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IN VITRO IHBITION OF PSEUDOMONAS FLUORESCENS BACTERIA BY USING CURRY TREE (MURRAYA KOENIGII) CRUDE EXTRACT

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ABSTRACT
Pseudomonas fluorescens is a bacterium that frequently infects fish living in freshwater environments. These bacteria can cause diseases that result in mass fish mortality. Besides, antibiotic treatment of the deseases results in antibiotic-resistant bacteria and antibiotic residues. Thus, alternative efforts utilizing natural ingredients such as curry tree (Murraya koenigii) extract were conducted. Curry tree contains active antibacterial compounds. A completely randomized design (CRD) was used in this study, with doses of (A) 125 ppm, (B) 150 ppm, (C) 175 ppm, (D) 200 ppm, and (E) 225 ppm, with a positive control using 30 ppm tetracycline antibiotics, and a negative control without extract. Each treatment was carried out three times. The crude curry tree extract (M. koenigii) inhibited the growth of P. fluorescens bacteria, forming a linear pattern with the equation y= 0.033x + 3.30 and the coefficient value R^2= 0.82. Treatment (E) with 250 ppm had the largest clear zone diameter with an average inhibition zone of 11.20±1.05 mm. Meanwhile, treatment (A) with 125 ppm had the smallest clear zone with an average of 7.63±0.98 mm. This study found that administering curry tree extract (M. koenigii) affected the inhibition of P. fluorescens bacteria. In this study, the best dose of curry tree extract was 225 ppm, with an average inhibition zone diameter of 11.20 mm.

KEY WORDS
Curry tree (Murraya koenigii), Pseudomonas fluorescens, infection, fish.

In the field of aquaculture, it is strongly associated with a wide range of problems. According to Aristotle et al. (2015), a disease is one of the threats to fish. Fish disease is a problem that fish agriculturalists try to avoid at all costs, as the attack of this disease leads to suboptimal harvests and mass fish mortality, resulting in significant losses.

P. fluorescens is included in the pathogenic bacteria in various types of fish, for example, in tilapia and carp (Zhang et al., 2020). This explanation is in line with the statement of Andayani et al. (2019) that the bacterium *P. fluorescens* was considered as a causative factor in the bacterial disease Hemorrhagic septicemia of cultured fish. This bacterium is a significant pathogen of freshwater fish and an opportunistic pathogen for other types of fish cultured in brackish and marine waters around the world. Treatment of this disease is mainly done by using antibiotics.

To avoid failure in fish farming and spreading disease, it is necessary to take steps to prevent and control the disease. Yulvizar et al. (2014) explained that the problem of disease caused by pathogenic bacteria could be overcome by giving antibiotics as an effort of chemotherapy to eliminate the disease. Because pathogenic bacteria are becoming more resistant to chemicals (antibiotics), an increase in antibiotic use may be followed by an increase in pathogenic diseases. Antibiotics cause pathogenic chromosomal mutations or the acquisition of plasmids.

According to Setyowati et al. (2014), one action that can be taken to reduce the use of antibiotics in fish farming is the use of natural ingredients, which are aimed to leave no residues in the fish's body and are safe for the surrounding environment. Curry tree crude extract (*Murraya koenigii*) is an alternative herbal medicine to control the presence of *P. fluorescens*.

Despite its numerous advantages, *M. koenigii* is still primarily used as an ornamental plant in the garden. Fadila et al. (2020) explained that the antibacterial properties of curry tree extract, both against gram-positive and negative bacteria, were due to carbazole alkaloid compounds. In addition to extracts, the curry tree is also known to produce essential oils. These oils are active in inhibiting bacteria such as *Proteus mirabilis*, *Corynebacterium pseudotuberculosis*, *Listeria innocua*, *Enterococcus faecalis*, *Salmonella Typhimurium*, *Streptococcus pyogenes*, and *Shigella dysenteriae*.

Based on these facts, this study aimed to determine the in vitro effect of crude curry tree extract (*M. koenigii*) on *P. fluorescens* bacteria. This research is envisioned to benefit the community's ability to overcome P. fluorescence-caused diseases in agricultural fields.

METHODS OF RESEARCH

The research was carried out at the Central Laboratory of Life Sciences (LSIH), Universitas Brawijaya, Malang, from March-April 2022.

This study employed an experimental method with a completely randomized design (CRD). The study used 5 treatments with 3 replications and 2 controls. The treatment used was curry tree extract (*M. koenigii*) as an antibacterial in inhibiting the growth of *P. fluorescens* bacteria with different doses. The dose for each treatment was: Treatment A (125 ppm), B (150 ppm), C (175 ppm), D (200 ppm), and E (225 ppm). The positive control consisted of 30 ppm tetracycline antibiotics and the negative control without any extract given.

The tools used in this research were autoclave, jar, beaker glass, blender, blue tip, film bottle, bunsen, petri dish, funnel, erlenmeyer, measuring cup, hot plate, incubator, caliper, Laminary Air Flow (LAF), ose needle, refrigerator, 100-1000 µl micropipette, tray, tweezers, test tube rack, rotary evaporator, rotary flask, spatula, sprayer, test tube, digital scale, analytical balance, vortex mixer, tweezers, triangle, volume pipette, rag, sterilization basket, and spectrophotometer.

The materials used in this study were curry trees (*M. koenigii*), *A. hydrophila* bacteria, MHA media, TSA media, TSB media, distilled water, 70% alcohol, DMSO 10%, ethanol 96%, denatured alcohol, cotton, paper discs, filter paper, plastic wrap, aluminum foil, chloramphenicol, label paper, tissue, rubber, and technical NaCl.

The main parameter in this study was the observation of the inhibition zone around the paper disc. The supporting parameter in this study was the use of an incubator temperature that helped bacteria to grow optimally.

Preparation of Curry Tree (Murraya koenigii) Extract. Curry trees were extracted by maceration methods and dissolved in 96% ethanol solvent because they can bind the compounds in the extract. The ratio of powder and solvent used is 1:10, in which 1 is 100 grams of powder and 10 is 1000 ml of ethanol. Curry trees were extracted by soaking in 96% ethanol at room temperature for 24 hours. The filtrate is then separated from the residue by filtration. After that, the filtrate was re-macerated for 3x24 hours. Furthermore, evaporation was carried out with a Rotary Vacuum Evaporator at a temperature of 60^{o}C to produce an extract in the form of a paste.

Sterilization of Tools and Materials. After being washed with soap, the tools utilized in this research were subsequently dried. After that, paper wrappings were placed around each tool. The tube and Erlenmeyer were similarly wrapped, but cotton was added to the top of them before being sealed. The autoclave was filled with sufficient distilled water. Then, the tools wrapped in waste paper were placed into the autoclave, then closed diagonally. The autoclave steps were as follows:

- Turn on the switch, press the ON button, turn the temperature to maximum and close the steam valve in the autoclave;
- At a temperature of 121^{o}C and a pressure of 1 atm, wait for 15 minutes by opening or closing the steam valve at the top of the autoclave lid;
- Let it still until the temperature reaches 0^{o}C, then, the steam valve is opened, and the cover on the autoclave can be opened symmetrically.

Preparation of PSA Media. PSA (Pseudomonas Selective Agar) media was weighed as much as 0.48 gr. Then, the PSA media was dissolved using 10 ml of distilled water in Erlenmeyer and homogenized using a hotplate. The test tube was then covered with cotton and aluminum foil and sterilized using an autoclave at 121^{o}C for 15 minutes. The sterile media was then placed at a 30^{o} position and allowed to solidify. The agar media was tilted and then struck with bacterial isolates.

Preparation of Bacterial Culture TSB Media. TSB (Tryptic Soy Broth) media was weighed 0.3 grams and dissolved with 10 ml of distilled water which was then homogenized with a hot plate. The media was then sterilized by autoclave at 121^{o}C for 15 minutes. The regenerated bacteria were then cultured on sterile TSB media.

Preparation of MIC (Minimum Inhibitory Concentration) Test Media. TSB media was weighed as much as 1.65 grams, then dissolved with 55 ml of distilled water and homogenized with a hot plate. The homogeneous medium was transferred to 11 test tubes of 5 ml each. The doses used in the MIC test were 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.3 ppm, 15.6 ppm, 7.8 ppm, 3.9 ppm, and 1.9 ppm. The test tube was covered with cotton and then wrapped in aluminum foil. Next, the test tube was autoclaved to sterilize the media at 121^{o}C for 15 minutes and at a pressure of 1 atm.

Preparation of MHA Media for Discs Test. MHA media was weighed as much as 3.8 grams, mixed with 100 ml of distilled water, and homogenized with a hot plate. The media was sterilized by autoclave at 121^{0}C for 15 minutes. The treatment doses of curry tree crude extract used were A (125 ppm), B (150 ppm), C (175 ppm), D (200 ppm), and E (225 ppm). Sterile media was poured into sterile Petri dishes, each as much as ± 25 ml. The treatment was carried out in Laminar Air Flow (LAF) to reduce the outside contamination risk and wait for it to solidify.

RESULTS AND DISCUSSION

The disc test aims to determine the antibacterial effect of curry tree extract (*M. koenigii*) on *P. flourescens* bacteria. The disc test was carried out by immersing the disc paper into the extract with doses according to different treatments for 15 minutes. In this study, the disc test used treatment doses of A (125 ppm), B (150 ppm), C (175 ppm), D (200 ppm), and E (225 ppm), as well as positive and negative controls. The positive control used a 30 ppm tetracycline antibiotic, and the negative control only used disc paper that was not soaked in the extract or antibiotics. Furthermore, it was put in a petri dish containing *P. flourescens* and then incubated for 24 hours to observe the clear zone and 48 hours to determine the antibacterial properties of curry tree extract (*M. koeginii*). The clear zone around the paper disc indicates the inhibitory effect of the extract used. The results of the disc test can be seen in Figure 1.

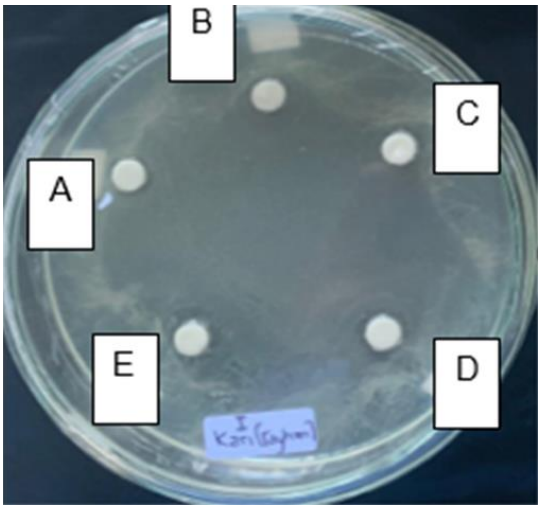

Figure 1 – Disc Test Result

The figure above shows a clear zone around the surface of the paper disc that has been soaked with curry tree extract (*M. koenigii*) using different treatment doses. The results obtained were different diameters of the inhibition zones. The diameter and average inhibition zone data of curry tree extract (*M. koenigii*) on the growth of *P. flourescens* bacteria can be seen in Table 1.

Table 1 – Average Result of Bacterial Inhibition Zone

Treatment	Average Inhibition Zone Diameter (mm)
A (125 ppm)	7.63[a]
B (150 ppm)	8.43[a]
C (175 ppm)	9.00[ab]
D (200 ppm)	10.20[c]
E (225 ppm)	11.20[d]

In table 1 above, the average results of the inhibition zones are obtained. In treatment A (125 ppm), the average inhibition zone was 7.63±0.98 mm. In treatment B (150 ppm), the average was 8.43±0.23 mm. Treatment C (175 ppm) was 9.00 ±0.10 mm. Treatment D (200 ppm) was 10.20±0.72 mm; for treatment E (225 ppm), the average inhibition zone was 11.20±1.05 mm. The results of this study indicated that a dose of 225 ppm (treatment E) showed the highest inhibition zone, 11.20 ± 1.05 mm. Meanwhile, treatment A at a dose of 125 ppm showed the lowest average result of the inhibition zone, which was 7.63±0.98 mm. So, this is in accordance with the statement of Alfiah et al. (2015); the size of the inhibition zone or the clear zone formed will depend on the size of the concentration of the extract given. The greater the concentration, the stronger the extract's ability to inhibit bacterial growth.

Polynomial orthogonal regression calculations were carried out to determine the relationship between the treatment parameters of the inhibitory test of curry tree extract on *P. fluorescens* bacteria. The following is a graph of the orthogonal polynomial calculations presented in Figure 2.

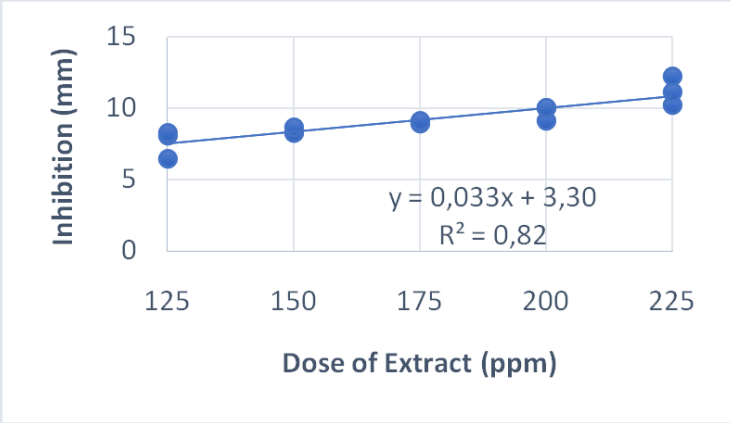

Figure 2 – Graph of Orthogonal Polynomials Test Results

The graph above shows the relationship between the inhibition zone on curry trees and P. fluorescens bacteria, forming a linear section with the equation y = 0.033x + 3.30 and the coefficient value R2 = 0.82. So, it can be said that the administration of curry tree crude extract (M. koenigii) on the dependent variable (inhibition) was 82%, while external variables could cause the remaining 18%.

The graph above shows that the clear zone will also get more significant with every increase in a dose—the greater the concentration, the stronger the extract's ability to inhibit

growth. The size of the inhibition zone can also be influenced by other factors such as temperature during incubation, time of installation of discs, and the distance between paper discs.

In this study, the crude extract of curry tree (*M. koenigii*) was revealed to inhibit the growth of *P. fluorescens* bacteria. On 24-hour observation, a clear zone was seen around the disc. This study's treatment doses of 125, 150, 175, 200, and 225 ppm resulted in a linear regression pattern. At 48 hours of observation, the clear zone around the disc paper could still be seen, but it showed a smaller clear zone compared to 24-hour observations. Thus, it was concluded that the curry tree extract (*M. koenigii*) was classified as bacteriostatic, indicating that it could only inhibit but did not stop the growth of *P. fluorescens* bacteria. Research from Septiani et al. (2017) also confirmed the current study's results, explaining that the decrease in the inhibition zone could be caused by bacteriostatic antibacterial activity. Bacteriostatic is the nature of antibiotics that can only inhibit, not kill, which is only temporary (reversible).

This research could not be separated from the role of active compounds in curry tree extract (*M. koenigii*). This active compound is a crucial inhibitor of the growth rate of *P. fluorescens* bacteria. Among the compounds in curry tree extract are alkaloids, flavonoids, and tannins. This explanation is in accordance with the Phytochemical Tests performed at the UPT Herbal Materia Medica Laboratory in Batu. According to Kusumastuti, et al. (2021), the mechanism of alkaloids as antibacterial is by interacting with bacterial cell walls, damaging cell walls that can be related to DNA from bacteria, causing protein synthesis to fail. Rastina, et al. (2015) explained that flavonoids could cause damage to the permeability of bacterial cell walls, lysosomes, and microsomes due to the interaction of flavonoids with bacterial DNA. Flavonoids have lipophilic properties, so it is possible to damage bacterial cell membranes. In addition, alkaloid compounds are known to be antimicrobial against bacteria, fungi, viruses, and protozoa. Unita and Voon (2016) explained that tannins had been found to inhibit protein synthesis in the form of irreversible complexes. This ability may be due to secondary metabolites in the extract. The flavonoids, saponins, and tannin compounds in *Murraya koenigii* are known for their antimicrobial properties.

The MIC (Minimum Inhibitory Concentration) test was carried out to determine the smallest extract dose to inhibit P. fluorescens bacteria using a crude extract of curry tree (Murraya koenigii). This study used doses based on the results of the MIC method according to Ruangpan and Kitio (1992) at doses of 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.3 ppm, 15.6 ppm, 7.8 ppm, 3 .9 ppm, and 1.9 ppm. The results of the MIC Test are presented in Figure 3.

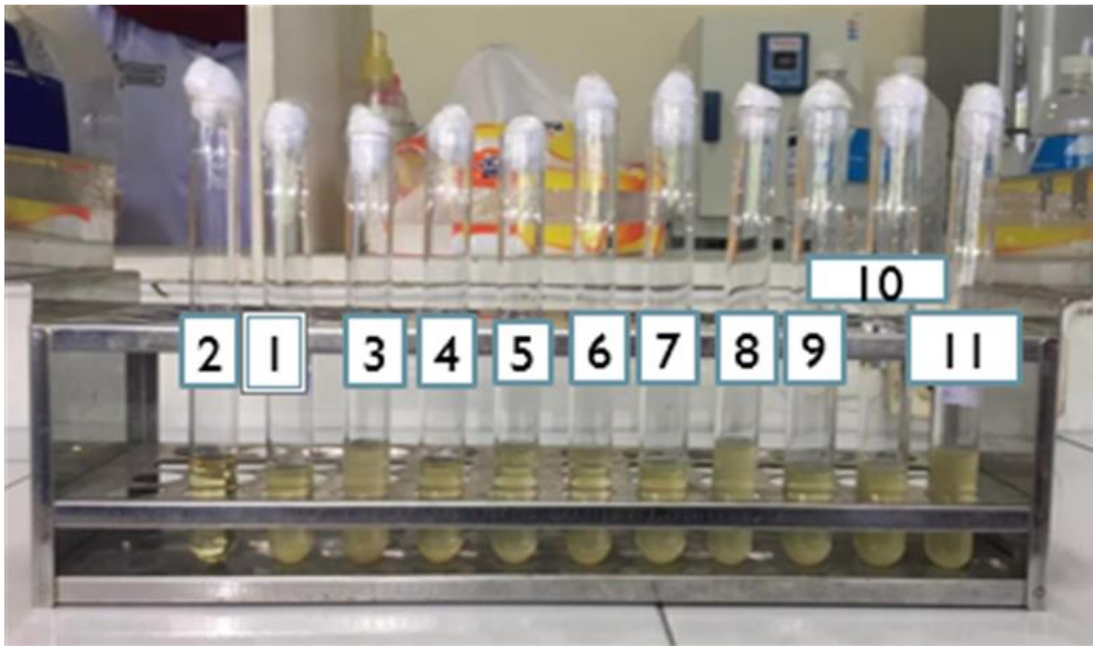

Figure 3 – MIC Test Results: Tube 1: Control (+), Tube 2: Tube (-), Tube 3: 1.9 ppm, Tube 4: 3.9 ppm, Tube 5: 7.8 ppm, Tube 6: 15.6 ppm, Tube 7: 31.3 ppm, Tube 8: 62.5 ppm, Tube 9: 125 pp, Tube 10: 250 ppm, Tube 11: 500 ppm

Based on turbidity observations and spectrophotometer results, a dose of 125 ppm was able to create absorbance values close to positive control values with turbidity levels close to positive control. The results of the spectrophotometer measurements are presented in Table 2.

Table 2 – MIC Test Observation Results

No	Dose (ppm)	Absorbance	Color
1	500	0.176	Clear
2	250	0.214	Clear
3	125	0.266	Clear
4	62,5	0.287	Slightly vague
5	31,3	0.292	Slightly vague
6	15,6	0.303	Slightly Vague
7	7,8	0.324	Vague
8	3,9	0.430	Vague
9	1,9	0.470	Vague
10	K(+)	0.257	Clear
11	K(-)	0.524	Vague

Notes: Tube no 3: minimum concentration of curry tree to inhibit the growth of P. fluorescens bacteria at a dose of 125 ppm; K(+): Positive control using 30 ppm tetracycline antibiotic; K(-): Negative control without using extract.

At a dose of 125 ppm, the results can inhibit the growth of *P. fluorescens* bacteria. That ability is due to the essential role of antibacterial compounds in curry tree (*Murraya koenigii*) extract. Based on the results of phytochemical tests, the antibacterial compounds in curry tree extract inhibit the growth of *P. fluorescens* bacteria, including active compounds such as alkaloids, flavonoids, and tannins. This result is in line with the research results of Sukma, et al. (2018); curry trees contain alkaloids, terpenoids, saponins, flavonoids, and tannins which are healing compounds from wounds.

CONCLUSION

Based on the research findings, it is possible to conclude that the use of crude curry tree extract (*M. koenigii*) inhibits the growth of *P. fluorescens* bacteria and that the extract is bacteriostatic. The best dose was obtained in Treatment E at 225 ppm, with an average inhibition zone diameter of 11.20 mm and a maximum inhibition zone diameter of 12.3 mm.

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