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Antibacterial Efficacy of Turmeric (*Curcuma domestica*) Rhizome Infusion Against *Aeromonas hydrophila* and Its Toxicity

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Abstract

Aeromonas hydrophila bacteria can cause Motile Aeromonas Septicemia (MAS), a disease that impacts freshwater fish. The turmeric rhizome contains numerous bioactive compounds that act as antibacterials and might be utilized to inhibit A. hydrophila. This research aimed to identify the phytochemicals in turmeric (*Curcuma domestica*) infusion, evaluate its antibacterial activity against A. hydrophila, and define the toxicity effect of turmeric rhizome infusion. The agar dilution method was used to measure antibacterial activity, whereas the Brine Shrimp Lethality Test (BSLT) was used to assess toxicity. The research confirmed the presence of phytochemicals such as tannins, alkaloids, flavonoids, phenols, and saponins in a turmeric rhizome infusion. According to the findings, the infusion from turmeric rhizome effectively inhibited the growth of A. hydrophila at concentrations between 750-1000 ppm. In toxicity tests, the LC50 value of the turmeric rhizome infusion against shrimp larvae was 381.18 ppm. Hence, the turmeric rhizome infusion has the potential to be further developed for the prevention and treatment of fish infected with A. hydrophila in freshwater fish farming.

Keywords: : *Aeromonas hydrophila*; Brine Shrimp; *Curcuma domestica*; Infusion; Turmeric.

Introduction

Aeromonas hydrophila is responsible for significant economic losses and high mortality rates of fish farmed in aquaculture (Kari et al., 2022). It is one of the most common causes of Motile Aeromonas Septicemia (MAS) in freshwater fish (Stratev and Odeyemi, 2017). In 2018, MAS caused the sudden death of 14,000 gouramis (Osphronemus goramy) caused by A. hydrophila (Antara, 2018). A. hydrophila bacterium is highly pathogenic and spreads rapidly (Dewi et al., 2011).

Infections caused by A. hydrophila are primarily treated with antibiotics (Woo et al., 2022). These drugs can inhibit or kill bacteria, making them efficient for treating bacterial infections. However, the excessive use of antibiotics in aquaculture can result in the emergence of antibiotic-resistant bacteria, which can pose a significant threat to both animals and humans (Manyi-Loh et al., 2018). Antibiotics are also associated with several potential adverse effects, including toxicity, environmental damage, and the elimination of organisms that are helpful to the body (Polianciuc et al., 2020).

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In order to limit the usage of antibiotics in aquaculture, plant extracts are widely recommended for treating bacterial infections. This method can reduce the antibiotics needed to fight bacteria that cause disease (Pękala-Safińska et al., 2021). Using herbs with potential antibacterial properties against A. hydrophila is crucial in preventing the spread of diseases in aquaculture (Semwal et al., 2020). Several medicinal plants, including Alpinia purpurata, Boesenbergi apandurata, Zingiber zerumber, Moringa oleifera, and Hibiscus sabdariffa, have been reported to prevent pathogenic A. hydrophila (Hardi et al., 2016; Sari et al., 2017; Rosidah et al., 2018; Bariyyah et al., 2019; Kenconojati et al., 2019).

One example of a medicinal plant that has several advantages is turmeric. Turmeric is a popular traditional remedy for humans. It is known for its antimicrobial, anti-inflammatory, and antioxidant properties and its ability to promote immunity against diseases (Hewlings et al., 2017; Sharifi-Rad et al., 2020). Turmeric rhizome has been used to treat bacterial diseases in fish, particularly MAS disease (Riauwaty et al., 2021). The rhizome's antibacterial properties, along with its environmentally-friendly nature, affordability, and accessibility, make it a potential candidate for drug development (Dewi, 2011; Wardani et al., 2012). Studies indicate that using turmeric extract (*Curcuma domestica*) obtained through the maceration process using methanol solvent effectively hinders the growth of A. hydrophila, which infects catfish (Karmila et al., 2017). However, the methanol-based maceration method is costly. Therefore, this research uses infusion as an alternative extraction approach with lower operational costs.

A biological toxicity test is one way to figure out if a substance could be used as a replacement for a new drug. A toxicity test can be done through the Brine Shrimp Lethality Test (BSLT) method, a preliminary assessment designed to evaluate the degree of toxicity of a substance to living organisms. This approach offers several advantages, including its cost-effectiveness, rapidity, simplicity of use, and the fact that it does not require sterile conditions (Hamidi et al., 2014; Banti et al., 2021). Additionally, the BSLT method provides a 95% confidence level. In this study, a toxicity test of turmeric infusion was carried out using the BSLT method with Artemia salina Leach as a model organism. This study aims to evaluate the potential effectiveness of turmeric infusion in inhibiting A. hydrophila growth and its toxicity to Artemia salina.

Research Methods

The research was conducted at the Laboratory of Microbiology, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan, in March-September 2022. Turmeric rhizomes were obtained from the Giwangan traditional market in Yogyakarta. Artemia salina was obtained from Animal Structure and Physiology Laboratory, Universitas Ahmad Dahlan.

Preparation of Turmeric Rhizome Simplicia

Selected turmeric (*Curcuma domestica*) rhizomes were picked, which entailed picking rhizomes with firm skin that did not peel off easily and had a shiny appearance when cut. Following selection, the rhizomes were carefully washed and sliced before being dried in an oven at 60°C for two to three days until a constant weight was achieved. The dried rhizomes were then ground into a fine powder.

Preparation of Infusions

In order to prepare turmeric rhizome infusion, 100 g of turmeric rhizome were combined with 100 mL of distilled water and boiled at 90°C for 15 minutes. The mixture was filtered using filter paper, and sterile distilled water was added to get the total volume to 100 mL.

Screening of Phytochemical Content

Phytochemical tests were conducted on turmeric rhizome infusion, including alkaloids, flavonoids, tannins, saponins, and phenols, based on the method described by Setyowati et al. (2014) and Cobra et al. (2019). Hydrochloric acid was added to the turmeric rhizome infusion to test for the presence of alkaloids, and the formation of a brown precipitate at the bottom of the tube indicated the presence of alkaloids. For flavonoid testing, 1% FeCl3 solution was added to the turmeric rhizome infusion, and a color change to green, red, dark black, blue, or purple indicated the presence of flavonoids. To test for saponins, 5 mL of distilled water was added to 5 mL of the turmeric rhizome infusion and shaken, and the presence of stable froth after standing for 30 minutes indicated the presence of saponins. To test for phenols, 5 mL of the turmeric rhizome infusion was added to 5 mL of FeCl3 solution, and the formation of a green to blue-black colour indicated the presence of phenols. Tannin testing was conducted by dissolving 1 mL of turmeric rhizome infusion in methanol, adding 2-3 drops to 1% FeCl3 solution, and observing the formation of a yellow precipitate.

Determination of Antibacterial Activity

The antibacterial activity test was conducted using the modified macrodilution method. Various amounts of extract at concentrations of 250, 500, 750, and 1000 ppm were prepared in Nutrient Broth media to determine the antibacterial activity of the extract. The standardized bacterial culture (100 µL) using the MacFarland 0.5 solution was distributed aseptically in each tube. The positive control consisted of media supplemented with chloramphenicol (0.1 mg/mL) without extract, while the negative control contained only the media. The tubes were left in an aerobic incubator at 37 °C for 24 hours. The number of bacteria in each tube was determined through the total plate count using the serial dilution technique. Each experiment was conducted three times, and the number of colonies on the plates was counted. The antibacterial activity was calculated using the following formula:

% Inhibition = (number of colonies in the control-number of colonies in the treatment)/(the number of colonies in control) × 100

Toxicity Test by Brine Shrimp Lethality Test Method

Artemia salina shrimp eggs (cysts) were soaked in distilled water for 1 hour. Eggs were moved in a container divided into two chambers, connected by small holes. The incubation room was kept dark while an aerator and light were provided as a source of oxygen to attract the shrimp larvae toward the brighter chamber. Healthy larvae swim to a bright chamber because shrimp larvae are attracted to light. After 48 hours, the shrimp larvae were ready to be tested.

The turmeric rhizome infusion was diluted to 250, 500, 750, and 1000 ppm concentrations. Ten healthy and active Artemia salina shrimp larvae, which were 48 hours old, were randomly selected and placed into test bottles containing different turmeric rhizome extract concentrations. A control solution was also included without the addition of any extracts. Each concentration was repeated four times, and a drop of yeast suspension was added to feed the shrimp larvae. The test bottles were then placed under light for 24 hours, after which the number of live shrimp larvae was counted using a magnifying glass. Any immobile larvae that sank or floated when touched with a spatula were classified as dead. The formula used to determine the percentage of mortality is as follows:

% M = (No-Nt)/No x 100%.

where,

Nt = number of dead larvae

M = Mortality

No = total number of larvae

Data analysis

The data obtained from the results were analyzed descriptively and presented as tables. Furthermore, the LC50 value was analyzed through probit analysis using SPSS Statistics software.

Research Results and Discussion

The active components in the samples were explored through phytochemical screening. The rhizome is the most commonly utilized component of the Curcuma genus. This portion contains various compounds, such as bioactive nonvolatile curcuminoids and volatile hydrocarbon compounds (Shafiri-Rad et al., 2020). A phytochemical analysis of the turmeric rhizome infusion revealed the presence of alkaloids, flavonoids, saponin, phenol, and tannin (Table 1). These findings are in line with Grover et al. (2021), who reported that the turmeric rhizome extracted using polar solvent (ethanol) contains compounds such as alkaloid, flavonoid, saponin, phenol, and tannin. Extraction with polar solvents yields the maximum number of phytochemical constituents compared to non-polar solvents, providing a more robust basis for their potency (Grover et al., 2021).

An antibacterial activity test was conducted to determine the antibacterial activity of turmeric rhizome infusion against A. hydrophila through cell counting after macrodilution method. Table 2 shows the inhibitory ability of the turmeric rhizome infusion against A. hydrophila. According to the findings, an infusion with a concentration of 750 ppm can inhibit almost all (98%) A. hydrophila bacteria. The presence of phytochemical compounds in the infusion is responsible for its antibacterial activity against A. hydrophila. Alkaloids inhibit bacterial growth by inhibiting nucleic acid and protein synthesis, altering cell membrane permeability, damaging cell walls and membranes, restraining metabolism, and inhibiting efflux pumps (Yan et al., 2021). Flavonoids exhibit antibacterial properties through various mechanisms, such as hindering nucleic acid synthesis, impairing cytoplasmic membrane function, inhibiting energy metabolism, preventing attachment and biofilm formation, obstructing porin on the cell membrane, altering membrane permeability, and reducing pathogenicity (Xie et al., 2015). The antibacterial mechanism of saponins has been attributed to their ability to increase the permeability of the bacterial cell membrane, owing to their detergent-like properties. This increased permeability allows saponins to exert their effects on the bacterial cell wall by causing damage to both the membrane and cell wall (Khan et al., 2018). Phenolic compounds have unique biological activities due to their structure and have been found to possess potent antimicrobial properties. Each phenolic compound subclass has different mechanisms, including impeding microbial cell wall biosynthesis, protein synthesis, nucleic acid synthesis, metabolic pathways, and disrupting cell membrane integrity (Ecevit et al., 2022). Tannins' antibacterial properties result from their ability to penetrate bacterial cell walls, reach the internal membrane, disrupt cell metabolism, and ultimately cause cell death. Nonetheless, the effect of tannins on Gram-negative bacteria is relatively slower due to the presence of a bilayered membrane (Kaczmarek, 2020).

Additionally, curcumin, a primary bioactive component found in turmeric, has been shown to possess potent antioxidant, anti-inflammatory, antibacterial, antifungal, and antiviral properties (Adamczak et al., 2020). Previous studies have identified various pharmacological mechanisms demonstrating curcumin's broad range of antimicrobial properties (Sharifi et al., 2020). Curcumin is known to inhibit bacterial DNA replication and modify gene expression, damage the cell membrane of bacteria, and decrease their motility (Tyagi et al., 2015). However, an investigation by Adamczak et al. (2020) found that curcumin exhibits a significantly more potent impact on Gram-positive than Gram-negative bacteria. The difference in the structure and composition of cell walls between Gram-positive and Gram-negative bacteria is responsible for the stronger effect observed on Gram-positive bacteria than on Gram-negative bacteria (Zheng et al., 2020). A. hydrophila is a freshwater Gram-negative bacterial pathogen that can cause disease by producing virulence factors such as adhesins, cytotoxins, hemolysins, lipases, and proteases (Beaz-Hidalgo and Figueras, 2013). Besides that, A. hydrophila can form biofilms, use specific metabolic pathways, and regulate virulence factor expression through quorum sensing (Rasmussen-Ivey et al., 2016). The study conducted by Tanhay et al. (2020) confirmed that curcumin could inhibit quorum sensing and biofilm formation in A. hydrophila. Therefore, developing a turmeric infusion to inhibit the proliferation of A. hydrophila in freshwater is of considerable significance.

Toxicity analysis is a test that determines the presence of toxic substances in a material and establishes the maximum allowable use of a plant as a traditional medicine (Khasanah et al., 2020). The BSLT test determined the Median Lethal Concentration (LC50), which is the concentration that can kill 50% of test animals within a certain time frame. Because it is simple, inexpensive, and reliable, the toxicity test method using the BSLT test is frequently used for pre-screening active compounds in plants. According to the results presented in Table 3, the treatment with an infusion concentration of 1000 ppm exhibited the highest larval mortality. Moreover, as the infusion concentration increased, the larval mortality level also increased.

The mortality of Artemia larvae is related to the presence of phytochemicals in the infusion of turmeric rhizomes. The turmeric rhizome infusion contains alkaloid compounds that could cause brine shrimp larvae to die. These compounds can diffuse through the larvae's cell membrane, affecting the biochemical and physiological of the larvae (Sharififar et al., 2017). The larvae's non-selective filter system allows toxic substances to enter their digestive tracts, causing damage to their enzymes, fats, cell membranes, and nucleic acids, ultimately leading to death (Jamil et al., 2019). The flavonoids in the infusion contribute to larval death by causing stomach poisoning and inhibiting their taste receptors, leading to hunger and death (Nur et al., 2019). Probit analysis indicated that 381,18 ppm of the turmeric rhizome infusion was lethal to 50% of shrimp larvae. Based on the findings of this study, a concentration of <400 ppm is considered safe to use, even though the complete elimination of A. hydrophila requires a higher concentration (750-1000 ppm). However, further studies on the toxicity of the turmeric rhizome infusion on fish are necessary to assess its effectiveness in preventing or treating fish infected with A. hydrophila bacteria.

Table 1
Phytochemical compounds of turmeric rhizome infusion

No	Identification	Chemical	Stain Color	Results
1	Alkaloid	Hydrochloric acid	Brown precipitate	+
2	Flavonoid	$FeCl_3$ 1%	Blue-black precipitate	+
3	Saponin	Aquades	Foam	+
4	Phenol	$FeCl_3$	Blackish blue	+
5	Tannin	methanol,$FeCl_3$	Yellow precipitate	+

Table 2
Antibacterial activity of turmeric rhizome infusion against Aeromonas hydrophila

Turmeric Rhizome Infusion (ppm)	Log10 CFU/mL	% Inhibition
0	5,05	0
250	3,43	32
500	1,22	76
750	0,11	98
1000	0,00 *	100

**no visible growth*

Table 3
The mortality rate and LC50 value of Artemia salina larvae treated with turmeric rhizome infusion

Infusion Concentration (ppm)	Mortality (%)	LC_{50} (ppm)
Control	0,00	
250	45,00	
500	71,67	381,18
750	85,00	
1000	96,67	

Conclusion

The infusion derived from the rhizomes of turmeric (*Curcuma domestica*) contains tannins, alkaloids, flavonoids, phenols, and saponins. A concentration of 750 ppm of the turmeric rhizome infusion can eliminate nearly all the *Aeromonas hydrophila* bacteria (98%). In shrimp larvae (Artemia salina), the turmeric rhizome infusion's lethal concentration 50 (LC50) is 381.18 ppm. Additional studies are necessary to determine the efficacy of the turmeric rhizome infusion in treating freshwater fish infected with *Aeromonas hydrophila*.

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4

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