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Antibacterial Activity of Guava Leaf Extract on The Growth of *Aeromonas Hydrophila*

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Abstract**:** *Research on the benefits of guava leaves has been widely carried out because of their ability as an antibacterial and antifungal agent, especially in aquaculture. This study aims to determine the antibacterial activity of guava leaf extract against the growth of the pathogenic bacteria Aeromonas hydrophila in vitro. The method used to determine the antibacterial activity of guava leaf extract is the disc diffusion method. The activity test used a completely randomized design (CRD) consisting of 4 treatments with concentrations of guava leaf extract, namely 30%, 50%, 100%, and aqua dest as a control. Guava leaf extract was dissolved using 96% ethanol by the maceration method. Each treatment concentration was tested for its inhibitory power by calculating the apparent zone diameter that appeared against Aeromonas hydrophila bacteria. The data obtained were analyzed using analysis of variance with a 95% confidence level and, if significantly different, continued with the LSD test. The in vitro antibacterial activity test showed that guava leaf extract had a very significant effect on the growth of Aeromonas hydrophila bacteria ($p<0.01$) with a concentration of 50%, resulting in the most effective inhibition Aeromonas hydrophilla bacteria growth with a clear zone formed 18.33 mm. Based on the study results, it can be concluded that in vitro guava leaf extract with a concentration of 50% can inhibit the growth of Aeromonas hydrophila bacteria with the most significant clear zone yield*.
Keywords: Antibacterial Activity; *Aeromonas hydrophila*; Bacteria; Guava; Leaves;

1. INTRODUCTION

Disease attack is an obstacle that is very often experienced by fish farmers. *Aeromonas hydrophila* bacteria, like pathogenic bacteria, often attack freshwater fish that cause disease (MAS) Motile Aeromonas Septicemia (Wahjuningrum et al., 2010). This disease is very malignant because it can cause death and reach more than 60% within seven days after infection (Apriliyanti et al., 2013). Looking at the impact of this disease will be very detrimental to cultivators; it is indispensable to prevent or treat the attack of this MAS disease. Prevention efforts can be made by controlling water quality and providing quality feed while in treatment using chemicals and antibiotics.

Some of the chemicals used are persistent, so they are not readily biodegradable and are not environmentally friendly. The use of antibiotics in overcoming MAS is quite effective but will increase the resistance of bacterial isolates to antibiotics. In addition, repeated use of antibiotics will result in the accumulation of antibiotics in fish body tissues, especially bones, which can harm humans who consume them (Fajri et al., 2016).

The use of chemicals and antibiotics is not recommended, especially for consuming fish. Therefore, treatment and prevention efforts can be

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made using natural ingredients in the surrounding environment. One of the plants that can be used is the leaves of the guava plant. Research on the benefits of guava plants has been widely carried out. The study results revealed that very high chemical compounds were found in all parts of the guava plant. Among the parts of the guava plant, the leaves contain the most chemical compounds among other plant parts (Hargono, 2003). So earlier, people used the leaves a lot as a source of medicinal ingredients to treat diarrhea, as an anti-inflammatory, dysentery, and other digestive disorders (Yuliani et al., 2003).

Based on research by Yuliani et al., (2003) and Qonita et al., (2019), guava leaves are often used as medicine because the leaves contain 7.092 - 12.66% tannin compounds, flavonoids, essential oils, and alkaloids. These chemical compounds have antibacterial activity, namely the ability to inhibit the growth of pathogenic bacteria (Sine & Fallo, 2016). Flavonoid compounds, tannins, alkaloids, and essential oils in guava leaves work as antibacterial because they can denature and coagulate bacterial cell proteins, damage cell walls and cytoplasmic membranes, prevent cell division, shrink cell membranes to change cell permeability function, damage constituent components peptidoglycan in the bacterial cell wall layer and volatile active substances can kill bacteria.

Qonita et al., (2019) reported that guava leaves with a concentration of 10% could inhibit *E.coli* and *V. cholerae* bacteria with an inhibition zone reaching 8.17 mm while Nuryani et al., (2017) reported a concentration of 25% guava leaf extract was able to inhibit the growth of pathogenic bacteria *S. aureus* by forming a clear zone of 23.2 mm, and a concentration of 60 mg/ml guava leaf ethanol extract was able to inhibit the growth of *Bacilus subtilis* bacteria with an average clear zone formed of 23.5 mm (Yulisma, 2018). However, in vitro studies of guava leaves in inhibiting the growth rate of *A. hydrophila* bacteria have not been widely carried out.

Research carried out by Sine & Fallo, (2016) is to conduct an in vitro test of guava leaf extract against the growth of *A. hydrophila* bacteria using the maceration method with solvent and several times shaking at room temperature. To extract all active compounds that have antibacterial potential, they are necessary to use a solvent that has the potential to produce the most significant percent yield. Azis et al., (2014) stated that ethanol is the solvent that attracts the most chemical compounds from Indian bay leaves, resulting in the most significant percent yield. Based on the description above, the purpose of this study was to test guava leaf extract using ethanol as a solvent to determine its use as an antibacterial against the growth of *A. hydrophila* bacteria.

2. RESEARCH METHOD

Time and Place

The research was carried out from February to April 2021 at the Fisheries Laboratory, Dharmawangsa University, Medan.

Experimental Design

This research was conducted using an experimental method, namely by conducting direct trials with various concentrations of guava leaf extract against *Aeromonas hydrophila* bacteria causing MAS disease by looking at its inhibition in vitro using a Completely Randomized Design consisting of four treatment levels and three replication levels (aqua dest solution as control; 30%; 50%; 100%). The concentration of treatment in this study refers to previous studies that used guava leaf extract to inhibit the growth of Streptococcus mutans bacteria (Tampedje et al., 2016).

Determination of Guava Leaf Extract

The extraction process uses the maceration method with 96% ethanol as solvent. Guava leaves are taken only on old leaves. Guava leaves washed under running water are then dried using an oven at 45^0C for 16 hours. The dried leaves were blended until smooth and sieved to a size of 1 mm. A total of 750 g of Simplicia was dissolved in 3.75 L of ethanol solvent, in which the ratio between Simplicia and solvent was 1:5. Then the maceration process was carried out for three days and filtered every day using Whatman filter paper 125 mm and compressed with a vacuum rotary evaporator at a temperature of 40 ^{0}C.

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Preparation of Turbidity Standard for Bacterial Test Solution (Mc. Farland's Solution)

9.5 ml of 1% H_2SO_4 solution was mixed with 0.5 ml of 1.175% $BaCl_2$.2H2O solution in an Erlenmeyer and then shaken until a cloudy solution was formed with absorbance values in the range of 0.08 to 0.13 (Tampedje et al., 2016). According to Tampedje et al., (2016), the standard solution of the bacterial test suspension was at a concentration of 1.5x108 CFU/mL, whose turbidity was equivalent to 0.5 McFarland's standard solution. This turbidity is used as a standard for the turbidity of the bacterial test suspension. The inoculated bacteria test were taken with a sterile ose needle and then suspended into a tube containing 2 ml of 0.9% NaCl solution until the turbidity was the same as the standard turbidity of Mc. Farland.

Testing the Inhibitory Zone of Guava Leaf Extract on the Growth of *Aeromonas Hydrophila* Bacteria

Bacterial inhibition was tested using the agar diffusion method by sticking a paper disc. Concentration variations were carried out by diluting the guava leaf ethanol extract from a higher concentration using a percentage ratio of % (v/v) concentration. The concentrations tested were guava leaf extract with concentrations of 30%, 50%, 100%, and 10 µl of sterile distilled water (negative control). Inoculated 50 µl of *Aeromonas hydrophila* bacteria on TSA media using the spread plate technique according to the standard cell density of the Mc Farland solution for the turbidity of the test bacteria (10^8 CFU/mL). Sterile disc paper was placed aseptically on the agar surface, then 10 µl of guava leaf extract was dripped onto the disc paper and incubated at room temperature for two days at 37 ^{0}C. The clear zone formed indicates that bacterial growth is inhibited by the antibacterial active ingredients in the guava leaf extract and is expressed by the diameter of the inhibition zone. The inhibition zone formed was measured twice, namely the measurement based on the center diagonal line, and then the results were averaged using the formula (D1 + D2): 2 (Paliling et al., 2016).

Data Analysis

The size of the inhibition zone of guava leaf extract was analyzed descriptively by comparing the diameter of the clear zone formed between treatments, while to determine the effect of guava leaf extract on the growth of *A. hydrophila* bacteria, it was analyzed using the analysis of variance (ANOVA) and if it had a significant effect, continued with further tests. LSD (*Least Significant Difference*) to determine the difference in the effect of each treatment in order to obtain the best treatment among all existing treatments.

3. RESULTS AND DISCUSSION

Extract Yield

Extraction of guava leaves using the maceration method. Maceration is an extraction method by which chemical compounds in the cytoplasm are dissolved into the solvent due to cell walls and membranes rupturing due to the pressure difference inside and outside the cell (Chandra et al., 2018). Simplicia guava leaves weighing 750 g that have gone through the drying process are immersed in 96% ethanol polar solvent with a ratio of 1:5 between simplicia and solvent. The thick extract of guava leaves obtained was blackish-brown in color. The results of guava leaf extraction can be seen in table 1.

Table 1. Guava Leaf Extraction Results

Pelarut	Berat Simplisia	Berat Hasil Ekstraksi	Rendeman	Warna
Etanol 96%	750 gram	50 gram	6,7 %	Hitam

The yield of guava leaf extract was 6.7%. Several previous studies showed higher yields compared to this study. Research by Qonita et al., (2019) using 96% ethanol as a solvent resulted in a 12.5% guava leaf extract yield using the maceration method. However, based on the research of Suhendra et al., (2019), the optimum yield of weed rhizome extract in 70% ethanol solvent (14.13%) decreased at 90% ethanol solvent concentration (11.09%). Yield is the secondary metabolite level extracted by the solvent. The higher the yield value, the more secondary metabolites extracted. The yield of the

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extract can be influenced by the solvent used and the difference in solvent concentration. The difference in solvent concentration will affect the polarity of the solvent. The same solvent with different concentrations also has different polarities (Widarta & Arnata, 2017).

The yield value obtained in this study was lower than previous studies that used the same extraction method, namely maceration, but the solvent concentration was different. The chemical compounds in guava leaves decreased at 90% ethanol concentration. This statement agrees with the results obtained by Suhendra et al., (2019) that chemical compounds in weed rhizomes will increase their solubility up to 70% ethanol concentration and decrease after 70% ethanol concentration. The polarity of ethanol will increase along with the decrease in its concentration in water, which means that the polarity of ethanol is 70% higher than pure ethanol (Widarta & Arnata, 2017).

Inhibitory Power of Guava Leaf Extract on *Aeromonas Hydrophila* Bacteria Growth

Testing the antibacterial activity of guava leaf extract using the agar diffusion method (Kirby-Bauer) with the help of disc paper against the test bacteria (*Aeromonas hydrophilla*). Testing the antibacterial activity of guava leaf extract using a negative control, namely distilled water, as a comparison to see the effect of the extract on the diameter of the inhibition zone formed. The antibacterial activity of guava leaf extract against test bacteria is presented in Table 1, while the picture of the antibacterial activity of guava leaf extract is shown in Figure 1.

Table 1. Antibacterial activity of guava leaf extract against *A. hydrophila* . bacteria

Perlakuan	Ulangan (mm)			Min	Max	Rata-rata
	I	II	III			
P0	0	0	0	0	0	0
P1	16	18	19	16	19	17,7
P2	17	21,5	16,5	16,5	21,5	18,3
P3	12	15	16,5	12	16,5	14,5

Ket= P0=kontrol negatif; P1=ekstrak daun jambu biji konsentrasi 30%; P2= ekstrak daun jambu biji konsentrasi 50%; P3=ekstrak daun jambu biji konsentrasi 100%

The inhibition zone formed resulted from the activity of the antibacterial compound of guava leaf extract in inhibiting the growth of pathogenic bacteria (Table 1). This study was conducted during the incubation time of 24 hours, so it cannot be concluded whether guava leaf extract is bactericidal. Sine & Fallo, (2016) stated that bactericidal activity is the ability of antimicrobial substances to kill bacterial growth, characterized by a clear zone that stops around the disc and is no longer overgrown by bacteria after 48 hours of incubation (Figure 1).

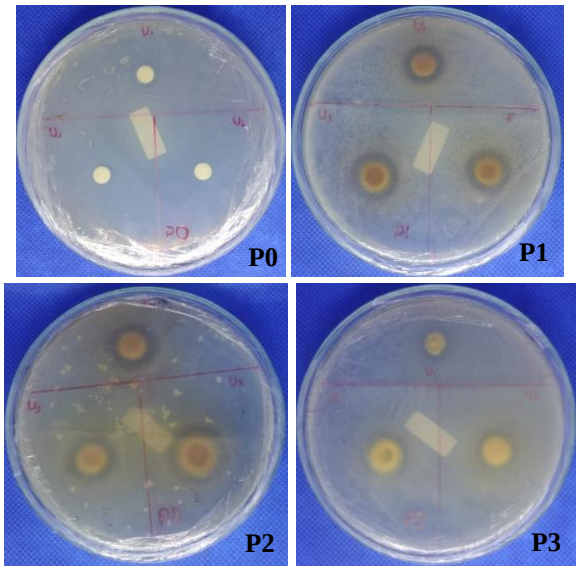

Figure 1. Clear zone of antibacterial activity of guava leaf extract (P0=negative control; P1=guava leaf extract 30% concentration; P2=guava leaf extract 50% concentration; P3=guava leaf extract 100% concentration).

After 24 hours of incubation, there was a clear zone in each extract. The clear zone is an area that is not overgrown with bacteria due to the influence of the antibacterial activity of the extract around the disk, where the size of this zone is influenced by the size of the dose tested. Based on the results of the research that has been carried out, it can be seen that the concentration of 0% in the form of aqua dest as control is that there is no inhibition zone formed, which means that the clear zone formed is purely due to the influence of guava leaf extract not from the solvent. At a concentration of 30%, an inhibition zone of 17.67

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mm was formed, at a concentration of 50%, an inhibition zone of 18.33 mm was formed, and at a concentration of 100%, and inhibition zone of 14.5 mm was formed. The diameter of the inhibition zone at concentrations of 30%, 50%, and 100% can be categorized as strong, and this is based on the opinion of Susanto et al., (2017) in Chandra et al., (2018), the criteria for the strength of antibacterial power are the inhibition zone <5 mm is categorized as weak, the inhibition zone is 6-10 mm is categorized as moderate, the inhibition zone is 11-20 mm is categorized as strong and the inhibition zone >20 mm is categorized as very strong.

The results of the analysis of the diversity of the clear zone data with the ratio of the dose of guava leaf extract to the growth of *A. hydrophila* with a 95% confidence level showed a significantly different data significance with p-value <0.005, which means that guava leaf extract had a very significant effect on the growth of bacteria *A. hydrophila*. The results of further analysis using LSD on the effect of guava leaf extract dose on the growth of *A. hydrophila* showed that there was a significant difference between treatments, namely, at a concentration of 50% (18.33mm) it produced an inhibition zone that was not significantly different with a concentration of 30% (17.67mm). This means that a 50% dose of guava leaf extract showed the same effect as a 30% dose in inhibiting the growth of *A. hydrophila* bacteria. However, at a concentration of 50% (18.33mm), the results were significantly different from a concentration of 100% (14.5mm); this means that a concentration of 50% of guava leaf extract gave the highest inhibition compared to a concentration of 100% and at a concentration of 50% (18,33mm) showed a very significant difference at a concentration of 0%. This means that the inhibition is formed due to the influence of the antibacterial activity of a 50% dose of guava leaf extract.

Antibacterial activity formed from guava leaf extract can occur due to secondary metabolites contained in the leaves. One of the secondary metabolites found in plants is a phenolic compound. Classes of phenolic compounds whose known structures include flavonoids, tannins, terpenes and steroids, saponins, alkaloids, and essential oils (Sine & Fallo, 2016). Based on research Nofita et al., (2021), the highest chemical compound levels in Australian guava leaves are phenolic compounds, flavonoids, and tannins. de Araújo et al., (2014) stated that the phenolic extract from the plant *E. tirucailli* L. was active as an antimicrobial against *S. epidermidis, E. Faecalis* and *P. aeruginosa* which activity was related to its ability to form complex compounds with extracellular proteins on the cell wall and disrupt microbial membranes. Where phenolic and polyphenolic compounds are toxic to microbes.

The flavonoid compounds in guava leaves work as antibacterial because they can denature and coagulate bacterial cell proteins so that bacterial cells die. In addition, flavonoids can also damage cell walls and cytoplasmic membranes, preventing bacterial division so that bacteria cannot reproduce (Evans, 2009). In addition to flavonoids, secondary metabolites with antibacterial properties contained in guava leaves are tannins. Qonita et al., (2019) stated that tannins have antibacterial action by inactivating bacterial adhesins, inhibiting enzyme activity, and inhibiting protein transport in the cell envelope. In addition, tannins have the power of toxicity by damaging bacterial cell membranes and, when bound to iron, will disrupt bacterial growth (Fratiwi, 2015). From the content of these compounds, the guava leaf extract can inhibit the growth of *A. hydrophila* bacteria.

In general, the diameter of the clear zone tends to increase in proportion to the increase in the concentration of the extract. Still, the results obtained in this study are different from previous studies conducted by (Tampedje et al., 2016). In this study, guava leaf extract was obtained with concentrations of 30%, 50%, and 100% can inhibit the growth of Streptococcus mutans bacteria. The greater the attention of the extract, the higher the inhibitory power produced. These results were different because the characteristics of the test bacteria used were different from the bacteria when this research was conducted, where Streptococcus mutans was gram-positive (+) while *A. hydrophila* was gram-negative (-). According to Rastina et al., (2015), this is because the gram-positive (+) cell wall consists of several

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layers of peptidoglycan, which forms a thick and rigid structure and contains a cell wall substance called teichoic acid, while the gram-negative (-) cell wall consists of one or more thin walls of peptidoglycan so that it tends to be weaker to physical shocks such as the administration of antibacterial or antibiotic agents.

At the highest concentration, namely 100% concentration, it did not produce the highest inhibition zone because the extract at 100% concentration was very concentrated, so it was suspected that the substances in the extract were not completely dissolved and would cause saturation, so the extract could not work well in inhibiting bacterial growth. Seme et al., (2020) also stated that the concentration of extracts that were too concentrated would result in the active substances contained in the extracts not being able to diffuse into bacterial cells. This is also supported by Maleki et al., (2008) opinion that the concentration of the extract that was too concentrated made it difficult for the extract to diffuse optimally into the inoculum medium. Dani et al., (2012) also argued that higher extract concentrations could lead to saturation, causing the active compounds contained in the extracts not to dissolve completely.

4. CONCLUSION

Based on the results of an in vitro test of guava leaf extract on the inhibition zone of the bacteria that cause MAS disease (Motile Aeromonas septicemia), it can be concluded that at a concentration of 0%, no inhibition zone of bacteria causes MAS disease (Motile Aeromonas septicemia). At a concentration of 30%, It had formed an inhibition zone with a diameter of 17.67 mm. At a concentration of 50%, It had created an inhibition zone with a diameter of 18.33 mm. At a concentration of 100%, it formed an inhibition zone with a diameter of 14.5 mm. A concentration of 50% is the best concentration in inhibiting the growth of bacteria that cause MAS disease (Motile Aeromonas septicemia).

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