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The Effect of Giving Jackfruit (*Artocarpus heterophyllus*) Leaves Crude Extract as An Alternative Antibacterial of *Edwardsiella tarda* Bacteria *In Vitro*

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Abstract

Barriers to cultivation activities are disease attacks, one of which is caused by infection with the bacterium *Edwardsiella tarda*. Treatment efforts can be made using natural ingredients such as jackfruit (*A. heterophyllus*) leaves. This study aims to determine the content of antibacterial compounds in the jackfruit (*A. heterophyllus*) leaves crude extract and their effect on *Edwardsiella tarda* bacteria in vitro. The inhibition test used a disc test with five different concentrations of jackfruit (*A. heterophyllus*) leaves crude extract with three replications: 75 mg/L, 150 mg/L, 225 mg/L, 300 mg/L and 375 mg/L with a comparison using two types of controls (positive with 5 mg/L chloramphenicol antibiotics and negative with no treatment) using Tryptone Soy Agar (TSA) media and incubated for 2x24 hours. The results of phytochemical screening proved that the jackfruit (*A. heterophyllus*) leaves crude extract contains flavonoids, alkaloids, tannins and saponins. Treatment E with a dose of 375 mg/L gave the highest inhibition of 7.80 mm and treatment A with a dose of 75 mg/L gave the lowest inhibition of 4.38 mm. The relationship between the treatment given to the test parameter in the form of the resulting inhibitory power obtained the equation $y = 0.0111x + 3.6297$ with an R^2 value of 0.974 which means 97% of the treatment given in the form of crude extract of jackfruit (*A. heterophyllus*) leaves affects inhibition of bacteria *E. tarda.*

INTRODUCTION

Edwardsiellosis is caused by *Edwardsiella tarda* infection. *E. tarda* is a gram-negative bacterium, (0.3-1.2) x (1.0-6.03) μm in size, facultatively anaerobic, having flagella and not forming spores. This bacteria also attack systemic in various age groups and various fish populations (Firma *et al*., 2012). Treatment efforts that can be done are the use of antibiotics, however, the massive use of antibiotics in fish farming will naturally increase antibiotic resistance. Not only resistant bacteria but also can transfer resistance genes that carry plasmids and move to other bacteria that have never been exposed to antibiotics (Wimalasena *et al*., 2018).

Alternative efforts that can be done are the use of natural ingredients in the form of crude extract of jackfruit (*A. heterophyllus*) leaves. Antibacterial compounds contained in the Jackfruit (*A. heterophyllus*) leaves crude extract include flavonoids, alkaloids, tannins and saponins (Amadi *et al*., 2018). The content of antibacterial compounds in the extract works synergistically in causing the lysis of bacteria (Maryani and Rosdiana, 2020). Jackfruit (*A. heterophyllus*) leaves crude extract has been shown to inhibit several bacteria such as *Staphylococcus aureus* (Majid *et al*., 2019), *Escherichia coli* (Gurning *et al*., 2019) and *Salmonella enterica* (de Sousa *et al*., 2021).

The use of jackfruit (*A. heterophyllus*) leaves crude extract to inhibit *E. tarda* bacteria in vitro or in vivo has never been done. This study aims to determine the content of antibacterial compounds in Jackfruit (*A. heterophyllus*) leaves crude extract and their effect on *E. tarda* bacteria in vitro.

METHODOLOGY

Place and Time

This research was conducted at the Aquaculture Laboratory, Fish Disease and Health Division, Faculty of Fisheries and Marine Sciences, Brawijaya University, from January to February 2022.

Research Materials

Research materials used for the preparation of the extract were jackfruit (*A. heterophyllus*) leaves obtained from UPT Materia Medica Batu, Malang, Pro-Analyst ethanol (PA) solvent, Dimethyl sulfoxide (DMSO), Whatman filter paper No. 42, Erlenmeyer, rotary evaporator, aluminum foil, plastic wrap and spatula.

Research materials used to test the antibacterial activity were *E. tarda* bacteria obtained from the Faculty of Medicine, Brawijaya University, Malang, aquadest, Tryptone Soy Agar (TSA), Tryptone Soy Broth (TSB) and disc paper with 6 mm diameter.

Research Design

The method used in this research is experimental with an experimental design using a completely randomized design. The variables used in this study were Jackfruit (*A. heterophyllus*) leaves crude extract as the independent variable and the growth of *E. tarda* bacteria as a dependent variable. This study used five different dose treatments (75 mg/L, 150 mg/L, 225 mg/L, 300 mg/L and 375 mg/L), positive control used 5 mg/L chloramphenicol antibiotics and negative control with three repetitions.

Work Procedure

Preparation of Jackfruit Leaves Crude Extract

Dark green jackfruit (*A. heterophyllus*) leaves that were picked randomly in the order of 3-5 from the shoots (Abadi *et al*., 2021) were collected, washed and then dried. Dried jackfruit leaves are then blended to form a powder (Adnyani *et al*., 2017). 200 grams of Jackfruit leaves powder was macerated with 1000 mL of ethanol as a solvent (ratio 1:5). Maceration was carried out 1x24 hours at a temperature of ± 25-28 °C. Macerat is separated by filtering using filter paper. The filtering process is repeated at least twice using the same type and amount of solvent. The next step is that all the macerate is collected and then evaporated using a rotary evaporator at a temperature of 35-40 °C until a brownish-green thick extract is obtained and then the yield is calculated (Kusumawati *et al*., 2017).

Phytochemical Screening

Preparation of test solutions to be used for phytochemical tests made with 5 mg of jackfruit (*A. heterophyllus*) leaves crude extract dissolved in 10 mL of 70% ethanol (Padmasari *et al*., 2013).

Flavonoid content was tested by using 1 ml of Jackfruit (*A. heterophyllus*) leaves crude extract was put in a test tube and then dissolved in 1-2 ml of 50% hot methanol. Mg metal and 4-5 drops of

concentrated HCL were added. An orange or red colored solution is formed, indicating the presence of flavonoids.

Alkaloid content was tested by using 2 mL of the test solution evaporated on a porcelain dish. The residue formed was dissolved with 0,5 mL of 2 N HCL. The resulting solution is divided into 3 test tubes. The first tube works as a blank added with HCL 2 N, the second tube is added 3 Dragendorff's reagent drops and the third tube added 3 Mayer's reagent drops. There are positive results alkaloids are characterized by the formation of a precipitate orange in the second tube and a yellow precipitate in the third tube.

Triterpenoid and Steroid content was tested by using 2 mL of test solution evaporated in a porcelain dish. The residue dissolved in 0.5 mL of chloroform, after that added with 0,5 mL of anhydrous acetic acid. After that add 2 mL of concentrated sulfuric acid through the tube wall. The presence of triterpenoids is indicated by the formation of a brownish or violet ring on the solution boundary, while the presence of steroids is indicated by the formation of a blue ring greenish.

Tannin content was tested by using 1 mL of the test solution reacted with a 10% solution of iron (III) chloride, the presence of Tannins indicated by the formation of color dark blue or greenish black.

Saponin content was tested by using 10 mL of the test solution in the test tube shaken vertically for 10 seconds then left for 10 seconds. Stable 1-10 cm high foam formation for not less than 10 minutes, indicates the presence of saponins and with the addition of 1 drop of 2N HCl, the foam does not disappear.

Preparation of Bacteria

E. tarda isolates were obtained from the Faculty of Medicine, Brawijaya University, Malang. 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

Inhibition test of jackfruit (*A. heterophyllus*) leaves crude extract against *E. tarda* bacteria using the diffusion method with 6 mm paper discs. The disc test was carried out by looking at the clear zone formed around the disc paper and measured using a caliper (Maftuch *et al*., 2015). The treatment given was a jackfruit (*A. heterophyllus*) leaves crude extract with five different doses (75 mg/L, 150 mg/L, 225 mg/L, 300 mg/L and 375 mg/L) and 2 controls (positive treatment with 5 mg/L of chloramphenicol and negative with no treatment).

Scanning Electron Microscope (SEM)

Observation of the morphology of *E. tarda* using the Scanning Electron Microscope (SEM) test aims to determine the structure changed of the bacterial cell wall due to the treatment of jackfruit (*A. heterophyllus*) leaves crude extract (Zahrah *et al*., 2018). The SEM test used two treatments, the first treatment was normal *E. tarda* bacteria and the second treatment was *E. tarda* bacteria after treatment using jackfruit leaves (*A. heterophyllus*) crude extract with doses 375 mg/L.

Data Analysis

The results obtained will be carried out ANOVA test (<0.05) to determine the effect of jackfruit (*A. heterophyllus*) leaves crude extract on *E. tarda* bacteria. To determine the relationship between treatment (jackfruit leaves crude extract) and test parameters (zone of inhibition) an orthogonal polynomial test was performed.

RESULTS AND DISCUSSION

The Yield of Jackfruit Leaves Crude Extract

The purpose of calculating the yield is related to the active compound from a sample. Yield shows that if the amount of yield increases, the number of active compounds contained in the sample also

increases (Hasnaeni *et al.*, 2019). The yield value of macerated jackfruit (*A. heterophyllus*) leaves using 70% ethanol as solvent is presented in Table 1.

Table 1. The yield of jackfruit leaves crude extract.

Solvent Type	Total Solvent (ml)	Sample Weight (gr)	Extract Weight (gr)	Yield (%)
Ethanol 70%	1000	200	27.8	13.9

Note: The yield value of 13.9% (Table 1) was obtained from the comparison of the final weight of the extract produced after extraction with the amount of simplicia before maceration then multiplied by 100% (Setyaningtyas *et al*., 2017).

Phytochemical Screening

The results of the phytochemical screening Jackfruit (*A. heterophyllus*) leaves crude extract proved to contain flavonoids, alkaloids, tannins, triterpenoids and saponins are presented in Table 2.

Table 2. Phytochemical screening results of jackfruit leaves crude extract.

Compound	Result	Characteristics
Flavonoid	+	Orange, Brick Red, Pink, Dark Red
Alkaloid		
-Dragendrof	-	Orange sediment
-Meyer	+	White sediment
-Bouchardat	+	Chocolate sediment
Tannin	+	Blackish Brown, Blackish Blue
Terpenoid		
-Steroid	-	Bluish green
-Triterpenoid	+	Orange, Brownish Orange
Saponin	+	Permanent foam

Flavonoids, alkaloids, saponins and tannins function as antibacterial compounds (Hamzah *et al*., 2013). According to Sivagnanasundaram and Karunanayake (2015), the results of phytochemical screening on jackfruit leaves extract contain antibacterial compounds in the form of flavonoids, alkaloids, phenols and terpenoids. According to Kusumawati *et al*. (2017), the ethanolic extract of jackfruit leaves contains secondary metabolites that have antibacterial activity because these compounds cause inhibition of cell walls, changes in cell membrane permeability, inhibition of protein synthesis and inhibition of nucleic acid synthesis. The dominant compound in jackfruit leaf extracts according to Sharma *et al*. (2015) was catechin-type flavonoid compounds. The LC-MS (Liquid Chromatograph Mass Spectrometry) test on jackfruit leaves contain flavonoids (12.43 mg/100 g), alkaloids (3.91 mg/100 g) and tannins (0.07 mg/100 g) (Amadi *et al*., 2018).

Antibacterial Activity Test

The results of inhibition zone measurements showed different results for each treatment. There was an increase in the diameter of the inhibition zone along with the increase in the dose of the given extract which is presented in Table 3.

Table 3. The crude extract disc test results of *A. heterophyllus*.

Concentration (mg/L)	Average Inhibition Zone Diameter (mm)		Classification of Response Zone
	24 hours	48 hours	
75	4,38±0,12	4,11±0,12	Weak
150	5,30±0,18	5,00±0,18	Moderate
225	6,37±0,12	6,08±0,12	Strong
300	6,78±0,18	6,48±0,18	Strong
375	7,80±0,15	7,50±0,15	Very Strong
Control (-)	0,00±0,00	0,00±0,00	Weak
Control (+)	13,70±0,35	13,70±0,35	Very Strong

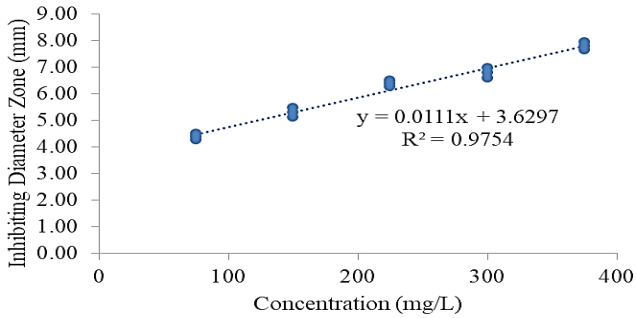

Figure 1. Antibacterial activity of jackfruit leaves crude extract against *E. tarda*.

From Table 3 above, the best treatment with the highest average inhibition zone of 7,80±0,15 mm was obtained by treatment E (375 mg/L) including the very strong category while the lowest average inhibition zone was 4,38±0,11 mm obtained by treatment A (75 mg/L) was categorized as weak. Other treatments such as treatment B (150 mg/L with an average inhibition zone of 5,30±0,18 including the moderate category and treatments C (225 mg/L) and D (300 mg/L) with an average inhibition zone of 6,37±0,12 and 6,78±0,18, respectively, were included in the strong category.

The treatment with negative control used distilled water that did not provide an inhibition zone because it did not have antibacterial compounds. Treatment with positive control in the form of 5 mg/L chloramphenicol antibiotics resulted in an average inhibition zone of 13,70±0,35 mm. The results of the inhibition zones produced were different for each treatment due to several factors such as the sensitivity of the organism, culture media, incubation conditions and speed of extract diffusion (Jamaludin *et al*., 2017).

The relationship between the treatment given to the test parameter in the form of the resulting inhibitory power obtained the equation y = 0,0111x + 3,6297 with an R^2 value of 0.974 which means 97% of the treatment given a crude extract of jackfruit (*A. heterophyllus*) leaves affect inhibition of bacteria *E. tarda.*

Giving extracts to *E. tarda* bacteria based on the table above is only bacteriostatic, which is only able to inhibit bacterial growth (Sukandar *et al*., 2014). It is because the average yield of the inhibition zone at 48 hours has decreased compared to 24 hours. Several factors can also cause the decrease in the average inhibition zone during the 48-hour incubation period, according to Kusuma *et al*. (2017) such as a decrease in the quality of the extract due to damage and reduction of antibacterial compounds and being contaminated with microbes such as yeast and mold growth. Another factor can come from the nature of the bacteria itself. In accordance with Taukoorah *et al*. (2016) statement, although it is only bacteriostatic, using natural ingredients as a treatment provides many advantages. It offers fewer side effects, is cost-effective, and can be used for a long time.

Radulovic *et al*. (2013) explained that bioactive compounds from plants in general have different ways of influencing bacteria. The main target of all active compounds is the cell membrane. The process of active compounds entering the bacteria begins with entering through the membrane (the entry of bioactive into the cell) and then the active compounds will interact with the bacterial cell components.

The mechanism of action of flavonoid compounds is by forming complex compounds with extracellular and dissolved proteins so that they can inhibit and damage the cytoplasmic membrane of microbial cells, and inhibit microbial energy metabolism (Rasyidah, 2021).

Saponins work as antibacterial by reducing surface tension, resulting in cell leakage and causing intracellular compounds to be released. This compound will diffuse through the outer membrane and cell wall, after which it binds to the cytoplasmic membrane and causes the cytoplasm to leak and consequently the cell dies (Ngajow *et al*., 2013).

The mechanism of action of alkaloids in inhibiting microbial growth is by destroying the peptidoglycan-forming components in cells so that the cell wall layers are not fully composed and result in cell death (Cushnie *et al*., 2014).

The mechanism of action of tannins in the opinion of Redondo *et al*. (2014) stated that tannins can inhibit microbial growth by inactivating microbial adhesins, enzymes, and transport proteins on cell membranes.

Scanning Electron Microscope (SEM)

The results of the SEM assay showing the changes in bacterial cell structure post-treatment using the extract are presented in Figure 2.

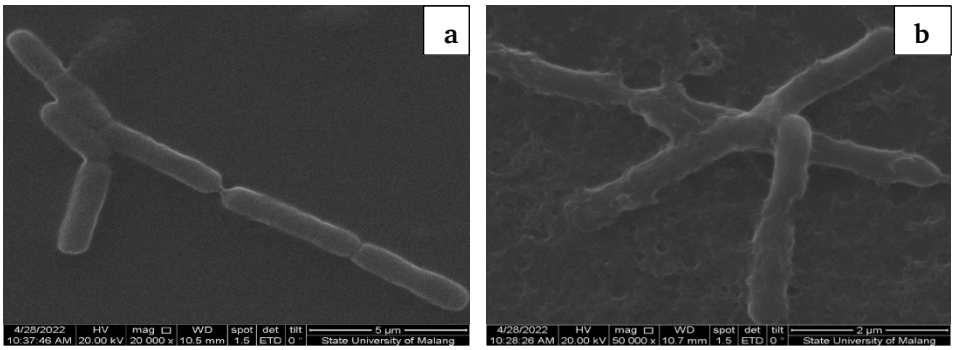

Figure 2. Morphology result of *E. tarda* after the SEM test was carried out. Note: A = No Treatment; B = Provision of Jackfruit (*A. heterophyllus*) leaves crude extract.

The changes in the morphology of *E. tarda* bacteria observed using SEM proved that the antibacterial compounds in the extract worked on *E. tarda* bacteria. One of these compounds is flavonoid which according to Armanda *et al*. (2017) stated that the mechanism of action of flavonoids is by denaturing bacterial cell proteins so that the typical characteristics of bacteria are lost. Flavonoids also cause changes in organic components and nutrient transport that result in toxic effects on bacteria. Flavonoids interact with bacterial DNA which aims to damage the hydrogen bridge bonds of the double-stranded DNA strands. Flavonoid compounds will contact the DNA in the cell nucleus and through the difference in polarity between the lipids that make up DNA and the alcohol groups on the flavonoid compounds a reaction will occur, thereby damaging the lipid structure of DNA and the bacterial cell nucleus will also lyse and die.

In addition, jackfruit (*A. heterophyllus*) leaves crude extract also

contains other ingredients such as alkaloids. The mechanism of antibacterial compounds by alkaloids according to Marina *et al*. (2015) can interfere with the formation of the constituent components of peptidoglycan in bacterial cells so that it can cause bacteria to lysis.

Tannins are antibacterial compounds that work through cell membrane reactions, the inactivation of enzymes and the inactivation of the function of genetic material. The way the tannin compounds work as an antibacterial is by entering the bacterial wall that has been lysed due to the activity of saponin and flavonoid antibacterial compounds, causing the tannin antibacterial compound to work by coagulating the protoplasm of bacterial cells (Risfianty and Indrawati, 2020).

Another compound, namely saponins, has a way of working according to Berlian *et al.* (2016) namely by lowering the surface tension of bacteria, resulting in increased permeability or leakage of bacterial cells and followed by the release of intracellular compounds. Another way of working is by interacting with the sterol membrane. The main effect of saponins on bacteria is the release of proteins and enzymes from the cells.

CONCLUSION

The crude extract of jackfruit leaves is bacteriostatic due to a decrease in inhibition after 48 hours of incubation. In addition, the dose of extract that gave the highest average inhibition was in treatment E (375 mg/L) of 4,38 ± 0,11 mm and was included in the strong category. The crude extract of jackfruit leaves has been shown to damage *E. tarda* bacteria through the results of the SEM test that has been carried out. Suggestions for further research prospects that necessary to carry out an LC50 test to determine the toxicity effect of the extract and then proceed with a vivo test to determine the effect of the crude extract of jackfruit (*A. heterophyllus*) leaves on fish infected with *E. tarda*.

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