Antibacterial Ability of *Rhizophora mucronata* Leaf Extract Against Bacterial Infections of *Edwardsiella tarda*

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Abstract

Edwardseilla tarda is a group of gram-negative bacteria with facultative anaerobic properties that can infect both cultivated and wild fish. *Edwardseilla tarda* infection causes considerable economic losses in the world. *Rhizopora mucronata* is a plant that is known to have the ability to produce secondary metabolites such as alkaloids and flavonoids. This study aims to determine the ability of *R. mucronata* to inhibit the growth of *E. tarda* as indicated by the presence of an inhibition zone and the absorbance value in the tube dilution test. The method used in this study is the disc diffusion test and the tube dilution test. The results of the phytochemical analysis showed the presence of flavonoid and alkaloid compounds in the crude extract of *R. mucronata* leaves. A concentration of 15.6 $mg.L^{-1}$ showed a minimal inhibitory response in inhibiting the growth of *E. tarda* bacteria. In comparison, the concentration of 56 $mg.L^{-1}$ showed the highest inhibitory response in inhibiting the growth of *E. tarda* bacteria. It indicates that the compounds contained in *R. mucronata* can be used as alternative ingredients in medicine to treat *E. tarda* bacterial infections.

Keywords: Alkaloid, Antibacterial, *E. tarda,* Flavonoid, *R. mucronata.*

INTRODUCTION

Edwardsiella tarda belongs to the group of facultative anaerobic gram-negative bacteria. It has a motile rod shape and belongs to the Enterobacteriaceae family [1]. The *E. tarda* bacteria is a relatively pathogenic severe agent that can infect cultured and wild fish [2]. Fish infected by *E. tarda* bacteria show several clinical symptoms, such as abnormal swimming movements, physiological (hematological) changes, and some organ damage due to infection [3]. The infection of *E. tarda* can affect various species of aquatic organisms, which can cause mass mortality in aquaculture. The *E. Tarda* infection is reported to have caused considerable economic losses worldwide [4]. Treatment of bacterial infection with *E. tarda* usually uses antibiotics [5]. Using antibiotics continuously for a long time can cause resistance in microorganisms [6]. Several studies have been conducted on the plasmid *E. Tarda*, which showed resistancy to several antibiotics, such as sulphonamide, tetracycline, streptomycin, kanamycin, and chloramphenicol [7]. Using chemical antibiotics also causes residue problems that will accumulate in aquatic organisms. The accumulation of residues in aquatic organisms can harm the health of humans who consume them [8].

It is necessary to reduce the use of synthetic antibiotics to prevent the problem of bacterial resistance. Therefore, it is necessary to do further research on natural ingredients as antibacterial. Some plants can produce secondary metabolites with antibacterial abilities [9]. One of the plants known to produce secondary metabolites that show antibacterial properties is mangrove plants of *R. mucronata* that belongs to the Rhizoporaceae family, distributed in the Indo-Pacific region. It can also be found in riverbanks and seaside areas [10]. Mangrove of *R. mucronata* contains several biochemical ingredients, such as alkaloids, tannins, saponins, and flavonoids which have a role in suppressing bacterial growth [11]. Therefore, the ability of *R. mucronata* leaf extract to inhibit the growth of *E. tarda* was assessed in this research.

MATERIAL AND METHOD

This research was conducted in December 2021-June 2022 in the laboratory of fish parasites and diseases (Faculty of Fisheries and Marine Sciences Universitas Brawijaya), fish reproduction laboratory (Faculty of Fisheries and Marine Sciences Universitas Brawijaya), Laboratory of Organic Chemistry, Faculty of Science and Technology., Malang State Islamic University. Materia Medica Batu, Batu City, East Java. Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya Malang.

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***R. mucronata* Leaf Extraction**

Samples of *R. Mucronata* were extracted using the maceration method. Toral of 100 g *R. mucronata* leaf powder was soaked in 400 mL methanol for 3x24 hours. The macerated sample was then filtered using Whatman paper no. 42. The filtered sample was put into a rotary vacuum evaporator to form a paste.

Bacterial Preparation

The bacteria used were obtained from the laboratory of fish parasites and diseases, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya. Bacteria were stored on Trypticase Soy Broth (TSB) media at -30°C.

Antibacterial Assay

Antibacterial testing was carried out using tube dilution to determine the minimum inhibitory response and the disk diffusion method to determine antibacterial sensitivity. First, the extract was dissolved in 10% DMSO with an initial concentration of 1000 $mg.L^{-1}$, 500 $mg.L^{-1}$, 250 $mg.L^{-1}$, 125 $mg.L^{-1}$, 62.5 $mg.L^{-1}$, 31.3 $mg.L^{-1}$, 15.6 $mg.L^{-1}$, 7.8 $mg.L^{-1}$, 3.9 $mg.L^{-1}$, and 1.9 $mg.L^{-1}$. The positive control used the addition of synthetic antibiotics (oxytetracycline), and the negative control only with the addition of *E. tarda* bacteria.

The antibacterial test using the tube dilution method was carried out by inoculating 0.1 mL of *E. tarda* bacteria on Trypticase Soy Broth (TSB) media and adding the dissolved extract concentration. Each tube was incubated at 30°C for 24 hours, and then observed the Optical Density with a spectrophotometer Thermo type Genesys 20 at a wavelength of 600 nm.

Disk assay was carried out using Muller Hinton Agar (MHA) media. The cup containing MHA was inoculated with 0.1 mL bacteria. The blank disk was dripped with *R. mucronata* leaf extract with a predetermined concentration and incubated for 24 hours at 30°C. The zone of inhibition obtained in the observation was then classified [12] as Table 1.

Tabel 1. Classification of zones of inhibition (Davis and Stout [18].

No	Diameter (mm)	Classification
1	<5	Weak
2	6-10	Medium
3	11-20	Strong
4	>20	Very Strong

Phytochemical Analysis

Phytochemical analysis were carried out to determine the presence of active ingredients in the *R. mucronata* extract. Phytochemical testing aims to test the presence of several compounds, such as saponins, alkaloids, flavonoids, and tannins. The flavonoid test used the Shinoda method. Alkaloids were tested by adding Mayer, Dragendorff, and Bouchardat reagents [13]. The saponin test method uses the foam test method [14]. At the same time, the tannin testing method is carried out by adding a ferric chloride solution [15].

Data Collection

Each treatment was repeated three times. The quantitative data of the inhibition zone and the growth of optical density were analyzed using one-way ANOVA, followed by Duncan's test using the SPSS 26 program.

RESULT AND DISCUSSION

Antibacterial Assay

The results in Table 2 show the antibacterial test data using the tube dilution method as indicated by the absorbance value. The lowest Optical Density was indicated by treatment with a dose of 15.66 $mg.L^{-1}$ with an Optical density of 0.077. The lower Optical density value indicates the inhibitory response produced by the *R. mucronata* extract. The cloudy bacterial media indicates the growth of bacteria. It indicates that the extract is ineffective enough to inhibit bacterial growth [16].

Table 2. Tube Dilution Test Result

No	Consentration ($mg.L^{-1}$)	Optical Density
1	1000.0	0.866
2	500.0	0.807
3	250.0	0.747
4	125.0	0.426
5	62.5	0.200
6	31.3	0.085
7	15.6	0.077
8	7.8	0.219
9	3.9	0.690
10	1.9	0.859
11	K(+)	0.015
12	K(-)	1.114

Notes: K(+) added oxytetracycline and K(-) *E. tarda* inoculum

To determine the sensitivity of the crude extract of *R. mucronata* leaves in inhibiting the growth of *E. tarda* bacteria, disk testing was performed (Fig. 1). The disk test results are presented in Table 3. The results of the disk diffusion test of *R. mucroata* leaf crude extract showed an increase in the inhibition zone from a concentration of 16 $mg.L^{-1}$ to 56 $mg.L^{-1}$ increased from 7.788 ± 0.336 mm to 10.18 ± 0.926 mm. The value of the inhibition zone produced by the *R.*

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mucronata extract was indeed lower than that of the + control. However, there is a possibility that if the concentration of the extract is increased to a particular concentration, the tilapia used can be close to and even in accordance with the + control used. This result is in accordance with the research conducted by Nurdiani *et al.* [17].

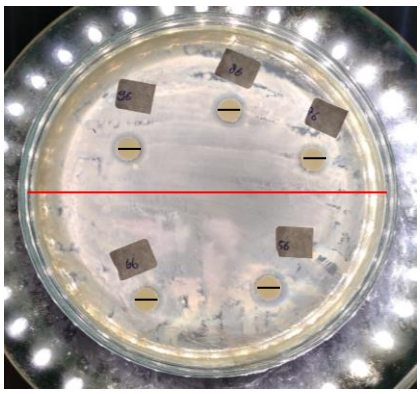

Figure 1. Disk Diffusion Test Result

Legend:

——— : Diameter of petri disc (60 mm)

___ : Diameter of blank disc (6 mm)

The study showed the antibacterial ability of the leaf extract of *R. mucronata*, which was tested on gram-negative bacteria such as *Escherichia coli*. The *E. coli* bacteria belong to Enterobacteriaceae, the same as *E. tarda* [18].

Table 3. Disk Diffusion Test Results

No	Concentration ($mg.L^{-1}$)	ø inhibition zone (mm)	Inhibition zone classification
1	16	7.788 ± 0.336	Medium
2	26	8.525 ± 0.361	Medium
3	36	8.328 ± 0.364	Medium
4	46	8.928 ± 0.484	Medium
5	56	10.18 ± 0.926	Strong
6	Control (+)	13.565 ± 1.351	Strong

Notes: ø = average diameter

The 56 mg.L-1 treatment's inhibition zone produced a strong inhibitory response. Differences in the size of the inhibition zone usually occurred due to the thickness of the agar medium used. The extract's ability to diffuse and the interaction between the medium and the content of the active ingredient also lead to different sizes of inhibition zone [19].

Phytochemical Analysis

The results of phytochemical testing of crude extract of *R. mucronata* leaves are presented in Table 4. Phytochemical analysis was carried out to qualitatively determine the content of active ingredients in a plant [20]. Phytochemical test results showed that the extract of *R. mucronata* showed the presence of active ingredients of flavonoids and alkaloids. According to Mangiro *et al.* [21], *R. mucronata* is rich in active ingredients such as flavonoids, alkaloids, saponins, and triterpenoids. However, in phytochemical testing of crude extract of *R. mucronata* leaves, only two active ingredients were found: flavonoids and alkaloids.

Table 4. Phytochemical Analysis Results

Bioactive compound	Characteristics	Explanation
Flavonoid	Orange, brick red, dark red	(+) Positive
Alkaloid	Brown sediment	(+) Positive
Tannin	Dark brown/dark blue	(-) Negative
Saponin	Stable Foam	(-) Negative

The different types of active ingredients found in the crude extract of *R. mucronata* leaves can be caused by the environmental conditions of the sample origin. *R. mucronata* will adapt to extreme environmental conditions by producing several bioactive ingredients [22]. Flavonoids are secondary metabolites found in nature, especially in green plants.

Flavonoids are generally used as drugs to treat infections of diseases [23]. Flavonoids also have antibacterial abilities, namely the destruction of membrane proteins, interfering in the protein synthesis process, and inhibiting the ATP transfer process [24,25].

Alkaloids are secondary metabolites produced by plants as a defense mechanism against pathogenic infections [26]. Alkaloids are also known to have antibacterial abilities by inhibiting the performance of the topoisomerase enzyme in bacterial cells [27]. In addition, several alkaloids, such as pergularinine and tylophorinidine alkaloids, can inhibit nucleic acid synthesis in bacteria [28].

The *R. mucronata* leaf crude extract has the ability to inhibit bacterial growth. It can be seen from the antibacterial test using the tube dilution method at 15.6 $mg.L^{-1+}$ extract concentration shows an OD value close to control (+) using oxytetracycline antibiotics. It indicates the ability of the extract to inhibit the growth of *E. tarda* bacteria. In addition, antibacterial activity can also be observed in tests using the disk diffusion method. The disk diffusion test showed the formation of an inhibitory zone around the blank disk that had been added with *R. mucronata* extract at a certain concentration. The presence of flavonoid and alkaloid compounds in the *R. mucronata* extract could cause the inhibitory ability of these bacteria.

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As stated by Rarasari *et al.* [29], flavonoids are antibacterial agents that have the potential to inhibit bacterial growth. The flavonoids could damage the permeability of bacterial cell walls, destroying lysosomes, microsomes, and bacterial cells when interacting with DNA. In addition to flavonoids, alkaloids are other active ingredients that play a role in inhibiting bacterial growth. Alkaloids are secondary metabolites produced by some plants. Alkaloids are generally known to have the ability to inhibit bacterial growth by various mechanisms, such as damaging bacterial cell walls, interfering with nucleic acid synthesis, and inhibiting bacterial metabolism [30]. Pfoze *et al.* [31] explained that alkaloids could inhibit the growth of gram-positive bacteria better than gram-negative bacteria. It is because gram-negative bacteria generally have an extra membrane outside the bacterial cell wall that functions as a cell defense.

The flavonoid compounds in the crude extract of *R. mucronata* leaves extract can damage the cell walls of *E. tarda* bacteria. It is due to the ability of flavonoids to dissolve and interact with extracellular proteins [5]. In comparison, the interaction of alkaloid compounds with the bacterial cell membrane of *E. tarda* can cause damage to the bacterial cell wall. When interacting with bacterial DNA, alkaloids can cause damage to the bacterial DNA structure [32].

CONCLUSION

Rhizopora mucronata leaf crude extract contains active ingredients in the form of flavonoids and alkaloids. It is evidenced by the results of the phytochemical test, which showed positive results on the flavonoid and alkaloid tests. The bacterial sensitivity test of *R. mucronata* extract showed that the lowest dose of *R. mucronata* extract that could inhibit bacterial growth was 15.6 $mg.L^{-1}$. The highest inhibition zone was indicated by a 56 $mg.L^{-1}$ concentration which showed a strong inhibitory response. Further research is suggested on applying *R. mucronata* leaf extract in aquaculture.

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