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ANTIBIOFILM ACTIVITY OF MANGOSTEEN (Garcinia mangostana L.*) LEAF EXTRACT AGAINST COLLECTION OF BACTERIAL ISOLATES FROM DIABETIC ULCERS*

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Abstract

Mangosteen (*Garcinia mangostana* L.) is a plant that contains many benefits and has the potential to be a medicinal plant in treating various diseases. Mangosteen leaves contain flavonoids, saponins, tannins, alkaloids and terpenoids which are known to have antibacterial and antibiofilm properties. This anti-biofilm agent is an alternative treatment for diabetic wound infections where cases of antibiotic resistance have increased. One of the causes of resistance is the biofilm formed by infectious bacteria. This research is a type of laboratory experimental research which aims to determine the anti-biofilm activity of mangosteen leaf extract against biofilms formed by a collection of bacterial isolates from diabetic wounds. The antibiofilm activity test carried out consisted of a cell attachment prevention test, a biofilm formation inhibition test, and a biofilm destruction test using the crystal violet staining method using the tube method and measuring optical density values on a UV-Vis spectrophotometer. The concentrations of mangosteen leaf extract used are 60%, 80% and 100% as well as control – and control +. The results of the antibiofilm test showed that mangosteen leaf extract had the best activity in inhibiting biofilm formation, preventing cell attachment, and destroying biofilm, respectively. The three most optimal activities were found at a concentration of 100%, with an inhibition percentage of 50.51%, prevention of 32.56%, and destruction of 5.63%.

Keywords: *Antibiofilm; Garcinia mangostana L.; Bacterial Isolates; Diabetic Ulcers*

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INTRODUCTION

Diabetic wounds are a complication caused by damage to nerves and blood vessels due to high blood glucose levels, which triggers the appearance of wounds (Yusnita et al., 2021). In Indonesia, the prevalence of diabetic wounds reaches 15% with a 30% risk of amputation and a 32% mortality rate. This disease is also known to be 80% of the causes of hospital admissions (Sulistyowati, 2015).

If diabetic wounds are left and not treated immediately, they will make the wounds worse and become easily infected with bacteria. This is supported by Lellu (2021), that high levels of glucose can inhibit the flow of blood to the hands and feet so that the blood supply that should carry leukocyte cells that fight infection in these areas is reduced. This condition can cause diabetic wounds to become infected, develop into gangrene, amputation, and even death if treatment is not appropriate.

Based on the opinion of Afonso et al. (2021), bacteria involved in diabetic wound infections are known to be able to worsen wounds in the long term and make healing difficult due to their ability to form biofilms. According to Schilcher and Horswill (2020), biofilm is an extracellular polymer matrix secreted by a population of biofilm components, either a homogeneous or heterogeneous population of bacteria that live growing in the matrix. The formation of bacterial biofilms can cause antibiotic resistance which often results in the effectiveness of therapy not being achieved and prolonged chronic infections. Efforts to overcome this problem can be done by utilizing plants that have the potential to act as antibiofilms, one of which is Mangosteen leaves (*Garcinia mangostana* L.).

Based on research by Rosalina and Mahendra (2021), methanol extract of Mangosteen leaves has been proven to have antibacterial properties against bacteria that cause diabetic wounds. The phytochemical identification results obtained also showed that the extract contained flavonoids, alkaloids, polyphenols, tannins and saponins. The compound content in Mangosteen leaves is known to act as an anti-biofilm. This is in accordance with the statement of Slobodníková, et al., (2016), that the content of polyphenolic compounds from plants, such as flavonoids, phenolic acids and tannins can show anti-biofilm effects.

Based on this description, this research was carried out which aimed to determine the anti-biofilm activity of Mangosteen leaf extract against bacteria that cause diabetic wound infections which were tested on a collection of clinical isolates originating from diabetic wounds.

Thus, it is hoped that the antibacterial ability of Mangosteen leaves against bacteria that cause diabetic wounds and their compounds can become an alternative anti-biofilm agent that can prevent adhesion, inhibit the formation or destroy bacterial biofilms in diabetic wounds.

RESEARCH METHODS

This research is a type of laboratory experimental research carried out to determine the anti-biofilm activity of Mangosteen leaf extract against a collection of bacterial isolates obtained from diabetic wounds using the crystal violet staining method using the tube method and measuring optical density values on a UV-Vis spectrophotometer.

The materials used in this research were Mangosteen leaves (*Garcinia mangostana* L.), Samples were obtained from Bongo IV village, Paguyaman subdistrict, Boalemo Regency, Gorontalo Province. Sampling was carried out when the plants were fresh in the morning. The mangosteen leaves selected as research samples were determined at the Botany Laboratory, Biology Department, FMIPA, Universitas Negeri Gorontalo. A collection of diabetic wound bacteria isolates is P1 (Control -), P2 (Extract 60%), P3 (Extract 80%), P4 (Extract 100%) and P5 (Control +), Congo Red Agar (CRA), Nutrient Agar (NA), Nutrient Broth (NB), Luria Bertani Broth (LBB), Brain Heart Infusion Broth (BHIB), 2% glucose, 10% dimethyl sulfoxide (DMSO), 1% crystal violet, sodium hypochlorite, phosphate-buffered saline (PBS), 0.9% NaCl, methanol, 96% ethanol, 95% alcohol , iodine, safranin, distilled water, 70% alcohol, filter paper, cotton, and aluminum foil.

Preparation of Test Bacteria

The test bacteria used in this research were diabetic wound bacteria isolates obtained from clinical isolates from the collection of the Microbiology Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Gorontalo. The isolate was rejuvenated by inoculating it on Nutrient Agar culture media and then incubating it at 37ºC for 24 hours.

Detection Of Biofilm Formation Of Test Bacteria Using The Congo Red Agar Method

The test was carried out as described by Kırmusaoğlu (2019), namely by inoculating rejuvenated bacteria on CRA media and then incubating for 24 hours at 37°C. Positive results in this test are indicated by the formation of black colonies with the consistency of dry crystals.

Macroscopic and Microscopic Characterization of Test Bacterial Isolates

Test bacterial isolates that were positive for forming biofilms were incubated on Nutrient Agar media for 24 hours and then macroscopically observed the morphology of the growing bacterial colonies, starting from the shape, edges, elevation and color of the colonies. Meanwhile, microscopic characterization was carried out using the Gram staining method, as described in the research of Sarijowan et al. (2022). Observations were carried out under a microscope to see the Gram type of bacteria after staining along with the cell shape. Gram positive bacteria will be purple while Gram negative bacteria will be red.

Media Optimization and Biofilm Formation Time Using the Tube Method

The first optimization carried out was culture media using Nutrient Broth (NB), Luria Bertani Broth (LBB), and Brain Heart Infusion Broth (BHIB). 1 mL of bacterial suspension was inoculated in 3 test tubes, each tube containing 1 mL of NB, LBB and BHIB media with the addition of 2% glucose to each medium. Then all tubes were incubated for 24 hours at 37°C. The bacterial culture that had been grown in the test tube was discarded and rinsed 3 times with sterile PBS solution and then dried. Next, the biofilm formed on the tube walls was stained with 1% crystal violet and left for 15 minutes, then rinsed again 3 times with sterile PBS to remove excess dye that did not dissolve in the biofilm. Positive results are indicated by the formation of purple spots on the walls and bottom of the tube. The denser the spots formed indicates the greater or stronger the biofilm formation.

The time optimization stages carried out are the same as the media optimization stages, but using culture media with varying optimization results and incubation times, namely 24 hours, 48 hours and 72 hours.

Making Mangosteen Leaf Extract (*Garcinia mangostana* L.)

The method used is total maceration using methanol solvent. The stage begins with weighing 500 grams of simplicia powder and then placing it in a maceration vessel. Next, 2000 mL of methanol was added until all the powder was completely submerged then macerated for 3×24 hours with occasional stirring for 1 hour. After that, the residue and filtrate are separated by filtering the macerate with filter paper. All filtrate is evaporated on a rotary evaporator for 3x24 hours until a thick extract is obtained, then the yield percentage is calculated using the following formula:

$$\% \text{ Yield} = \frac{\text{Weight of Thick Extract}}{\text{Weight of Simplicia Powder}} \times 100\%$$

Making Test Sample Solutions

The methanol extract of Mangosteen leaves (*Garcinia mangostana* L.) obtained was then made into 3 concentrations, namely 60%, 80% and 100% by diluting it using 10% DMSO solvent.

Antibiofilm Testing of Mangosteen Leaf Extract (*Garcinia mangostana* L.) against Diabetic Wound Bacterial Isolates

1. Cell Adhesion Prevention Assay

This test consists of three activities, namely preventing cell attachment, inhibiting biofilm formation, and destroying biofilm. These three activity tests were carried out according to research by Kining et al. (2016) with several modifications.

The cell adhesion prevention test begins by inserting 2 mL of sample solution of 60%, 80% and 100% concentration into 3 test test tubes. A total of 2 mL of the best media from optimization which was added with 2% glucose as a negative control and sodium hypochlorite as a positive control was also added to 2 other test tubes. Next, the tube was incubated for 1 hour at 37°C. After incubation, the contents of the test tube are discarded. Next, 1 mL of bacterial suspension and 1 mL of media with the addition of 2% glucose were added to each test and control tube. The tube was re-incubated for an optimized time at 37°C then rinsed 3 times using sterile PBS and dried. 1% crystal violet solution was added to each tube as much as 2 mL and left for 15 minutes at room temperature. The tube was rinsed again 3 times with sterile PBS and dried. After that, 2 mL of 96% ethanol solvent was added to each tube and left for 15 minutes. Optical density (OD) values were read at a wavelength of 595 nm with a UV-Vis spectrophotometer. The percentage of prevention of cell adhesion (%PPS) obtained from Mangosteen leaf extract is calculated using the following formula:

$$\%PPS = \frac{\text{OD Negative Control-OD Sample}}{\text{OD Negative Control}} \times 100\%$$

2. Biofilm Formation Inhibition Test

The biofilm formation inhibition test began by inserting 1 mL of 60%, 80% and 100% sample solution into 3 test tubes, and adding 0.5 mL of bacterial suspension and 0.5 mL of the best optimized media with the addition of 2% glucose, respectively. . Another tube containing 1 mL of bacterial suspension and 1 mL of media without sample administration was used as a negative control, while 0.5 mL of bacterial suspension and 0.5 mL of media with 1 mL of sodium hypochlorite were used as positive controls. Next, the tube was incubated for an optimized time at 37°C. Then the contents of the tube were removed and rinsed 3 times using sterile

PBS and then dried. To each tube, 2 mL of 1% crystal violet was added and left for 15 minutes at room temperature. The tube was rinsed again 3 times with sterile PBS and dried. After that, 2 mL of 96% ethanol solvent was added to all tubes and left for 15 minutes. The OD value was read at a wavelength of 595 nm with a UV-Vis spectrophotometer. The percentage of inhibition of biofilm formation (%PPB) obtained was then calculated using the same formula as in the cell attachment prevention assay.

3. Biofilm Destruction Test

The biofilm destruction test began by inserting the bacterial suspension and the best optimized media with 1 mL of 2% glucose added each into each test and control tube and then incubating for the optimized time at 37°C. After incubation, the contents of the tube were removed and rinsed 3 times with sterile PBS and then dried. Next, 2 mL of 60%, 80% and 100% sample solutions were put into each test tube. In the control tube, 2 mL of media was added with the addition of 2% glucose for the negative control and 2 mL of sodium hypochlorite for the positive control. All tubes were incubated again for 1 hour at 37°C then rinsed 3 times with sterile PBS and dried. 1% crystal violet solution was added to each tube as much as 2 mL and left for 15 minutes at room temperature. The tubes were rinsed again 3 times and dried, then 2 mL of 96% ethanol solvent was added to each tube and left for 15 minutes. The OD value was read at a wavelength of 595 nm with a UV-Vis spectrophotometer. The percentage of biofilm destruction (%PB) obtained was calculated using the same formula as in the cell adhesion prevention assay.

RESULTS AND DISCUSSION

Detection of Biofilm Formation of Test Bacteria Using the Congo Red Agar Method

As is known, there are 7 collections of diabetic wound bacterial isolates from the Microbiology Laboratory, Biology Department, Gorontalo State University. Of the seven isolates, 1 bacterial isolate was found to be positive, forming black colonies with the consistency of dry crystals. According to Hou et al. (2012), this change in colony color is caused by the production of polysaccharide intercellular adhesin (PIA) by bacteria which functions as an adhesive between bacterial cells so that they become colonies. This is also supported by Mariana et al. (2009), that the black colonies are an interaction of Congo red dye with PIA which is usually synthesized by bacterial strains that have the icaA and icaD genes. The presence of these two genes indicates that the isolate is capable of forming a biofilm.

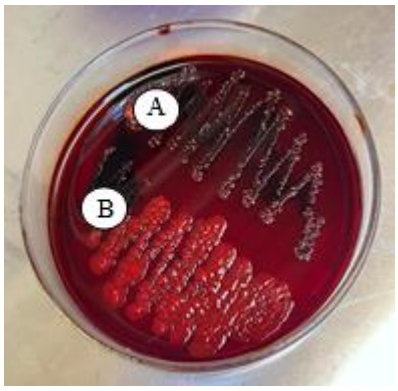

Figure 1. Biofilm Formation Detection Results. (A) Isolates that form biofilms, and (B) Isolates that do not form biofilms

Macroscopic and Microscopic Characterization of Test Bacterial Isolates

The bacterial isolates obtained were positive for forming biofilms in the Congo Red Agar test and were then characterized macroscopically and microscopically. In this study, the macroscopic characteristics of diabetic wound bacterial isolates were found to be round in shape, with flat colony edges, the elevation or growth height of the colony was convex, and the colonies were yellowish white. From the macroscopic characterization results were obtained which showed that the bacterial isolate was a type of Gram positive bacteria where the cells were purple and had the shape of coccus cells.

Media Optimization and Biofilm Formation Time Using the Tube Method

Based on the optimization that has been carried out, the best media for biofilm growth is BHIB, followed by LBB and NB (Figure 2). These results are in line with research by Wijesinghe et al. (2019), that the maximum growth of biofilms from mono-species or mixed bacteria is known to occur when using BHIB, then LBB, and NB media. It was further said that this was related to the composition of the media, where the highest nutrient concentration was also found in BHIB, followed by LBB and NB. In accordance with the opinion of Chen et al. (2012), BHIB contains higher levels of peptone, protein and salt than the other two media, where peptone and its infusion (calf brain and beef heart) provide nitrogen compounds, carbon, amino acids and vitamins, as well as dextrose as a source of energy. Overall it can facilitate planktonic and biofilm growth.

The optimal media obtained, namely BHIB, was then used in the optimization stage of biofilm formation time. In this research, formation optimization.

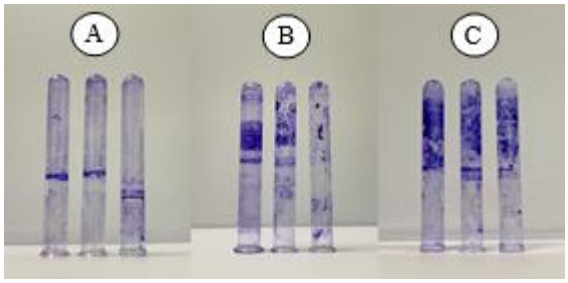

Figure 2. Results of Optimizing Incubation Time Using BHIB Media. During (A) 24 hours, (B) 48 hours, and (C) 72 hours.

The results of the biofilm that were carried out were the most optimal incubation time, namely 72 hours. The results of media optimization and time for biofilm formation were then used as a reference for culture media and incubation time in the anti-biofilm test for Mangosteen leaf extract (*Garcinia mangostana* L.).

Extraction of Mangosteen Leaves (*Garcinia mangostana* L.)

The extraction results obtained in this research were in the form of a percent yield of methanol extract of 14.42%. According to research by Putri et al. (2017), the yield percentage results have met the requirements, namely around 10-15%, which means the extraction process is going well. This also shows that the compounds contained in the Mangosteen leaf samples were extracted and dissolved well in the solvent used. The explanation from Hasan et al. (2022), that the higher the extract soaking, the higher the content of compounds attracted to a sample.

Antibiofilm Testing of Mangosteen Leaf Extract (*Garcinia mangostana* L.) against Diabetic Wound Bacterial Isolates

In the cell adhesion prevention activity test, concentration measurement results were obtained at 60%, 80%, and 100% (Table 1) with an average OD value of 1.668, 1.450, and 1.191 respectively, while the positive and negative controls obtained an average of 0.146. and 1,766.

Table 1. Results of measuring optical density values to prevent cell adhesion

Treatment	Repetition			Average OD Value
	1	2	3	
Control (-)	1,771	1,759	1,768	1,766
Extract 60%	1,670	1,594	1,739	1,668
Extract 80%	1,582	1,398	1,370	1,450
Extract 100%	1,051	1,253	1,269	1,191
Control (+)	0,197	0,131	0,111	0,146

The data from the calculation of the percentage of prevention is shown in the 60% extract at 5.55%, at the 80% extract at 17.89%, and at the 100% extract at 32.56% (Figure 3). Based on the results obtained, the extract concentration is inversely proportional to the OD value but directly proportional to the percentage of prevention. This shows that the higher the extract concentration, the lower the OD value, but the percentage of cell adhesion prevention is also higher.

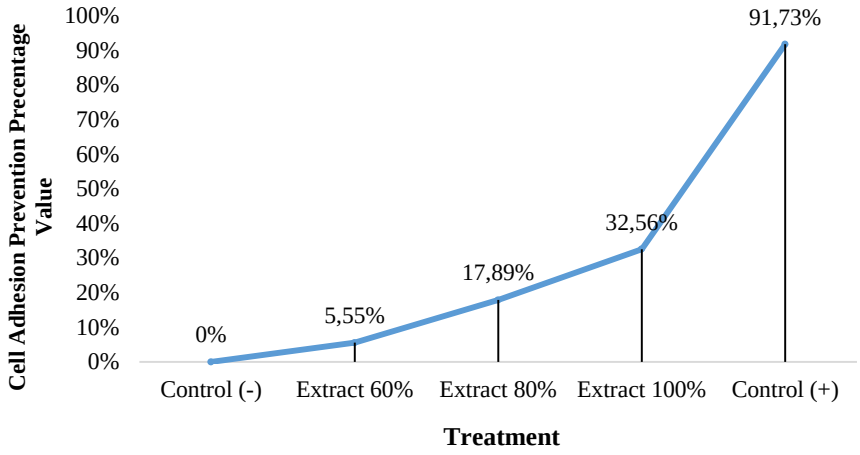

Figure 3. Percentage of Cell Adhesion Prevention Activity of Mangosteen Leaf Extract

The mechanism for preventing cell attachment of diabetic wound bacterial isolates by Mangosteen leaf extract is thought to be through the anti-adhesive effect of the tannin and flavonoid compounds contained in the extract. According to Kining et al. (2016), tannins and flavonoids have the potential to inhibit the icaA and icaD genes which synthesize intercellular adhesion polysaccharide which plays an important role in the cell adhesion process. Additionally, Lahiri et al. (2019) argue that tannins can also bind cell adhesin receptors on the surface of bacteria so that the bacteria's ability to adhere is reduced.

In testing the inhibitory activity of biofilm formation, the average OD values for concentrations of 60%, 80%, and 100% were 1.668, 1.334, and 0.874, respectively, while the positive and negative controls had an average of 0.230 and 1.766 (Table 2).

Table 2. Results of Measurement of Optical Density Values for Inhibition of Biofilm Formation

Treatment	Repetition			Average OD Value
	1	2	3	
Control (-)	1,772	1,769	1,758	1,766
Extract 60%	1,675	1,695	1,633	1,668
Extract 80%	1,330	1,356	1,347	1,344
Extract 100%	0,860	0,954	0,808	0,874
Control (+)	0,291	0,217	0,183	0,230

Based on the OD value, the results of calculating the percentage of inhibition shown in the 60%, 80%, 100% extract were also obtained, namely 5.61%, 23.89% and 50.51% respectively (Figure 4).

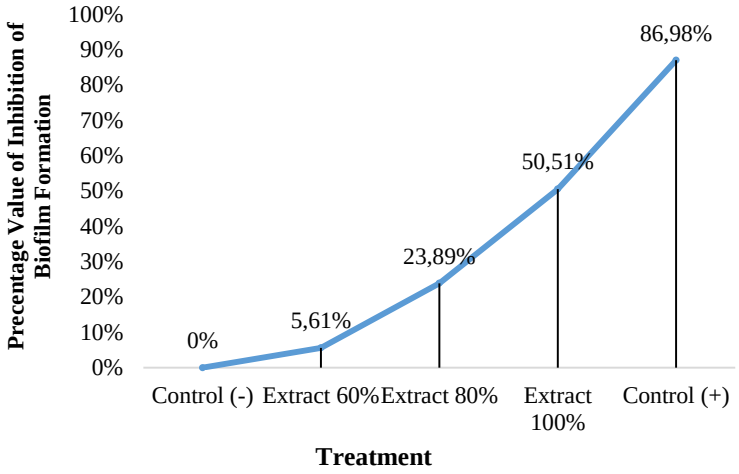

Figure 4. Percentage of Activity Inhibiting Biofilm Formation

Similar to prevention, the results of inhibiting biofilm formation show that the higher the extract concentration, the percentage of inhibition also increases, as indicated by the decreasing OD value.

Compounds in Mangosteen leaf extract which are able to inhibit quorum-sensing and biofilm production can inhibit biofilm formation like the ability of alkaloid compounds. According to Jain and Parihar (2018), alkaloids are able to reduce initiator genes for forming biofilms and also inhibit quorum-sensing in battery cells. As for Kining et al. (2016) explained that flavonoids and tannins can also prevent the regulation of the icaA and icaD genes which play an important role in the process of biofilm EPS formation.

The final test was the biofilm destruction activity with the average OD value of the 60% extract concentration being 1.759, the 80% extract being 1.742, and the 100% extract being 1.660, while the positive and negative controls had an average of 0.379 and 1.759 (Table 3).

Table 3. Results of Measurement of Optical Density Values of Biofilm Destruction

Treatment	Repetition			Average OD Value
	1	2	3	
Control (-)	1,758	1,760	1,759	1,759
Extract 60%	1,755	1,762	1,760	1,759
Extract 80%	1,752	1,726	1,748	1,742
Extract 100%	1,669	1,654	1,658	1,660
Control (+)	0,357	0,361	0,421	0,379

The results of calculating the percentage of destruction shown in extracts of 60%, 80%, and 100% were respectively 0%, 0.97%, and 5.63% (Figure 5). Based on the results obtained, it is known that Mangosteen leaf extract has a weaker ability to destroy biofilms compared to the other two anti-biofilm activities.

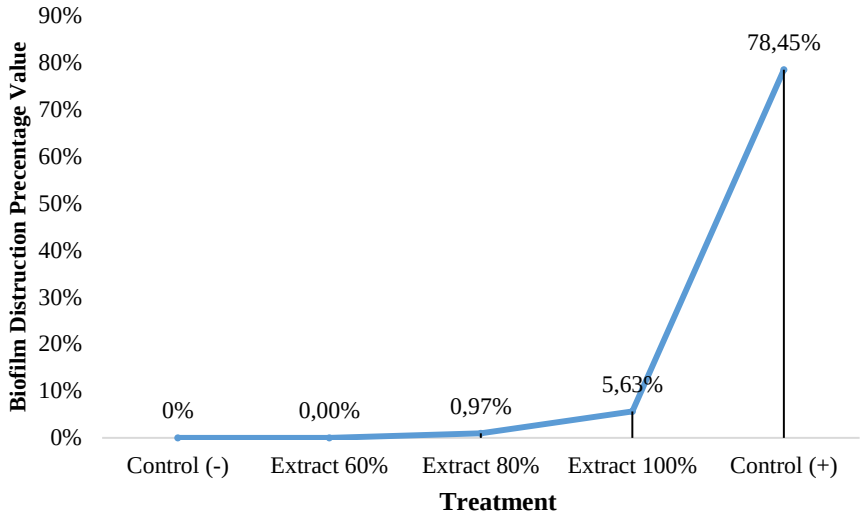

Figure 5. Percentage of Biofilm Destruction Activity

The biofilm destroying activity of an extract is demonstrated by the compound content which is able to penetrate the EPS matrix layer and destroy the biofilm. The content of Mangosteen leaf extract which is known to have this ability is saponin. As stated by Andrade et al. (2019), that saponin can affect the EPS matrix of bacterial biofilms, resulting in a decrease in the amount of EPS and changes in membrane integrity which in turn causes the cell wall to become unstable. According to Slobodníková et al. (2016), flavonoid and tannin compounds can also have a destructive effect by changing the structure of the proteins that make up the biofilm, causing EPS to denature and the biofilm to be degraded.

CONCLUSION

Based on the research results, it can be concluded that Mangosteen leaf extract (*Garcinia mangostana* L.) has antibiofilm activity against a collection of bacterial isolates from diabetic wounds. The optimum concentration of Mangosteen leaf extract in providing anti-biofilm activity is at a concentration of 100%, with a percentage of cell adhesion prevention of 32.56%, inhibition of biofilm formation of 50.51%, and destruction of biofilms of 5.63%. Judging from the optical density measurement values and percentages, the best activity of Mangosteen leaf extract is in

inhibiting biofilm formation, followed by preventing cell attachment and destroying biofilm.

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