ODS column (2 mm × 150 mm, 3 µm particle size), maintained at a temperature of 35°C. An isocratic elution system was applied with 95% ethanol as the mobile phase at a flow rate of 0.5 mL/min, over a total run time of 60 minutes. Detection utilized Electrospray Ionization (ESI) in positive ion mode [M]+, with mass spectra recorded across an m/z range of 10–1500 and a scan time of 0.6 seconds per scan. Nitrogen gas was used as the desolvation gas during ionization. The resulting data were interpreted based on molecular ion peaks and fragmentation patterns. Compound identification analysis was carried out using the NIST database by comparing retention times and molecular mass spectra from the chromatograms. The analysis was performed in a single run for qualitative profiling of secondary metabolites. LC–MS analysis in this study aimed to qualitatively identify and determine the relative composition of compounds in ethanol extracts of *M. kauki* leaves. The data obtained were peak area percentages representing the relative abundance of each compound.

Optimization of biofilm formation time of *P. aeruginosa*

This optimisation aims to obtain the optimal incubation time for forming biofilm. The variations used were 24, 48, and 72 hours. Optimisation of biofilm formation time refers to the method used by Besan et al. (2023) with some modifications. A total of 200 µL of bacterial suspension was put into the well plate. Then, incubated for 24, 48, and 72 hours at 37°C. Following the incubation period, the microplate was rinsed three times using running water. Then, 200 µL of 1% crystal violet was added and incubated at room temperature for 15 minutes. Subsequently, it was rinsed three times with running water, followed by the addition of 200 µL of 96% ethanol to each well

and incubation at room temperature for 15 minutes. Then, the biofilm growth was read with an absorbance of 630 nm. The negative control used is growth media without bacteria. The average OD value that has been obtained is compared with the OD-cut value determined using the formula:

OD-cut (ODc) = Mean OD value of negative control + (3 × Deviation standard of negative control)

Biofilm inhibition activity test

The biofilm inhibition assay was conducted based on the method described by Kining et al. (2017), with slight modifications. A total of 80 µL of extract (concentrations of 62.5, 125, 250, 500, and 1000 ppm), 80 µL of HTR media, and 40 µL of bacterial suspension were placed in a microplate. Then, incubated at 37°C for the optimum time previously determined. The microplate was thoroughly washed three times using distilled water. A volume of 200 µL of 1% crystal violet solution was added to each well, followed by incubation at room temperature for 15 minutes. Then, washed with running water 3 times, and added 96% ethanol, as much as 200 µL, was added to each well and incubated at room temperature for 15 minutes. Next, the biofilm inhibition was read with an absorbance of 630 nm. The positive control used ciprofloxacin with a concentration of 0.625, 1.25, 2.5, 5, and 10 mg/mL.

%Biofilm inhibition = × 100%

Biofilm degradation activity test

The biofilm destruction test refers to the method used by Tobi et al. (2022) with modifications. The biofilm degradation assay was performed by adding 80 µL of HTR medium and 40 µL of a bacterial suspension standardized to 1.5×10^6 CFU/mL into each well, followed by incubation at 37°C for the predetermined optimal duration. After the incubation period, the contents of the wells were washed using water, and then the ethanol extract of *M. kauki* leaves at concentrations of 62.5, 125, 250, 500, and 1000 ppm was added as much as 80 µL, then the microplate was again incubated at 37°C for 90 minutes. The microplate was rinsed three times with sterile distilled water to remove non-adherent cells. A total of 200 µL of 1% crystal violet was put into the wells, then incubated at room temperature for 15 minutes. Then washed with running water 3 times, and added 96% ethanol was added to each well, up to 200 µL, and incubated at room temperature for 15 minutes. Next, the biofilm growth was read with an absorbance of 630 nm.

% Biofilm degradation = × 100%

Statistical analysis

The research results are presented in the form of OD values for each stage of biofilm formation, inhibition, and destruction. Statistical analysis was performed using SPSS version 25 (2017). The statistical test results can be seen in supporting file 2. A one-way ANOVA test was conducted to evaluate the differences between groups. Differences were considered significant if $p < 0.05$. Differences between groups were further analyzed using Tukey's post hoc test. A $p < 0.05$ indicated a significant difference in effect between treatments. The relationship between the concentration of the extract or control and the OD value was analyzed using Pearson's correlation test. $P < 0.05$ indicates a correlation between the concentration of the extract and control used and the level of biofilm inhibition or destruction.

RESULTS AND DISCUSSION

Total phenolic content of ethanol extract of *M. kauki* leaves

The determination of total phenolic content using the Folin-Ciocalteu reagent method. Folin-Ciocalteu reagent is common and often used because it is simple, sensitive, consistent, relatively accurate results, and does not require specific and sophisticated equipment (Agustin et al., 2022). The principle of this method is a reduction-oxidation reaction in which the phenolic compound acts as a reductant by donating electrons, while the Folin-Ciocalteu reagent acts as an oxidizer (Pérez et al., 2023). In this test, the standard used is

gallic acid. The absorbance measurement results of the standard solution concentration series obtained were then made into a calibration curve. According to the linear regression analysis of the gallic acid standard solution concentrations and their corresponding absorbance values, a regression equation of y = 0.0098x + 0.2303 was obtained, with a coefficient of determination (R^2) of 0.9976. Furthermore, the absorbance of the sample of ethanol extract of *M. kauki* leaves was substituted into the linear regression equation, resulting in a total phenolic content of 48.983 ± 0.059 mg GAE/g extract. This value is higher when compared to the research of Zakri et al. (2025) on the butanol fraction of 39.94 ± 4.01 mg GAE/g.

Total flavonoid content of the ethanol extract of *M. Kauki* leaves

The total flavonoid content was determined using a colorimetric assay involving the addition of aluminum chloride ($AlCl_3$). Where Al(III) is used as a complexing agent and does not require specific and sophisticated equipment (Agustin et al., 2022). This method is based on the principle of complex formation between aluminum chloride and the keto and hydroxyl groups located at the C4 and C5 positions of the flavonoid structure (Widyasari & Sari, 2021). The absorbance measurement results of the standard solution concentration series obtained were then made into a calibration curve. According to the results of linear regression analysis between the concentration of the gallic acid standard solution and its corresponding absorbance values, a regression equation of y = 0.005x - 0.0113 was obtained, with an R^2 value of 0.9997. By substituting the absorbance value of the *M. kauki* leaf ethanol extract into the equation, the total flavonoid content was calculated to be 21.260 ± 0.306 mg QE/g extract. This result is much greater when compared to the flavonoids in *M. zapota* extract, which is only 2.51 mg QE/g.

Identification of the chemical content of ethanol extract of *M. kauki* leaves using LC-MS

The results of LC-MS analysis showed that ethanol extracts of *M. kauki* leaves contained various compounds with varying relative compositions. The percentage of compounds was determined based on the peak area of the chromatogram, reflecting relative abundance rather than absolute content. According to LC-MS analysis, it can be identified that the content of chemical constituents present in the ethanol extract of *M. kauki* leaves includes phenolics, flavonoids, terpenoids, alkaloids, tannins, carbohydrates, steroids, vitamin C, and aromatic groups.

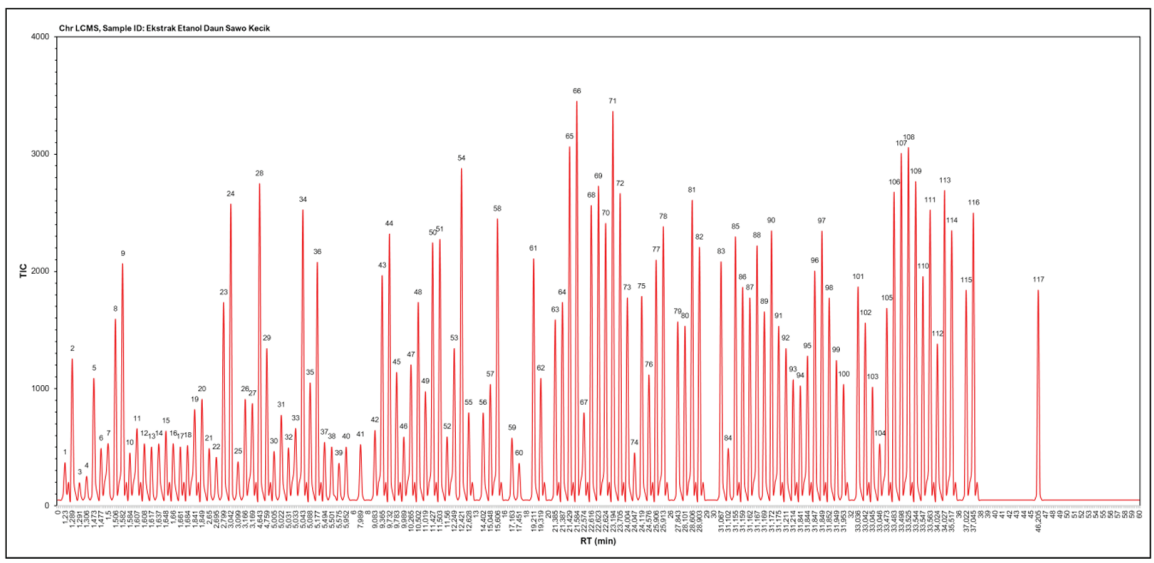


Figure 1. LC-MS chromatogram of the ethanol extract of *M. kauki* leaves

The identification results showed 117 compounds indicated by the LC-MS chromatogram in Figure 1. The phenolic composition of the ethanolic extract of *M. kauki* leaves is high, as much as 23.08%, while flavonoids are 46.15%, and other groups are 30.77%. This value is higher than the results of the total phenolic and flavonoid content tests that have been carried out. This is due to differences in measurement methods. Measurement of total phenolic and flavonoid contents using colorimetric methods with Folin–Ciocalteu and $AlCl_3$ reagents cannot always detect all phenolic and flavonoid compounds completely, so the results may be lower than the actual. Meanwhile, LC-MS has a more sensitive and selective ability, so it can detect and quantify more types of compounds, including compounds that are sometimes not detected by colorimetric methods. Furthermore, colorimetric assays provide cumulative values expressed as equivalents (mg GAE/g or mg QE/g), whereas LC–MS profiling is based on the detection of individual compounds and their relative abundance, which can lead to apparently higher values. Therefore, discrepancies between the two methods are expected and reflect fundamental differences in analytical principles rather than inconsistencies in the results.

The compounds identified by LC-MS (see supplementary file 1) are dominated by phenolic groups, especially flavonoids. The identified flavonoids can be classified based on their basic framework, such as flavone, flavan-3-ol, and flavanol. Flavonoids with a flavone basic framework include apigenin, luteolin, chryseriol, vitexin, and isovitexin. Compounds that belong to the flavan-3-ol group include epigallocatechin and its derivatives, namely epigallocatechin-3-*O*-coumarate, epicatechin-3-*O*-*p*-hydroxybenzoate, epigallocatechin-3-*O*-cinnamate, and epigallocatechin gallate, epiafzelechin, and its derivative, epiafzelechin-3-*O*-gallate. Epigallocatechin and epiafzelechin derivatives undergo esterification, where the -OH group at the C3 position reacts with phenolic acid to form an ester. Flavonoid compounds with flavonol basic skeletons include kaempferol-7-*O*-*β*-*D*-glucoside, kaempferol-3-*O*-*D*-glucoside, kaempferol-3-*O*-rhamnoside, kaempferitrin, quercetin, quercetin-3-*O*-rhamnoside, myricetin-3-glucoside, myricetin-3-*O*-*β*-*D*-galactopyranoside, and quercetin-3-*O*-rutinoside. These compounds, except quercetin, undergo glycosylation, where there is the addition of sugar groups to the flavonol hydroxyl group through glycosidic bonds.

In addition to flavonoids, there are non-flavonoid phenolic compounds identified in the ethanol extract of *M. kauki* leaves, such as phenolic acid. Compounds that include phenolic acids include gallic acid, cinnamic acid, syringic acid, benzoic acid, vanillic acid, ferulic acid, salicylic acid, and caffeic acid. This group of compounds is polar, so that it can be extracted by ethanol solvent.

The compounds identified by LC-MS were also found in other species of the Manilkara genus. In the *Manilkara zapota* species, caffeic acid, gallic acid, and epigallocatechin compounds were found (Baky et al., 2016). The extract also contained various other constituents, including salicylic acid, vanillic acid, ferulic acid, and quercetin, which were also identified in *Manilkara hexandra* (Roxb.) Dubard (Parikh & Patel, 2017b). This finding is in line with the concept of chemotaxonomy, which indicates that plant species with close taxonomic relationships may share similar chemical constituents (Uckele et al., 2021).

Optimization of *P. aeruginosa* biofilm formation

Optimization of biofilm formation was carried out to determine the time required for *P. aeruginosa* bacteria to form the best biofilm so that it can be used as an optimum condition for biofilm inhibition and destruction testing. In the incubation process, 3 variations of incubation time were carried out, namely 24, 48, and 72 hours. The *P. aeruginosa* bacterial suspension in this test was grown in liquid media. Media can encourage the growth and development of a microbe. Liquid culture media make it easier for bacteria to access nutrients (Bonnet et al., 2020).

The media used in this study is HTR media because it provides nutrients that support the optimal growth of heterotrophic bacteria, so that biofilm can form well on the surface of the microplate. The results of biofilm formation on the microplate were followed by measurement of the OD value. Measurement of OD value in this study was carried out to determine the strength of bacterial biofilm formation (Wang et al., 2024). An increase in OD value indicates an increase in suspension turbidity, which means that the number of microbial cells has increased. If used to measure the growth of biofilm formation, the higher the OD value, the more biofilm is formed on the surface (Turhan & Koca, 2024). OD value data of biofilm formation optimization results are presented in Figure 2.

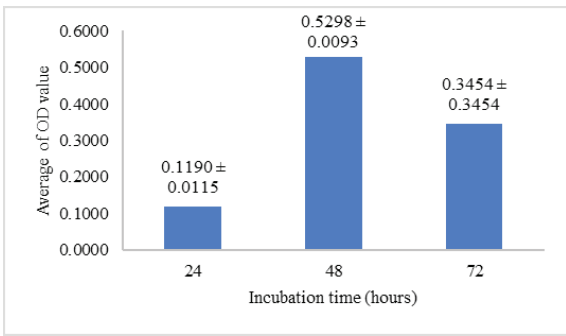


Figure 2. Average OD value on biofilm formation

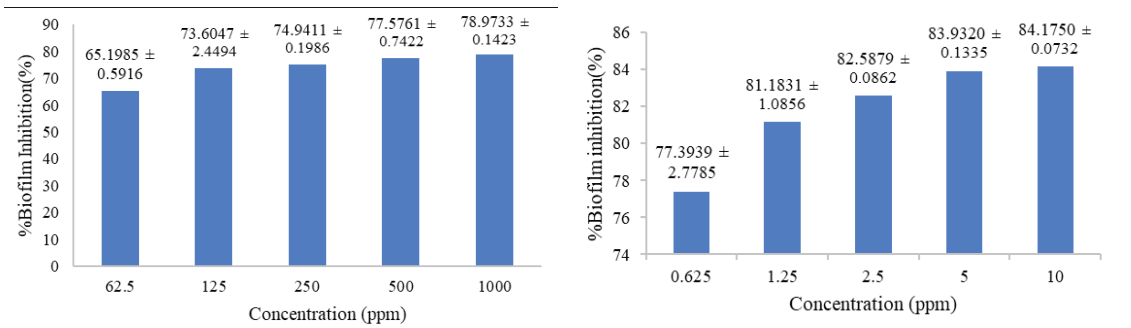
According to Figure 2, on the 24-hour mark, the average OD value of biofilm was 0.1190, on the 48-hour mark, an increase in the average OD of 0.5298, and on the 72-hour mark, a decrease in the average OD value of 0.3454. Thus, it is known that the incubation time of 48 hours showed the best formation and growth of *P. aeruginosa* biofilm with an OD value of 0.5298. This average was then compared with the ODCut value to determine the strength of biofilm formation. The ODcut value obtained was 0.0462. Therefore, the ability of the test bacteria to form biofilms is classified as strong, as it fulfills the criterion of 4×ODcut < OD isolate. This finding is consistent with the study conducted by Azimzadeh et al. (2025). that *P. aeruginosa* is a strong biofilm former with OD values meeting 4×ODcut < OD isolate.

Incubation for 48 hours had the highest OD value compared to 24 and 72 hours. Biofilm formation increased at 48 hours and a decrease on 72 hours. The increase in biofilm formation after 48 hours occurred because the bacteria were in the phase of active growth and biofilm maturation, where the bacteria multiplied, produced extracellular matrix, and formed a mature biofilm structure. After 72 hours, it began to decrease due to the dispersion phase, where some bacteria began to break away from the biofilm to find a new environment, or due to nutrient limitation and accumulation of waste products that inhibited further growth. At the beginning of biofilm formation, nutrients in the growth medium are abundant, supporting bacterial growth and multiplication. However, over time, these nutrients can be depleted, causing bacterial growth to slow or stop, so that the biofilm biomass no longer increases as quickly as before (Azizah et al., 2023). This cycle is a natural part of biofilm dynamics, where, after reaching maximum density, the biofilm will experience a decrease in biomass due to cell release, so that at that stage, the number of colonies attached to the well wall decreases. This is in accordance with the research of Chimi et al. (2024) that the best time for *P. aeruginosa* biofilm formation is 48 hours, where biofilm formation increases with incubation time up to 48 hours and decreases after 72 hours. Thus, it can be concluded that the time required for *P. aeruginosa* to be able to form the best biofilm for testing is 48 hours.

Biofilm formation inhibition activity

The biofilm inhibition assay was carried out to evaluate the ability of the ethanolic extract of *M. kauki* leaves to prevent biofilm formation by *Pseudomonas aeruginosa* during the initial phase of development. An inhibition test was conducted on several concentrations of extracts with a positive control of ciprofloxacin using the microtiter plate biofilm assay method. On the other hand, the negative control used is a suspension with media and solvent without the

addition of extracts. The OD value of the negative control was 0.4390. The OD value of the negative control illustrates the maximum amount of biofilm formed in the absence of extract treatment, so it is used as a reference in calculating the effectiveness of inhibition.



(a) (b)

Figure 3. (a) Percentage (%) inhibition of biofilm formation by (a) the ethanol extract of *M. kauki* leaves and (b) ciprofloxacin

Figure 3 shows the % inhibition of biofilm formation in the ethanol extract of *M. kauki* leaves and the ciprofloxacin control. The %inhibition value of biofilm formation continues to increase from low to high concentrations. This indicates that increasing the concentration of both the extract and the control results in a greater percentage of biofilm formation inhibition. This is because the higher the percentage of extracts and controls, the more antibiofilm substances they contain, so that they can prevent biofilm attachment better.

The highest percentage inhibition of ethanol extract is at a concentration of 1000 ppm, which is higher than the control at a concentration of 0.625 ppm. While the lowest % inhibition was at a concentration of 62.5 ppm. For the ciprofloxacin control, the highest biofilm inhibition was observed at 10 ppm, while the lowest was observed at 0.625 ppm. The biofilm inhibition percentages obtained from each concentration were subsequently used to calculate the IC_{50} value through non-linear dose–response regression curve analysis. The IC_{50} values of the ethanol extract of *M. kauki* leaves and ciprofloxacin are shown in Table 1.

Table 1. IC_{50} value of biofilm formation inhibition of ethanolic extract of *M. kauki* leaves and ciprofloxacin

Sample	IC_{50} (µg/mL)	Category
Ethanol extract of *M. kauki* leaves	134	Moderate
Ciprofloxacin	1.63	Very strong

The ethanol extract of *M. kauki* leaves has an IC_{50} of 134 µg/mL, indicating moderate antibiofilm activity. Ciprofloxacin showed significant antibiofilm activity with an IC_{50} value of 1.63 µg/mL, which is included in the very strong category. According to Souhoka et al. (2019), IC_{50} values <50 µg/mL are classified as very strong, 50–100 µg/mL as strong, 100–150 µg/mL as moderate, and 151–200 µg/mL as weak. The IC_{50} value is often used for biofilm formation inhibition tests; a lower IC_{50} value indicates greater effectiveness of the sample in inhibiting biofilm formation (Besan et al., 2023).

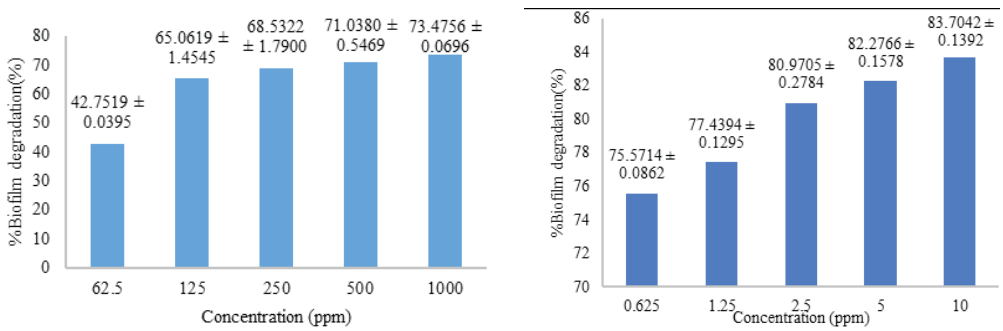
According to the LC-MS results on *M. Kauki* leaves, there is a fairly high concentration of epigallocatechin gallate (EGCG) at 1.549%. Referring to the results of research by Hao et al. (2021), it is

known that epigallocatechin gallate inhibits the formation of *P. aeruginosa* biofilm by more than 70% at a concentration of 256 µg/mL. This inhibitory effect increases with increasing EGCG concentration. EGCG inhibits the formation of *P. aeruginosa* biofilm through a mechanism in the QS system. Epigallocatechin gallate significantly reduces the production of C4-HSL and 3-oxo-C12-HSL, which are two major AHL molecules that activate the las and rhl regulatory systems. This decrease in AHL levels causes suppression of the transcription of important QS genes, including lasI, lasR, rhlI, rhlR, pqsA, and pqsR, which respectively encode QS synthase enzymes or transcription regulators. This directly impacts the reduced expression of other virulence genes, including lasB (encoding elastase), phzA, and phzM (involved in the biosynthesis of the toxic pigment pyocyanin). Additionally, EGCG significantly inhibits swimming and swarming motility, which are important initial stages for bacteria to spread and attach to the substrate surfaces before forming stable three-dimensional biofilms (Hao et al., 2021).

In the inhibition of biofilm formation, the ANOVA results for the extract and control showed p = 0.000, indicating a significant difference. The post hoc test showed that each OD value at the concentration of small *M. kauki* leaf ethanol extract and ciprofloxacin control was <0.05. The Pearson correlation test produced p = 0.000, indicating a significant correlation between the increase in concentration and the level of biofilm inhibition and destruction.

Biofilm degradation activity test

Biofilm degradation test was carried out by putting bacterial suspension and HTR media into a microplate and incubating for 48 hours. Then the solution in the plate was discarded and washed. Next, the extract and positive control were treated with various concentrations.



(a) (b)

Figure 4. Percentage of biofilm degradation (a) ethanol extract of *M. kauki* leaves and (b) ciprofloxacin

Figure 4 shows the % inhibition of biofilm formation in the ethanol extract of *M. kauki* leaves and the ciprofloxacin control. According to Figure 4, it is known that the % of biofilm destruction continues to increase from low to high concentrations. This indicated that increasing the concentration of both the extract and the control leads to a higher percentage of biofilm degradation. The higher the percentage of extracts and controls, the more antibiofilm substance content in it, so that it can destroy biofilm better.

The highest percentage of destruction of ethanol extract is at a concentration of 1000 ppm, which is higher than the control at a concentration of 0.625 ppm. While the lowest % destruction was at a concentration of 62.5 ppm. Meanwhile, for the ciprofloxacin control, the greatest level of biofilm destruction was observed at a concentration of 10 ppm, while the lowest occurred at 0.625 ppm.

In general, eliminating established biofilms using extracts is more challenging. Several studies have also

reported that preventing initial cell attachment is easier than disrupting already formed biofilms. These findings are consistent with the study conducted by Tobi et al. (2022), who conducted antibiofilm testing against *Staphylococcus aureus* bacteria, where the inhibition value was higher than destruction, namely 71.17% and 54%. This supports the notion that pathogens exhibit greater resistance to antimicrobial agents when embedded within biofilms, allowing infections to persist on various biotic and abiotic surfaces (Famuyide et al., 2019).

The biofilm formation inhibition percentage data were subsequently used to determine the IC_{50} value, which was calculated from the inhibition percentages at each concentration using non-linear dose–response regression curve analysis. Table 2 presents the IC_{50} values of the ethanolic extract of *M. kauki* leaves and ciprofloxacin.

Table 2. IC_{50} value of biofilm degradation inhibition of ethanolic extract from the leaves of *M. kauki* leaves and ciprofloxacin

Sample	IC_{50} (µg/mL)	Category
Ethanol extract of *M. kauki* leaves	108.40	Moderate
Ciprofloxacin	2.04	Very strong

According to Table 2, the ethanol extract of *M. kauki* leaves has an IC_{50} value of 108.40 µg/mL, which is included in the category of moderate antibiofilm activity. Meanwhile, ciprofloxacin showed significant antibiofilm activity with an IC_{50} value of 2.04 µg/mL, which is included in the very strong category. The IC_{50} value is often used for biofilm formation inhibition tests; a lower IC_{50} value indicates a higher efficacy of the sample in inhibiting biofilm formation.

Compounds present in the ethanol extract of *M. kauki* are capable of inhibiting and destroying biofilms through mechanisms such as degradation of the biofilm matrix, membrane leakage, and cell death (Besan et al., 2023). LC–MS analysis of *M. kauki* leaves revealed the presence of chlorogenic acid at a relatively high concentration of 1.674%. Chlorogenic acid has been reported by Wang et al. (2024) to exhibit biofilm-disrupting activity against *Pseudomonas putida*, achieving up to 70% biofilm destruction at a concentration of 25.4 mM. This biofilm-disrupting effect increased in a concentration-dependent manner. Chlorogenic acid disrupts biofilms by interfering with the physical structure of the extracellular polymeric substance (EPS) matrix, which consists of eDNA, exopolysaccharides, and proteins (Liu et al., 2025). Chlorogenic acid can bind and form hydrogen bonds with hydroxyl (–OH) and carboxylate ($–COO^-$) groups of polysaccharides or proteins within the EPS (Diniyah & Lee, 2020). This interaction leads to protein denaturation and disintegration of the EPS structure. Damage to the EPS compromises its ability to retain water and nutrients, protect against antibiotics, and maintain biofilm structural stability. The weakening of the biofilm may result in membrane disruption and leakage, as the compound inserts into the lipid layer, increasing membrane permeability. Increased membrane permeability can cause leakage of cytoplasmic contents, leading to the loss of essential components required for protein synthesis, DNA/RNA synthesis, and energy production, thereby disrupting fundamental cellular functions (Campos et al., 2025).

In the inhibition of biofilm destruction, the ANOVA results for the extract and control showed $p = 0.000$, indicating a significant difference. The post hoc test showed that each OD value at the concentration of small *M. kauki* leaf ethanol extract and ciprofloxacin control was <0.05. The Pearson correlation test produced $p = 0.000$, indicating a significant correlation between the increase in concentration and the level of biofilm inhibition and destruction.

CONCLUSION

This study concludes that the total phenolic and flavonoid contents in the ethanol extract of *M. kauki* leaves were 48.9830 mg GAE/g and 21.327 mg QE/g, respectively. *Pseudomonas aeruginosa* was identified as a strong biofilm-forming bacterium, with an

optimum formation time of 48 hours and an OD value of 0.5298. The ethanol extract of *M. kauki* leaves demonstrated inhibitory and disruptive activities against *P. aeruginosa* biofilms, with the highest inhibition and destruction percentages observed at a concentration of 1000 ppm, reaching 78.9733% and 73.4756%, respectively, and IC_{50} values of 134 µg/mL and 108.4 µg/mL.

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AUTHOR CONTRIBUTION STATEMENT

Writing original draft, experiment of TPC and TFC contents, antibiofilm experiment, analysis of data (LF). Writing review & Editing, Resources, Data curation, Supervision (TT).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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