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Effect of Bitter Leaf Extract (*Andrographis paniculata*) against *Edwardsiella tarda* Bacteria in vitro

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Abstract

This study aimed to determine the antibacterial activity of bitter leaf extract (*A. paniculata*) against the growth of *E. tarda* bacteria. This test was carried out using the Kirby Bauer (disc test) method using five different concentrations, namely 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, and 250 mg/L in TSA (Tryptone Soya Agar) and incubated for 2x24 hours using a temperature of 30 ºC. The positive control used was chloramphenicol (5 mg/L), while the negative control used PBS. Bitter leaf extract contains active compounds that have bacteriostatic antibacterial properties. After incubation for 24 hours, the concentration of 250 mg/L showed the highest inhibition zone with a diameter of 7.82 mm and decreased at 48 hours to 7.46 mm. After being incubated for 24 hours, the concentration of 250 mg/L obtained an effectiveness value of 57.08% which is the best effectiveness, and there was a decrease in the 48th hour to 54.45%.

INTRODUCTION

In fish farming activities, the problem commonly faced by farmers is Edwardsiellosis disease caused by the bacterium *E. tarda* (A'yunin *et al*., 2019). This is generally influenced by inadequate fish farming environmental conditions with high temperatures and high concentrations of organic matter (Davies *et al*., 2018). The spread of *E. tarda* bacteria occurs horizontally, namely through contact with one another or through water (Kerie *et al*., 2019). To overcome this problem, efforts are needed to prevent, treat and control *E. tarda*.

According to Pratiwi (2017), efforts to control this disease generally use antibiotics and chemicals. However, the use of antibiotics in the long term will have a negative impact on both the cultivated biota and the surrounding environment and require quite expensive costs (Polianciuc *et al*., 2020). This is reinforced by the statement of Indriani *et al*. (2014), which states that the continuous use of antibiotics will cause resistance to bacteria. Based on these problems, it is necessary to find alternatives to prevent disease problems without using antibiotics and other chemicals. One alternative that can be used is

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the use of natural ingredients as a treatment effort (Valladão *et al*., 2015).

The use of natural ingredients is recommended because it is environmentally friendly, non-carcinogenic, and inexpensive (Azhar *et al*., 2020). One of the natural ingredients that can be used is bitter leaf (*A. paniculata*) which has a variety of active ingredients that have antibacterial and other pathogenic activity. Bitter leaf contains bioactive compounds that are good for health (Ratnani *et al*., 2012). Bitter leaf (*A. paniculata*) has various kinds of active compounds in the form of saponins, flavonoids, alkaloids and tannins that can inhibit the activity of bacteria and other pathogens (Fardiyah *et al*., 2020). In several studies, bitter leaf extract has been proven as an antibacterial that can inhibit the growth of *Staphylococcus aureus* and *E. coli* (Retnowati *et al*., 2011; Sawitti *et al*., 2013). Based on this description, it is necessary to conduct further research and study the content of active compounds in bitter leaf (*A. paniculata*) as antibacterial and determine the optimum dose that can inhibit or kill the growth of *E. tarda* bacteria.

METHODOLOGY

Place and Time

This research was conducted in January - February 2022 at the Fish Health Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya Malang.

Research Materials

The tools used in this study were Erlenmeyer, rotary vacuum evaporator, vortex mixer, spatula, tray, funnel, digital scale, analytical balance, film bottle, test tube, test tube rack, petri dish, ose needle, suction ball, bunsen, heat plate, spectrophotometer, pipette, sprayer bottle, laminar airflow (LAF), oven, refrigerator, incubator, measuring cup, autoclave, beaker glass, section set, and syringe. The material used is bitter leaf (*A. paniculata*) obtained by Medica Care, Batu City, East Java. The solvent for maceration is ethanol with pro-analytical quality (PA), Whatman No. trademark filter paper. 42, aluminum foil and plastic wrap, DMSO and hydrobath. The bacteria used for this research activity were obtained from the Faculty of Medicine, Universitas Brawijaya. For culture and bacterial rejuvenation media in the form of Tryptic Soy Agar (TSA), Tryptone Soya Broth (TSB), Aquades, Alcohol, paper discs with a diameter of 6 mm.

Research Design

The method used in this study is an experiment to fully explain the content of bitter leaf and its benefits for alternative medicine. The research design used was a completely randomized design (CRD) with different doses. The tested dose consisted of 5 concentrations referring to the study of Riyadi *et al*. (2021), using multiples of 50 mg/L and two positive and negative control treatments with three replications.

Work Procedure

Preparation of Bitter Leaf Extract

Extract preparation refers to the method used by Budianto *et al*. (2015), with slight modifications. The bitter leaves were collected and cleaned of dirt that could not be seen with the naked eye and washed with running water until clean, and drained. Bitter leaf was dried then crushed and filtered to obtain a fine powder. The powder used was 100 grams macerated by simplicia soaked in 1 L of ethanol solvent (1:10 ratio) for 2x24 hours at room temperature. After the maceration process is complete, the extract is filtered using filter paper and the evaporation process is continued with a rotary evaporator vacuum at 40 ºC to form a paste.

Phytochemical Screening

The compound identification test was carried out to determine the active compound in the extract of bitter leaf (*A. paniculata*) and to determine the dominant compound in the extract of bitter leaf

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(*A. paniculata*). Phytochemical screening was carried out qualitatively by adding Methanol, HCl, Dragendroff, Mayer, $FeCl_3$, H_2SO_4 and observing the color change formed (Parbuntari *et al*., 2018). Target identification of compounds in bitter leaf extract (*A. paniculata*) are flavonoids, alkaloids, tannin, saponin and terpenoids.

Preparation of Bacteria

Pure isolates of *E. tarda* obtained from the Faculty of Medicine, Universitas Brawijaya Malang were stored in Trypticase Soy Agar (TSA) at a temperature of ± 4 °C and Trypticase Soy Broth (TSB) subcultures were stored overnight before use.

Antibacterial Activities Test

Testing the antibacterial activity of bitter leaf extract against *E. tarda* bacteria was carried out by Kirby Bauer method (disc test). The disc test was carried out to determine the inhibition of the administration of bitter leaf extract (*A. paniculata*) as an antibacterial of *E. tarda* by looking at the clear zone around the disc paper. Measurement of the clear zone for each dose of bitter leaf extract used a digital caliper in millimeters (mm). Growth inhibition response classification bacteria seen by zone diameter Inhibitory consists of 4 groups, namely response weak (diameter 5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (20 mm) (Azaldin *et al*., 2020).

Scanning Electron Microscope (SEM)

The SEM test was carried out to observe the morphological structure of bacteria due to the administration of the extract with 50.000x magnification (Sarwono, 2010). SEM using FEI Brand, Type: Inspect-S50. The first step that needs to be done is making preparations for *E. tarda* bacteria to be observed. The preparation of this preparation was carried out twice with different samples. The first sample was a pure isolate of *E. tarda* without extract, while the second sample was *E. tarda* which had been given an extract of bitter leaf (*A. paniculata*) at a dose of 150 ppm. Both sample preparations were observed using SEM (Scanning Electron Microscope). Analysis of the morphological damage of *E. tarda* bacteria was carried out by comparing photos of SEM observations between samples and seeing the picture of the damage to the bacterial cell wall.

Data Analysis

To determine the effectiveness of the inhibition on *E. tarda* bacteria using bitter leaf extract (*A. paniculata*) was carried out using the research method 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 results from the maceration process of bitter leaf extract using 70% ethanol solvent followed by evaporation can be seen in Table 1.

Table 1. Bitter leaf extract yield.

Type of Solvent	Total Solvent (ml)	Number of Samples (gr)	Number of Weight (gr)	Yield (%)
Ethanol 70 %	1000	100	11.15	11.15

Based on Table 1, the yield value obtained from the maceration and evaporation processes was 11,15%. This yield is not less than 9,2% according to Indonesian herbs Pharmacopoeia Requirements. This calculation is done by comparing the final weight of the extraction process with

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the amount of simplicia used at the beginning of the maceration process (Alegantina *et al*., 2013). The use of 70% ethanol solvent was chosen because of the nature of ethanol which can attract polar compounds, whereas the compounds contained in bitter leaf (*A. paniculata*) are polar. According to Riwanti *et al.* (2020), 70% ethanol is a solvent that is more polar than 96% ethanol and more non-polar than 50% ethanol so polar compounds will dissolve more in 70% ethanol.

Phytochemical Screening

The results of the phytochemical test of bitter leaf extract (*A. paniculata*) showed the presence of flavonoids, tannins, saponin, triterpenoids, and alkaloids as shown in Table 2.

Table 2. Results of phytochemical tests on bitter leaves.

Compound Identification	Characteristics	Result
Flavonoid	Orange, Brick Red, Pink, Dark Red	(+) Positive
Tannin	Dark Chocolate, Dark Blue	(+) Positive
Saponin	Foam Appearance	(+) Positive
Terpenoid		
Steroid	Bluish Green	(-) Negative
Triterpenoid	Orange	(+) Positive
Alkaloid		
Mayer	White precipitate	(+) Positive
Dragendrof	Orange precipitate	(+) Positive

Note: (+) there are active compounds, (-) there are no active compounds.

The results of the phytochemical tests carried out, the results showed that bitter leaf extract using 70% ethanol as a solvent contained flavonoid compounds, alkaloids, triterpenoid tannins, and saponins. The compounds obtained have antibacterial properties that have different functions. Flavonoids and saponins function to damage bacterial cell walls, causing lysis (Dima *et al*., 2016; Ernawati and Sari, 2015). Alkaloid compounds play a role in disrupting the components of the peptidoglycan permeability group in bacterial cells so that the cell wall layer is not fully formed and causes cell death (Wintola and Afolayan, 2015).

Tannins damage bacterial cell membranes and bind to one of the adhesin proteins in bacteria that are used as receptors. resulting in a decrease in bacterial adhesion and inhibition of protein synthesis for cell wall formation (Widowati *et al*., 2014). Terpenoid compounds function to inhibit the growth of membranes or cell walls so that it is possible not to form membranes or cell walls or to form imperfectly (Rialita *et al*., 2019). According to Rais (2015), the active compounds contained in bitter leaves have the potential as antioxidants associated with several activities such as anti-inflammatory and antibacterial.

Antibacterial Activity Test

The results of the disc test using bitter leaf extract against *E. tarda* bacteria showed inhibition of bacterial growth. The inhibition zones obtained varied depending on the dose of the extract used. The presence of a clear zone on the test media indicated the ability of bitter leaf extract to inhibit the growth of *E. tarda* bacteria. The measurement of the clear zone on the paper side of the disc was observed 24 and 48 hours after incubation. The measurement of the clear zone on the paper side of the disc observed for 24 hours is presented in Table 3.

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Table 3. The results of the disc test of bitter leaf extract (*A. paniculata*) 24 hours.

Concentration (mg/L)	Average Inhibition Zone Diameter (mm)	Inhibition Zone Response Qualifications
K-	0±0	Weak
50	4,32±0,092	Weak
100	5,33±0,061	Moderate
150	6,35±0,070	Moderate
200	6,83±0,061	Moderate
250	7,82±0,117	Moderate
K+	13,70±0,345	Strong

Note: Diameter 5 is weak, moderate 5-10 mm, strong 10-20 and very strong >20 mm (Azaldin *et al*., 2020).

The results of measuring the diameter of the inhibition zone for 24 hours showed that the number of doses of the extract given could increase the diameter of the inhibition zone. The extract concentration of 50 mg/L was included in the weak category with an average inhibition zone diameter of 4,32. Inhibition zones in the moderate category were obtained at doses of 100, 150, 200, and 250 mg/L, with an average diameter of 5,33 – 7,82 mm. The content contained in bitter leaf extract can cause damage to cell wall permeability, microsomes, and lysosomes and saponins can damage cell membranes and will lyse (Dima *et al*., 2016; Ernawati and Sari, 2015). According to Horváth *et al* (2016), several factors that can affect the size of the inhibition zone on bacterial cultured agar media including the size of the test plate, the number of compounds placed on the test plate, the type and concentration of agar, thickness and pH of the media, incubation temperature, colony density, incubation time. Another influencing factor is the type of bacteria used (Zeniusa *et al*., 2019). Inhibition zone measurements at 48 hours are presented in Table 4.

Table 4. The results of the disc test of bitter leaf extract (*A. paniculata*) 48 hours.

Concentrate (mg/L)	Average Barrier Zone Diameter (mm) +	Inhibition Zone Response Qualifications
K-	0 ± 0	Weak
50	4.03±0.085	Weak
100	5.04±0.068	Moderate
150	6.05±0.060	Moderate
200	6.6±0.050	Moderate
250	7.46±0.096	Moderate
K+	13.17±0.345	Strong

Note: Diameter 5 is weak, moderate 5-10 mm, strong 10-20 and very strong >20 mm (Azaldin *et al*., 2020).

There are two types, namely the nature of antibacterial, namely, bacteriostatic and bactericidal (Nemeth *et al*., 2015). The inhibition zone diameter in all treatments decreased at 48 hours after incubation. This is because the extract of bitter leaf (*A. paniculata*) is bacteriostatic. The decrease that occurred at 48 hours could also be caused by several other factors such as the nature of the bacteria itself, as well as the ability of the compound to suppress bacterial growth or the state of the active ingredient of the antibacterial compound used.

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Table 5. The value of the effectiveness of inhibition at 24 and 48 hours.

Concentrate (mg/L)	Inhibition effectiveness within 24 hours	Inhibition effectiveness within 48 hours
50	31.53%	29.41%
100	38.90%	36.78%
150	46.35%	44.16%
200	49.85%	48.17%
250	57.08%	54.45%
Average	44.74%	42.59%
Standard Deviation	00.98	0.097

A decrease in the effectiveness of bitter leaf extract in all treatments at 24 and 48 hours is presented in Table 5. The extract with a concentration of 250 mg/L which was the best dose obtained an inhibitory effectiveness value of 57.08% at 24 hours and decreased at 24 hours. to 48 in the form of 54.45%. According to Mardiana and Handayani (2017) the active compound contained in the extract of bitter leaf (*A. paniculata*) can inhibit nucleic acid synthesis, precipitate bacterial proteins and interfere with the peptidoglycan constituent components of bacterial cells so that the cell wall layer is not fully formed and causes cell death.

Scanning Electron Microscope (SEM)

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

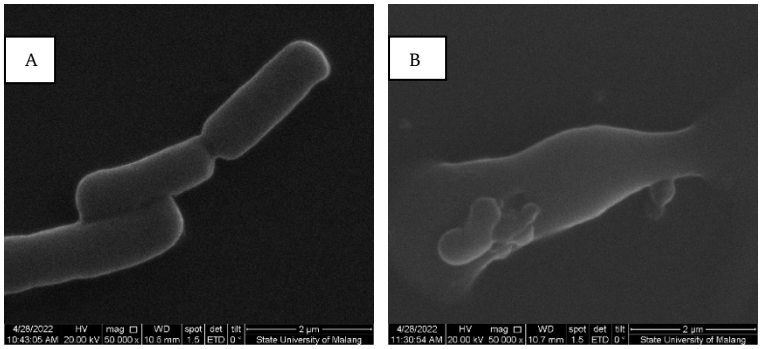

Figure 1. Description of the damage parameters of *E. tarda*.

Figure 1 A shows the morphology of *E. tarda* bacteria without any damage to the cell wall, while Figure 1 B shows a morphological image of *E. tarda* bacteria which is damaged in morphology so that it disrupts the metabolism of bacteria. This is due to the effect of giving bitter leaf extract (*A. paniculata*) which has antibacterial active compounds, such as flavonoids, alkaloids, tannins, and terpenoids. Inhibition of bacterial growth from flavonoid compounds and saponins can cause damage to bacterial cell walls and damage microsomes and lysosomes to cause lysis (Dima *et al*., 2016; Ernawati and Sari, 2015).

Alkaloid compounds will interfere with the components of the peptidoglycan permeability group in bacterial cells, so that the cell wall layer is not fully formed and causes cell death (Ajizah, 2004). Tannins will damage bacterial cell membranes and bind to one of the adhesin proteins in bacteria that are used as receptors, resulting in a decrease in bacterial adhesion and inhibition of protein synthesis for cell wall formation (Widowati *et al.*, 2014). Terpenoid compounds can inhibit the growth of membranes or cell walls so that they may not form a membrane or cell wall or form imperfectly (Rialita *et al*., 2019). The mechanism of terpenoids as antimicrobials

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is to react with porins (transmembrane proteins) on the outer membrane of the bacterial cell wall, forming strong polymeric bonds that cause porin damage (Wulansari *et al.*, 2020).

CONCLUSION

The active compound contained in the extract of bitter leaf (*A. paniculata*) is an antibacterial which has bacteriostatic properties as indicated by the decrease in the diameter of the clear zone after 48 hours of incubation. Treatment with a dose of 250 mg/L was the best dose to inhibit the growth of *E. tarda* bacteria. This was indicated by the higher inhibition zone value than other treatments and from the SEM test carried out there was morphological damage to the bacteria given the extract.

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