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Potential of Moringa (*Moringa oleifera*) Leaf Extract to Inhibit the Growth of Pathogenic Bacteria *Edwardsiella tarda*

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Abstract

This study analyzed the antibacterial activity of *Moringa oliefera* leaf extract against the growth of *Edwardsiella tarda* bacteria. This study aims to determine the antibacterial activity of Moringa leaf extract (*M. oliefera*) against the growth of *E. tarda* bacteria. Inhibition testing is done by diffusion method (disc test). The disc test used five variations of concentration of 75 mg/L, 150 mg/L, 225 mg/L, 300 mg/L, and 375 mg/L on TSA (Tryptone Soya Agar) media and incubated for 2x24 hours. As a positive control, an antibiotic in the form of chloramphenicol was used. (5 mg/L) Moreover, distilled water was used as a negative control. Moringa leaf extract contains natural active compounds, bacteriostatic antibacterial, due to decreased bacterial growth after 48 hours of incubation. The highest inhibition diameter of *E. tarda* was 12.95 mm at a concentration of 375 mg/L after 24 hours incubation and decreased by 11.02 mm after 48 hours incubation. The highest inhibitory effectiveness was at a concentration of 375 mg/L with an effectiveness of 58.8%, while the effectiveness of the decrease was 48.1% after 48 hours of incubation.

INTRODUCTION

The problem that is often faced by fish farming communities is disease attacks. With a disease attack, cultured fish experience high mortality or mortality in a short time, resulting in a large number of fish farmers experiencing considerable economic losses (Ashari *et al*., 2014).

Fish diseases that often attack aquaculture activities are from bacteria, one of which is *E. tarda*. According to Narwiyani and Kurniasih (2011), the factors influencing *E. tarda* infection are fish stress, primarily due to high stocking density, poor water quality conditions, and high content organic matter. Horizontal disease transmission of *E. tarda* bacteria is contact between one host and another or through the water. The external symptoms of fish attacked by edwardsiellosis in mild infections only show small wounds (Indriasari *et al*., 2020).

So far, to minimize deaths in *E. tarda* attacks by using chemicals or antibiotics classified as safe, and their use is following government regulations (A'yunin *et al*., 2019). However, the continuous use of chemicals can cause

new problems. Namely, it can increase environmental pollution, accumulate antibiotic residues in fish tissue, affect its growth and resistance to drugs (Maqsood *et al*., 2009; Kadlec *et al*., 2011). Therefore, other alternative materials are needed as candidates for antibiotic replacement drugs from natural ingredients, including Moringa leaves (*Moringa oliefera*).

Moringa plants are used for leaves, seeds, flowers, pods (fruit), bark, and roots with several active antibacterial compounds. According to Pandey *et al*. (2012), Moringa leaves have secondary metabolite compounds such as flavonoids, alkaloids, phenols, and tannins, inhibiting bacterial activity. Research on Moringa leaf extract as antibacterial from various sources has been done, so it is necessary to research to determine Moringa leaf extract antibacterial against *E. tarda*, which is the cause of disease in aquaculture.

The purpose of this study was to determine the in vitro antimicrobial activity of Moringa leaf extract against *E. tarda* bacteria.

METHODOLOGY

Place and Time

This research was conducted from December 2020 to January 2021 at the Fish Health Disease Laboratory of Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang.

Research Materials

The tools used in this research were Erlenmeyer, rotary vacuum evaporator, vortex mixer, spatula, tray, funnel, digital scales, analytical scales, film bottles, test tubes, test tube racks, petri dishes, ose needles, suction balls, Bunsen, hot plate, spectrophotometer, drop pipette, sprayer bottle, laminar airflow (LAF), oven, refrigerator, incubator, measuring cup, autoclave, beaker glass, aquarium, aerator, aeration stone, aquarium heater, section set, and syringe.

The materials used in the extraction activity were Moringa leaves (*M. oliefera*) obtained in the Dinoyo area, Malang, East Java. The Solvent for maceration is ethanol with pro-analysis quality (PA), filter paper with the trademark Whatman No. 42, aluminum foil and plastic warp, dimethyl sulfoxide (DMSO), and hydro bath. For bacterial culture and antibacterial inhibition test, the materials used were E. tarda bacteria obtained from the Fish Quarantine and Inspection Agency, Jakarta, culture media, namely Tryptic Soy Agar (TSA) Merck, Tryptone Soya Broth (TSB) Merck, aquades, alcohol, disc paper with a diameter of 6 mm.

Research Design

The method used in this research was experimental. The research design in this study was a completely randomized design (CRD). The treatments used in this study were five treatments with different doses, positive control, negative control, and three repetitions.

Work Procedure

Preparation of Moringa Leaf Extract (*M. oliefera*)

Moringa leaves were collected and cleaned. Then, Moringa leaves were dried by being aerated in the room until dry, then milled and sieved to obtain a fine powder (Susanty *et al*., 2019). A total of 100 g Moringa leaf powder was macerated using Simplicia soaked in 1000 ml of ethanol solvent (ratio 1:10) for 2x24 hours at room temperature ± 25-28 °C. The extract was filtered using filter paper then evaporated with a rotary vacuum evaporator at a temperature of 35-40 °C, and the yield was calculated (Hayati *et al*., 2012; Wardhani *et al*., 2018).

Phytochemical Screening

Phytochemical analysis was carried out according to the Harborne method (Putra *et al*., 2016). The aim is to observe the active compounds in the crude extract. The compounds analyzed included: flavonoids, alkaloids, phenolics, steroids,

tannins, and saponins with a density of 10^4 individual/mL.

Preparation of Bacteria

E. tarda isolate from the Fish Quarantine and Inspection Agency (BUSKIPM) Jakarta. These bacteria were stored in Trypticase Soy Agar (TSA) medium at ± 4 °C and sub-culture Trypticase Soy Broth (TSB) overnight before use.

Antibacterial Activity Test

The test for the antibacterial activity of Moringa leaf extract against pathogenic bacteria *E. tarda* using the diffusion method was carried out with the disc test. According to Fitriana *et al*. (2019), it was used to determine the test microbes' sensitivity to antimicrobial agents. The dose of Moringa leaf extract tested consisted of 5 concentrations (75 mg/L, 150 mg/L, 225 mg/L, 300 mg/L and 375 mg/L) and 2 controls (positive and negative). Chloramphenicol at a concentration of 5 mg / L was used as a positive control. Measurement of the clear zone from each dose of *M. oliefera* leaf extract using a digital caliper in millimeters units (mm).

Scanning Electron Microscope (SEM)

SEM observations are intended to determine the damage to the morphology of bacterial cells due to the administration of extracts containing active ingredients (Hariati *et al*., 2018). Preparation of *E. tarda* preparations was carried out with 2 treatments. The first treatment of *E. tarda* was normal, the second treatment of *E. tarda* was given Moringa leaf extract with a dose of 400 ppm. Then the two preparations were ready to be observed using SEM (Scanning Electron Microscope).

Analysis of damage *to E. tarda* bacteria was carried out by comparing photos from SEM observations between normal conditions and bacteria given Moringa leaf extract and seeing the picture of the damage to the bacterial cell wall. Based on the results of the damage analysis, it will be continued in the next stage.

Data Analysis

Phytochemical data analysis was carried out by observing the extract solution's color change in the phytochemical test. Data measurements were performed during the 24 and 48 hour incubation period for the disc test. The data obtained from the measurement results are tabulated and analyzed by measuring the disc test's resistance zone's diameter. The calculation of the extract inhibition effectiveness is calculated based on the equation (Hamzah, 2019).

$$E = \frac{D}{Da} \times 100\%$$

Where:

E = effectiveness of inhibition (%)
D = diameter of plant material extract inhibition zone (mm)
Da = diameter of antibiotic inhibition zone (mm)

RESULTS AND DISCUSSION

The Yield of Moringa Leaf Extract

The yield obtained from the maceration of Moringa leaf extract with 96% ethanol solvent is presented in table 1.

Table 1. Yield Moringa leaf extract.

Solvent Type	Total Solvent (ml)	Sample Weight (g)	Extract Weight (g)	Yield (%)	FHI requirements
Ethanol 96%	1000	100	9,51	9,51%	Not less 9.2%

The yield of 96% ethanol extract from *Moringa oleifera* leaves above is obtained from the final weight after extraction is complete compared to the number of simplicial used at the time of extraction (Alegantina *et al*., 2013). The

results from Table 1 indicate that the yield value obtained was 9.51%. These results meet the Indonesian Herbal Pharmacopoeia requirements; namely, the yield is not less than 9.2%. Determination of yield aims to determine the approximate amount of Simplicia needed to make a certain amount of thick extract (Kartikasari *et al*., 2014).

Phytochemical Screening

M. oleifera L. leaf extract's phytochemical test results showed alkaloids, flavonoids, triterpenoids, and tannins, as in Table 2.

Table 2. Phytochemical test results on *M. oliefera* L. leaves.

Compound Identification	Characteristics	Result
Flavonoid	Orange, Brick Red, Pink, Dark Red	(+) Positive
Alkaloid		
Meyer	White sediment	(+) Positive
Dragendorff	Orange sediment	(+) Positive
Bouchardat	Brown sediment	(-) Negative
Tannin	Blackish Brown, Blackish Blue	(+) Positive
Terpenoid		
Steroid	Bluish Green	(-) Negative
Triterpenoid	Orange, Brownish Orange	(+) Positive
Saponin	Permanent Foam	(-) Negative

Note: (+) = there is a chemical content, (-) = there is no chemical content.

Meanwhile, alkaloid and terpenoid compounds were not found (Table 1). Alkaloids, flavonoids, triterpenoids, and tannins are active compounds with various functions, including antibacterial activity.

According to Widowati *et al*. (2014), the mechanism for reducing microorganisms using Moringa leaf extract is caused by antibacterial phytochemicals, namely flavonoids and tannins saponins, and polyphenols with bacterial inhibition mechanisms. The mechanism of action is with antibacterial compounds, including inhibiting cell wall synthesis, inhibiting the integrity of bacterial cell wall permeability, inhibiting enzyme action, and inhibiting the synthesis of nucleic acids and proteins. Naturally, these active compounds are present in plants as a mechanism for protecting themselves from disease and damage from the external environment (Kenconojati and Rukman, 2019).

Antibacterial Activity Test

The antibacterial activity test results showed that Moringa leaf extract could inhibit bacterial growth. Inhibition of bacterial growth based on extract dosage. The clear zone indicates Moringa's leaf extract's ability to inhibit *E. tarda* bacteria's growth. The clear zone measurements around the disc paper were observed at 24 and 48 hours after incubation. The measurements of the clear zone around the disc paper in 24 hours are presented in Table 3.

Table 3. The results of the 24-hour Moringa (*M. oleifera*) leaf extract disc test.

Concentration (mg/L)	Average Inhibition Zone Diameter (mm)	Inhibition Zone Response Qualifications
K-	0 ± 0.00^{a}	Weak
75	$4{,}41 \pm 0.27^{b}$	Moderate
150	$5{,}28 \pm 0.58^{c}$	Moderate
225	$6{,}36 \pm 0.45^{d}$	Moderate
300	$6{,}67 \pm 0.21^{d}$	Moderate
375	$7{,}30 \pm 0.21^{e}$	Strong
K+	$15{,}99 \pm 0.10^{f}$	Very Strong

Note: Classification of inhibition zone diameter, weak = 3 mm, moderate 3-6 mm and strong > 6 mm (Pan *et al*., 2009).

The results of measuring the inhibition zone diameter at all doses with time intervals of 24 indicated that the number of extract doses given could increase the diameter inhibition zone. At extract concentrations of 75 mg/L, 150 mg/L, 225 m/L, and 300 m/L included in the moderate category with the mean diameter of the inhibition zone 4,41, 5,28, 6,36, and 6,67. The extract with a strong inhibition zone was 375 mg/L, with an average diameter of 7,30 mm. This is following the opinion of Pan *et al*. (2009) in determining the inhibition zone category is the result of the diameter of the inhibition zone minus the diameter of the 6 mm paper disc and the classification of bacterial inhibition zones, namely those with a diameter of 0-3 mm is in the weak category, 3-6 mm is in the medium category and >6 mm is classified as strong inhibition zones.

The results showed that the higher the concentration used, the greater the resistance zone formed. Flavonoid compounds are phenolic compounds that can cause protein denaturation and damage cell walls. The inhibition of the growth of bacterial colonies is thought to be caused by damage to the structural components of the bacterial cell membrane. Cell damage disrupts nutrient transport (compounds or ions) through cells so that bacterial cells lack the nutrients needed for growth (Sari and Mursiti, 2016). Tannins also have a target on cell wall polypeptides so that the formation of cell walls is less than perfect. This causes the bacterial cell to lyse due to osmotic and physical pressure so that the bacterial cell will die. Terpenoid compounds have a mechanism of action as antibacterial substances allegedly involving membrane damage by lipophilic compounds. Terpenoids can react with porins (transmembrane proteins) on the outer membrane of the bacterial cell wall, forming strong polymeric bonds and damaging the porin, reducing the permeability of the bacterial cell wall so that the bacterial cell lacks nutrients, inhibits bacterial growth, or dies (Haryati *et al.,* 2015)

Factors that affect the inhibition zone's size in bacteria are growth sensitivity, the reaction between the active ingredient and the medium and incubation temperature, environmental pH, media components, colony density, incubation time, and microorganisms' metabolic activity. Another factor affecting the clear zone's size is the amount of active substance in the solution (Surjowardojo *et al*., 2016).

Table 4. Results of *M. oliefera* leaf extract disc test in 48 hours.

Concentration (mg/L)	Average Inhibition Zone Diameter (mm)	Inhibition Zone Response Qualifications
K-	$0\pm0,00^{a}$	Weak
75	$4,48\pm0,57^{b}$	Moderate
150	$5,25\pm0,47^{bc}$	Moderate
225	$6,42\pm1,37^{cd}$	Moderate
300	$6,56\pm0,93^{cd}$	Moderate
375	$7,07\pm0,62^{d}$	Strong
K+	$16,23\pm0,53^{e}$	Very Strong

Note: Classification of inhibition zone diameter, weak = 3 mm, moderate 3-6 mm and strong > 6 mm (Pan *et al*., 2009).

The inhibition zone diameter measurement at all doses with a time interval of 48 hours has decreased is presented in Table 4. Only positive control (+) increased to 22.87 mm. The decrease in inhibition zone diameter is due to antibacterial substances that only inhibit bacterial growth but do not wholly kill

bacterial colonies (Kawengian *et al*., 2017).

According to Marfuah *et al*. (2018), Moringa leaf extract is bacteriostatic. The mechanism of action of antibacterial compounds is divided into two, namely bacteriostatic and bactericidal. If the antibacterial compound inhibits bacterial growth, it is included in the bacteriostatic group, whereas if it kills bacteria, it is included in the bactericidal group. The decrease in the inhibition zone size during the 48 hours incubation period can also be caused by several factors such as the nature of the bacteria itself and the compound's ability to suppress bacterial growth or the state of the active ingredient of the antibacterial compound used.

Table 5. The effectiveness of inhibition in 24 and 48 hours.

Concentration	Effectiveness of inhibition in 24 hours	Effectiveness of inhibition in 48 hours
75	32,5%	28,2%
150	38,4%	33,1%
225	44,6%	36,5%
300	45,6%	42,2%
375	58,8%	48,1%
Average	43,98%	35,00%
Standard Deviation	0,098	0,058

Based on table 5, the percentage of effectiveness of Moringa leaf extract, all doses decreased after 24 hours. The extract with a 375 mg/L concentration was the most effective dose with inhibitory effectiveness of 58.8% in 24 hours. In contrast, the decrease was only 48.1% after 48 hours. Simultaneously, the extract with a 75 mg/L concentration was the least effective dose with the effectiveness of inhibition of only 32.5% at 24 hours and 28.2% at 48 hours.

The effectiveness of the inhibition of Moringa leaf extract against *E. tarda* bacteria is caused by the content of bioactive compounds in Moringa leaf extract, which can damage the protein synthesis system, damage to the cell wall, which causes lysis resulting in cell wall damage that can interfere with the mechanism of bacterial cell wall synthesis (Hamzah, 2019). This result is supported by the statement of Roslizawaty *et al*. (2013) that the substance's concentration influences an antibacterial substance's effectiveness. Increasing the concentration of substances causes an increase in the content of active compounds that function as antibacterial so that their ability to kill bacteria is also more remarkable. Nemeth *et al*. (2015) stated bacteriostatic antibiotics are assumed to require phagocytic cells to clear bacteria and are therefore considered less effective without an efficient immune response. This theoretical model has resulted in the recommendation that severely ill and immunosuppressed patients with bacterial infections should be treated with bactericidal antibiotics.

Scanning Electron Microscope (SEM)

The SEM results show the effect of treatment on changes in the bacterial cell structure which is presented in Figure 1.

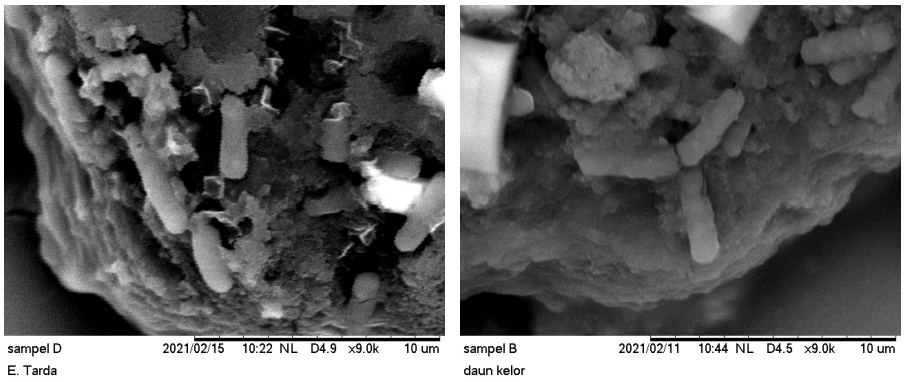

Figure 1. Summary of estimated growth parameters for *C. gariepinus*.

Figure A shows the morphology of *E. tarda* bacteria without any cell wall damage, while Figure B shows the morphology of *E. tarda* bacteria that undergoes lysis due to cell wall instability that disrupts bacterial metabolism. This is due to the effect of giving Moringa leaf extract which has antibacterial active compounds, such as flavonoids, alkaloids, tannins, and terpenoids.

Inhibition of bacterial growth from alkaloid antimicrobial substances by inhibiting enzymes that play a role in the DNA replication process. Inhibition of DNA replication will cause bacteria to not be able to divide so that it inhibits bacterial growth. Meanwhile, the alkaloids contained in the extract can interfere with the formation of cross-bridges of peptidoglycan constituent components in bacterial cells, so that the cell wall layer is not fully formed and causes certain cell death (Ernawati and Sari, 2015). Flavonoids work as antibacterial with some mechanism of action, including inhibiting nucleic acid synthesis, inhibiting cytoplasmic membrane function, and inhibit the energy metabolism of bacteria (Manik *et al*., 2014). According to Ngajow *et al*. (2013), Tannins have antibacterial activity related to their ability to inactivate microbial cell adhesion and inactivate enzymes and interfere with protein transport in the inner layer of cells.

CONCLUSION

After conducting the research, several conclusions were obtained: the bioactive extract of Moringa leaves (*M. oliefera*) is bacteriostatic as indicated by a reduction in the clear zone diameter after 48 hours of incubation. Besides, it was found that the effectiveness of Moringa (*M. oliefera*) leaf extract at a concentration of 375 mg/L against *E. tarda* bacteria and seen from the analysis of scanning electron microscopy, there was damage to *E. tarda* bacteria exposed to Moringa leaf extract.

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