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In Vitro Analysis of Antibacterial Activities of Curry Leaf (*Murraya koenigii*) Extract Towards Bacteria *Edwardsiella tarda*

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Abstract

This study analyzed the antibacterial activity of curry leaf extract (*Murraya koenigii*) on the growth of *Edwardsiella tarda* bacteria. This study aims to determine the bioactivity and antibacterial effectiveness of *M. koenigii* leaf extract against the growth of *E.tarda* bacteria. Inhibition test was carried out by delusion (MIC test) and diffusion (disc test) methods. MIC test used 5 variations of concentration: 1 mg/L, 10 mg/L, 100 mg/L, 500 mg/L and 1,000 mg/L on TSB (Tryptone Soya Broth) media; it was incubated for 24 hours. While the disc test used 5 variations of concentration: 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L on TSA (Tryptone Soya Agar) media and incubated for 2x24 hours. Chloramphenicol (5 mg/L) was used as a positive control, and distilled water was used as a negative control. *M. koenigii* leaf extract contains natural bioactive; it was bacteriostatic antibacterial due to bacteria's growth after 48 hours incubation. The highest inhibition diameter of the extract against *E.tarda* was 7,20 mm at a concentration of 500 mg/L after 24 hours incubation. The highest inhibitory effectiveness was at a concentration of 500 mg/L and the effectivity of inhibition was 56.3%, while it declined to 46,44% after 48 hours incubation.

INTRODUCTION

E. tarda is a pathogen bacterium from the Enterobacteriaceae family that causes edwardsiellosis in freshwater and seawater aquaculture(Choi *et al*., 2011). In the aquaculture industry, fish diseases especially those caused by Gram-negative bacteria (e.g., *E.tarda*), becomes a severe problem and reduces profitability substantially. *E. tarda* causes edwardsiellosis in various hosts such as fish, amphibians, reptiles, birds, and mammals (including humans. This pathogenic bacterium is reported to be a significant threat to many fish species that have substantial economic values around the world (Li *et al*., 2019).

Antibiotics such as tetracycline and kanamycin are quite effective in controlling infection of *E. tarda*. However, the overuse of antibiotics and the misuse of antibiotics evoke several serious challenges related to the resistance of *E. tarda* to antibiotics and the potential residual antibiotics in humans (Xu *et al*., 2019). According to Harikrishnan *et al*. (2020), the use of antibiotics and chemical substances in aquaculture also has the potential to cause disadvantages, such as

water toxicity problems, public health problems and environmental damage.

The use of natural bioactive from *M. koenigii* leaf extracts is an alternative that may tackle the problems. *M. koenigii* is a plant from the Rutaceae family; it is widely grown in tropical and subtropical areas. The leaves, stems and roots of this plant have been used as a traditional medicine to treat various diseases. Phytochemical investigations of *M. koenigii* have revealed various natural products, including alkaloids, sesquiterpenes, essential oils, and alkenes. There are various biological activities such as anti-inflammatory, anti-oxidative, nephroprotective, hepatoprotective, anti-listerial, and antibacterial activity in different parts of *M. koenigii* (Ma *et al*., 2019). Studies on *M. koenigii* leaf extract as antibacterial from various sources have been carried out, so it is necessary to research to determine the potential of *M. koenigii* as an antibacterial against *E. tarda* which is the cause of disease in aquaculture.

This study aimed to determine the in vitro antimicrobial and inhibitory power of *M. koenigii* leaf extract against *E. tarda* bacteria.

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 December 2020 to January 2021.

Research Materials

In this study, sample preparation tools included blenders, trays, spatulas and analytical scales. Extraction: Erlenmeyer, hot plate with a stirrer, measuring cup, beaker glass and evaporator. Bacterial culture: petri dish, bunsen, loop needle, incubator and laminar flow. Antibacterial activity test: disc paper, tweezers, micropipette, tip puppet, bunsen and loop needle. Observation of the inhibition zone for bacterial growth: electric calipers.

The materials used in this study were the mashed leaves of *M. koenigii*, ethanol 96%, alcohol, sterile distilled water, TSA (Tryptone Soya Agar), TSB (Tryptone Soya Broth), chloramphenicol 250 g, isolate of *E. tarda* bacteria obtained from BUSKIPM Jakarta.

Research Design

This research was conducted using experimental laboratory methods for in vitro testing. The number of treatments in this study was five concentrations with two kinds of controls, positive control dan negative control.

Work Procedure

The extraction method is carried out by the maceration method. The leaves of *M. koenigii* were put into a maceration container of 100 grams, then added with 1 litre of 96% ethanol. Maceration of *M. koenigii* with ethanol as a solvent was carried out at room temperature 24 hours with occasional stirring. Comparison of sample and ethanol is 1:10. After 24 hours, the obtained filtrate is evaporated using a vacuum rotary evaporator (Rastina *et al*., 2015). The evaporation process is carried out at a temperature of 40°C with a speed of 100 rpm. The results obtained from this extraction are the crude extract of *M. koenigii* in ethanol 96% (Ekawaty *et al.*, 2015).

Antibacterial activity test of *M. koenigii* leaf extract against pathogenic bacteria *E. tarda* uses liquid delusion and diffusion methods. The liquid delusion method is carried out by the MIC (Minimum Inhibition Concentration) test (Rinawati, *et al*., 2011). The doses of *M. koenigii* leaf extract that were tested consisted of 5 concentrations (1 mg/L, 10 mg/L, 100 mg/L, 500 mg/L and 1,000 mg/L). Chloramphenicol 5 mg/L was used as a positive control. The level of turbidity and its absorbance were measured using a spectrophotometer (wavelength of 570 nm).

The diffusion method is carried out by the disc test (Ulmursida *et al.*, 2017). The doses of *M. koenigii* leaf extract tested consisted of 5 concentrations (100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L). Chloramphenicol 5 mg/L was used as a positive control. Data measurements were performed during the 24 hours incubation period for the MIC test and 24 and 48 hours for the disc test.

Data Analysis

The results are tabulated and analyzed by comparing the sample's absorbance level on the MIC test and measuring the disc test's resistance zone diameter. The calculation of extract inhibition the effectiveness is calculated based on the equation (Arora and Kaur, 2007).

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

Where:

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

RESULTS AND DISCUSSION

The yield obtained from the maceration of *M. koenigii* leaf powder using ethanol as a solvent is presented in Table 1.

Table 1. The yield of *M. koenigii* extract.

Total Solvent (ml)	Sample Weight (g)	Extract Weight (g)	Yield (%)
1000	100	8.33	8.33

Table 1 showed that the maceration of 100 g *M. koenigii* leaf powder with 1,000 ml ethanol produced 8,33 g extract or 8.33% of yield. Isadora *et al.* (2016) stated that the process of withdrawal of material (extraction) occurs by the flow of solvent into the cell, so that it will cause swell protoplasm, and the cell content will dissolve according to its solubility. This high solubility is related to the polarity of the solvent and the polarity of the extracted compound.

Minimal Concentration Test of Extracts as Antibacterial

MIC test results are measured the absorbance value. The absorbance value indicates the ability of *M. koenigii* extract to inhibit the growth of *E. tarda* bacteria. The results of the measurement of the absorbance value are presented in Table 2.

Table 2. MIC test results.

Concentration (mg/L)	Absorbance
Control (-)	0,894
1	0,516
10	0,447
100	0,241
500	0,357
1.000	0,305
Control (+)	0,023

Note: Positive control (K +) using chloramphenicol 5 mg/L, negative control (K-) without treatment only bacteria.

The table showed that *M. koenigii* extract with ethanol solvent at a concentration of 100 mg/L had the lowest absorbance value with a value of 0.095 Å; it approached the positive control. Data in all concentrations showed that the absorbance level decreased along with the increase of concentration of extract given at each given dose. So, it can be seen that the growth of *E. tarda* bacteria can be

inhibited due to the antibacterial compounds in *M. koenigii* extract. It is related to Mustanir *et al*. (2019), which explains that *M. koenigii* is one type of herbal plant with the potential as an antibiotic ingredient.

The data explained that the *M. koenigii* extract at a dose of 100 mg/L was chosen as the reference for the minimum dose to determine the disc test dose. According to Putri *et al*. (2008), the spectrophotometer could not distinguish the turbidity level of pigment from the extract and the turbidity from bacterial cells so that the Optical Density (OD) value obtained was a combination of the two. The MIC test results are tested on the disc paper test to determine the conformity results.

The disc test is performed to measure the clear zone that forms around the disc paper. The clear zone indicates the ability of *M. koenigii* extract to inhibit the growth of *E. tarda* bacteria. The results of the measurement of the clear zone around the disc paper are presented in Table 3.

Table 3. The extract disc test results of *M. koenigii* in 24 hours.

Concentration (mg/L)	Average of 24 hours Inhibition Zone Diameter (mm)	Classification of Response Zone
K-	0	Weak
100	2,50	Weak
200	3,61	Moderate
300	4,70	Moderate
400	6,50	Strong
500	7,20	Strong
K+	13,80	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 at 24 hours intervals showed that the quantity of extract dose given could increase the inhibition zone diameter. At extract concentrations of 100 mg/L, it was in a weak category with an average inhibition zone diameter of 2.50 mm. Extract concentrations of 200 mg/L and 300 mg/L had an average of inhibition zone diameter 3.61 mm and 4.70 mm, respectively; it was in a moderate category. In comparison, the extract concentrations that had a strong inhibition zone were 400 mg/L and 500 mg/L with average inhibition zone diameter of more than 6 mm.

Forming a clear area around the disc paper indicates antibacterial activity. The small inhibition zone indicates low antibacterial activity, while the large inhibition zone indicates high antibacterial activity. The amount of activity is due to the active compounds in the extract. Antibacterial ingredients are compounds that can inhibit growth or kill bacteria. The quality or ability of these antibacterials is determined by the activity and spectrum of substances against bacteria. The ability of antibacterials to inhibit growth is influenced by (1) antibacterial concentration, (2) duration of contact with antibacterials, (3) ambient temperature, (4) bacterial characteristics (age, type, concentration and state of bacteria), (5) physical properties and chemistry and other types of compounds in it (Yuhana *et al*., 2008).

Table 4. The extract disc test results of *M. koenigii* in 48 hours.

Concentration (mg/L)	Average of 48 hours Inhibition Zone Diameter (mm)	Classification of Response Zone
K-	0	Weak
100	2,15	Weak
200	3,25	Moderate
300	4,33	Moderate
400	6,05	Strong
500	6,85	Strong
K+	14,75	Strong

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

Table 4 showed the inhibition zone diameter measurements for all doses at 48 hours intervals. The inhibition zone was declined at the 48-hour time interval. At the extract concentration of 100 mg/L, 200 mg/L and 300 mg/L the inhibition zone was reduced to 1.85 mm, 5.12 mm And 6.78 mm respectively. While the inhibition zone's diameter at the extract concentration of 400 mg/L and 500 mg/L, the inhibition zone's size declined by 0.52 mm and 0.48 mm. On the other hand, the inhibition zone's diameter at the positive control increased from 21.76 mm to 23.35 mm.

The pharmacodynamic aspects of antibacterial drugs include the nature of bacteriostatic or bactericidal and also time-dependent or concentration-dependent. When a decrease of the number of bacterial colonies is ≥99.9% (two levels decrease of Log10) then the antibacterial effect can be categorized into the bactericidal, but if it is less than these values are categorized as bacteriostatic. Time dependency is a category where the antibacterial effect is not affected even though the value of concentration continues to be raised. Only the duration of working time can affect the antibacterial effects. Concentration dependency is a category for the antibacterial activity that is unaffected by the duration of working time, which acts thus only based on the increase in concentration (Setiadhi *et al.*, 2018).

Antimicrobial is bacteriostatic if only inhibit bacterial growth when the compound's application continues. However, if it is stopped or exhausted, bacteria's growth will increase again, and indicated by bacterial colony growth. Conversely, it is bacteriocidal if clarity increases at the next incubation period because these compounds can kill and stop bacteria's physiological activity, even though this administration of these compounds is stopped (Soelama *et al.*, 2015).

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

Concentration (mg/L)	Effectiveness of inhibition in 24 hours (%)	Effectiveness of inhibition in 48 hours (%)
100	18.11%	14.50 %
200	26.15%	22.03%
300	34.05%	29.35%
400	47.10%	41.01%
500	52.17%	46.44%

The effectiveness of inhibition from the *M. koenigii* extract showed in table 5; it declined in all doses from 24 hours to 48 hours after incubation. The extract with a 500 mg/L concentration was the most effective dose with effectiveness of inhibition 52.17% in 24 hours. In contrast, it declined to just 46.44% after 48 hours. On the other hand, the extract with a 100 mg/L concentration was the most

ineffective dose with effectiveness of inhibition only 18,11% at 24 hours and 14.50% at 48 hours. The extract with concentration 200 mg/L, 300 mg/L and 400 mg/L showed the decline of inhibition effectiveness percentage after 48 hours about 4.12%, 4.70% and 6.09% respectively.

Roslizawaty *et al*. (2013) stated that antibacterial effectiveness is influenced by the extract concentration. The higher concentration of curry leaf extract will increase the antibacterial active compounds' abillity so that the inhibition of bacterial growth will be even more significant. The way the extract works to inhibit bacterial growth is by damaging or changing cell walls, cell permeability, protein molecules, nucleic acids, enzymes work, and can inhibit the synthesis of nucleic acids and proteins. Nemeth *et al*. (2015) stated bacteriostatic antibiotics are assumed to require phagocytic cells to clear bacteria and are therefore thought to be less effective without an efficient immune response. This theoretical model has led to the recommendation that severely ill and immunosuppressed patients with bacterial infections should be treated with bactericidal antibiotics.

CONCLUSION

The bioactivity of *M. koenigii* leaf extracts were bacteriostatic as indicated by a decrease of the inhibition zone diameter after 48 hours of incubation. Besides *M. koenigii* leaf extract showed the effectiveness of antibacterial activity against *E.tarda* bacteria at a concentration of 500 mg/L. Further research is needed regarding bioactive compounds that are more specific to *M. koenigii* leaves.

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