The activity of Muntingia calabura leaf extract against pathogenic bacteria in fish

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Abstract. Bacterial diseases have emerged as a significant issue within the fish ecosystem, and the use of antibiotics to treat these diseases has detrimental effects on both fish and the environment. Therefore, it is imperative to seek out natural products that are safe for combating fish diseases. This research aims to assess the antibacterial properties of an extract derived from Muntingia calabura against various bacteria, including A. hydrophila, A. salmonicida, E. ictaluri, P. aeruginosa, E. tarda, and V. alginolyticus. M. calabura extract was obtained by soaking with ethanol solvent. This extract has been tested for phytochemical content and characterized by FT-IR. The antibacterial activity of the M. calabura extract was evaluated using the agar diffusion method, employing concentrations of 100, 200, 300, 400, and 500 mg mL-1. The phytochemical analysis of the M. calabura extract revealed the presence of flavonoids, phenolic compounds, saponins, and terpenoids. The results of the FT-IR characterization of M. calabura extract contained O-H, C=O, C=C aliphatic, C=C aromatic and C-O groups. The inhibition tests demonstrated that the M. calabura extract effectively hindered the growth of A. hydrophila bacteria by a range of 8.1 mm to 14.2 mm, A. salmonicida by 9.2 mm to 10.9 mm, E. ictaluri by 8.8 mm to 11.5 mm, P. aeruginosa by 8.1 mm to 12.1 mm, E. tarda by 9.6 mm to 11.4 mm, and V. alginolyticus by 8.2 mm to 12.2 mm. In summary, the extract from M. calabura has the potential to effectively on paper plates and incubated at 30°C for 24 hours.

1. Introduction

Fish infections caused by pathogenic bacteria such as Aeromonas hydrophila, Edwardsiella ictaluri, Vibrio alginolyticus, Edwardsiella tarda, Pseudomonas aeruginosa, Aeromonas salmonicida, often occur in fish farming. This pathogenic bacterial infection can cause death in fish. A. hydrophila is a Gram negative bacteria, is opportunistic, causes Motile

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Aeromonas Septicemia (MAS) disease in fish [1, 2]. A. hydrophila infection can cause hemorrhage on parts of the body, especially on the chest, stomach and base of the fins [3].

A. salmonicida infects many salmonid fish [4]. A. salmonicida bacteria is an aquatic pathogen that can infect several types of fish [5], capable of causing hemorrhage, ulcerative lesions, bleeding and death [6]. E. ictaluri, a group of Gram negative bacteria, has been found to infect many catfish [7], especially fingerling sized fish [8]. Causing problems in the cultivation of Pangasianodon hypophthalmus fish, namely Enteric Septicemia of Catfish (ESC) [9]. *P. aeruginosa* is an opportunistic bacteria that infects many farmed fish [10]. Causes sepsis, gill necrosis, abdominal distention, splenomegaly, liver and kidney congestion [11].

Vibrio alginolyticus is known as a gram-negative opportunistic pathogen, endangering the development of the global aquaculture industry as well as human health. [12]. V. alginolyticus infection can cause exophthalmia, ulcers, septicemia in fish [13]. The bacterium E. tarda is an important pathogen of freshwater and marine fish, causing enormous economic losses to the aquaculture industry worldwide [14, 15]. E. tarda is an important aquatic pathogen for many species, especially farmed fish. E. tarda causes severe mortality in Paralichthys olivaceus fish and causes large economic losses [16].

To control bacterial diseases, antibiotics are often used, but inappropriate use of antibiotics can increase the risk of bacterial resistance [17] and have negative effects on fish [18]. As a substitute for antibiotics, natural products from plants that have antibacterial activity are sought, such as the Muntingia calabura plant.

M. calabura plants are often found in Indonesia. M. calabura It has been scientifically proven that the leaves have a number of pharmacological activities such as antidiabetic, antioxidant, antibacterial, antihelminthic, hypolipidemic and anti-inflammatory. The chemical content of cherry leaves is flavonoids, alkaloids, tannins, saponins and terpenoids. M. calabura leaves have the potential to be further developed into herbal medicinal preparations [19].

Secondary metabolite compounds contained in M. calabura leaves are thought to play a role in antibacterial activity. The content of these compounds can inhibit the growth of pathogenic bacteria Escherichia coli, Staphylococcus aureus, Bacillus subtilis [20], Streptococcus viridans, Staphylococcus aureus [21], Salmonella sp. [22], Streptococcus viridians [23], Staphlococcous epidermidis [24], Staphylococcus aureus, Bacillus cereus, Escherichia coli [25], Pseudomonas aeruginosa [26], these types of bacteria do not attack farmed fish. For this reason, it is necessary to study M. calabura leaf extract to inhibit the growth of pathogenic bacteria that infect fish. This study aims to determine the performance of M. calabura extract as an V. alginolyticus bacteria, A. salmonicida, antibacterial towards A. hydrophila, E. tarda, E. ictaluri, P. aeruginosa.

2 Material and Method

2.1 Extraction of *M. calabura* leaf

Muntingia calabura leaves were sourced from the grounds of the Faculty of Agriculture at the Islamic University of Riau in Pekanbaru, Indonesia. These leaves were subjected to a drying process in an oven at 40°C for a duration of 2 days. Subsequently, they were finely

ground using a blender. The resulting M. calabura powder was subjected to maceration with a 90% ethanol solvent for a period of 3 days. Afterward, the filtrate was concentrated by evaporating it using a rotary evaporator operating at 50°C with a speed of 50 rpm until the extract was obtained.

2.2 Phytochemical testing and Fourier transform infrared spectroscopy (FT-IR)

A chemical analysis was carried out on the M. calabura extract to detect the presence of different compounds, which include phenolics, saponins, flavonoids, alkaloids and terpenoids. In the alkaloid test, 250 μL of Mayer reagent ($HgCl_2$, Ki) was introduced to 10 mg of the M. calabura extract, resulting in the formation of a white precipitate. If Dragendorff reagent (Ki, Bi))was added, it turned orange, indicating a positive outcome. As for the flavonoid test, 0.05 g of magnesium and 10 drops of 37% HCl were mixed with 10 mg of M. calabura extract and stirred for 1 minute. A positive result was indicated by a change in color to red.

The phenolic test involved mixing 10 mg of M. calabura extract with 500 μL of 5% FeCl3, and a positive result was indicated by a change in color to blue. To test for saponins, 10 mg of M. calabura extract was shaken with 5 mL of aqua dest for 1 minute, followed by the addition of 150 μL of 1N HCl and another 1-minute shaking, with a positive result indicated by the formation of foam. For the terpenoid test, 10 mg of M. calabura extract was mixed with 3 drops of H2SO4 and 10 drops of CH3COOH, giving a positive result indicated by the formation of a red color.

To further Identify functional groups groups present in the M. calabura extracts, FT-IR spectroscopy analysis was conducted. This involved crushing 1 mg of each extract, adding it to KBr, and vigorously mixing until homogenized. The resulting mixture was then measured for infrared absorbance in the wavelength range 4500–450 cm, allowing for the identification of functional groups.

2.3 Inhibitory activity of M. calabura extract

The M. calabura extract obtained from the study underwent testing against pathogenic bacteria, specifically A. hydrophila, A. salmonicida, E. ictaluri, P. aeruginosa, E. tarda, and V. alginolyticus, using the agar diffusion method with 6 mm paper discs. To initiate the test, 1 mL of pathogenic bacteria inoculum (with an optical density between 0.08-0.1 at 600nm) was mixed with 15 mL of liquid agar nutrient medium. The resulting mixture was then heated to 50°C, thoroughly mixed, and poured into a petri dish. After the medium solidified with the pathogenic bacteria culture, Oxytetracycline antibiotic paper was used as a positive control, and a paper disc soaked in 30 ml of methanol was used as a negative control. The M. calabura extract was prepared, and different concentrations (100, 200, 300, 400, and 500 mg mL-1) were achieved by diluting it with methanol. Subsequently, 30 μL of each concentration was placed on paper plates and incubated at 30°C for 24 hours. The inhibitory activity of the M. calabura extract was evaluated based on the size of the clear zone that formed around each disc.

2.4 Data Analysis

The data collected was presented in a table and subjected to descriptive analysis by evaluating the size of the clear zones formed as an indicator of antibacterial effectiveness.

3 Results and Discussion

3.1 Phytochemical test and functional groups

The phytochemical test was conducted to detect the presence of active compounds within the M. calabura leaf extract. This test aimed to ascertain the primary group of active compounds responsible for its antimicrobial properties. The identification of compound groups in the M. calabura extract was achieved by observing changes in the extract's color following the addition of specific reagents.

When M. calabura extract was subjected to Mayer and Dragendroff reagents, there were no observable color changes or sediment formation. In contrast, when the sample containing alkaloids reacted with these standard reagents, it exhibited a transformation into white or orange, as reported in reference [27]. The phytochemical test involving M. calabura extract aimed to identify the presence of active compounds within the leaf extract and determine the primary group of active compounds responsible for its antimicrobial properties. The identification of compound groups within the M. calabura extract was based on the color change observed in the sample, which turned pink. This change in color to pink occurred due to the formation of benzopyrylium salt, resulting in a red hue, attributed to the reduction of polyhydroxy groups from flavanols by the presence of magnesium in hydrochloric acid. [28].

The presence of phenolic compounds in the M. calabura extract was confirmed by observing a color change to blue-black upon reacting with FeCl3. This color change is a result of the reduction process involving phosphomolybdic acid and phosphotungstate with the reagent, forming a blue color. The intensity of the blue color correlates with the concentration of phenolic compounds, with higher phenolic content leading to a darker blue coloration. This phenomenon was attributed to the reduction of heteropoly acid (phosphomolybdic phosphotungstate) by phenolic ions, resulting in the formation of a molybdenum-tungsten complex that intensified the color [29]. The development of the blue-black color was associated with the formation of sugar bonds in the sample.

When M. calabura extract was combined with HCl, it resulted in the production of foam. The presence of saponin in the sample was confirmed if the foam remained stable for approximately 5 minutes after the reaction with the reagent. In other words, if the foam persisted for about 5 minutes after the reaction with the reagent, it indicated a positive presence of saponin in the sample [30]. The foam generated in the sample following the addition of 1 drop of 1N HCl ranged in height from 1 to 5 cm.

The M. calabura extract, when subjected to Lieber-Burchard reagent, underwent a color transformation to red. This alteration in color to red was a result of acid oxidation facilitated by sulfuric acid. In this process, electrons from the hydrogen group were dissociated, leading to the extension of the compound's conjugation, which was visually represented by the red hue [31]. The transition in color to red observed in the sample was attributed to the presence of terpenoids, which are derivatives resulting from dehydrogenation and oxygenation of terpene compounds. Terpenes are hydrocarbon groups frequently synthesized by plants. The results of the FT-IR characterization of M. calabura extract contained O-H alcohol, C=O hydroxyl, C=C aliphatic, C=C aromatic and C-O alcohol groups.

3.2 Inhibitory Activity

Inhibition test results showed that M. calabura extract has the ability to inhibit the growth of A. hydrophila. bacteria by 8.1 mm to 12.1 mm. On A. salmonicida by 9.2 mm to 10.9 mm. On E. ictaluri by 8.8 mm to 11.5 mm. On P. aeruginosa by 8.1 mm to 12.1 mm. On E. tarda by 9.6 mm to 11.4 mm. On V. alginolyticus by 8.2 mm to 12.2 mm (Table 1). The inhibitory ability of M. calabura extract is classified as medium to strong.
The most significant outcome of the M. calabura extract's inhibitory activity was observed against the V. alginolyticus bacterium. Variations in the diameter of the inhibition zone could be attributed to the pathogenic bacteria's ability to resist the antibacterial compounds present in the M. calabura extract. The effectiveness of an antibacterial compound in restraining microorganism growth depended on both its concentration and the specific type of antimicrobial substance it produced. As the concentration of the extract increased, so did the quantity of active substances within it. Consequently, the effectiveness in inhibiting bacteria increased, resulting in a broader transparent region [32]. The formation of an inhibition zone on the pathogen culture medium shows that the M. calabura extract contains antibacterial compounds such as flavonoids, phenolic compounds, saponins and terpenoids.

Flavonoids exhibited the capacity to create intricate compounds possessing attributes capable of causing harm to bacterial cell membranes. These compounds engaged with extracellular proteins in the bacterial structure, leading to the release of intracellular components. Furthermore, flavonoids were proficient in channeling transduction energy towards the cytoplasm of bacteria, thus impeding bacterial mobility [33]. Flavonoid compounds hinder the growth of bacteria by attaching to adhesins, causing harm to membranes and cell walls, and deactivating enzymes. The structural components thought to be involved in the antibacterial activity were the beta rings and OH groups in flavonoids. Flavonoids were capable of halting nucleic acid synthesis and disrupting cytoplasmic membrane function by interfering with porin activity, formation. biofilm permeability, and interacting with various essential enzymes [34].

Table 1. Inhibition activity of M. calabura extract against pathogen bacteria

M. calabura Extract concentration (mg mL^{-1})	Inhibition zone diameter (mm)					
	A. hydrophila	A. salmonicida	E. ictaluri	P. aeruginosa	E. tarda	V. alginolyticus
10%	8.1±0.1	9.2±0.4	8.8±0.1	8.1±0.1	9.6±0.1	8.2±0.2
20%	9.2±0.2	9.2±0.1	9.7±0.2	9.2±0.2	10.1±0.4	9.1±0.1
30%	10.3±0.4	9.6±0.1	10.3±0.4	10.3±0.4	10.9±0.2	10.2±0.3
40%	11.2±0.3	10.3±0.1	10.9±0.6	11.2±0.3	11.2±0.1	11.1±0.1
50%	12.1±0.1	10.9±0.4	11.5±01	12.1±0.1	11.4±0.4	12.2±0.2

Phenolic compounds possess an aromatic ring with one or two hydroxyl groups and exhibit potent antimicrobial properties. Phenolics function by denaturing proteins and inhibiting bacterial ingestion. They also disrupt bacterial cell membranes by dissolving the fats in the cell walls [35]. Phenolic induce the release of bacterial flagella, inflict damage to the structure of cell walls and cell membranes, and result in the leakage of macromolecules from within the cells [36].

The mechanism by which saponin compounds act as antibacterials involves the reduction of cell wall surface tension. Saponins bind to the lipopolysaccharides in the

bacterial cell wall, which leads to an increase in cell wall permeability and a decrease in cell wall surface tension [37].

. Saponins possess both antibacterial and antifungal characteristics. Their antibacterial effect is achieved by disrupting membrane permeability, leading to an increase in permeability that results in the leakage of specific proteins and enzymes from bacterial cells [38]. When saponins interact with membrane sterols, they can damage cell wall structures, leading to the release of vital components from within bacterial cells.

Terpenoid compounds were believed to act as antibacterial agents by causing membrane damage through their lipophilic properties [39]. Terpenoids could interact with porin, which is a transmembrane protein located in the outer wall of bacterial cell membranes. This interaction led to the formation of robust polymer bonds that damaged the porin. As a consequence, the permeability of the bacterial cell wall was significantly reduced, causing bacterial cells to become deprived of nutrients and ultimately inhibiting or completely halting bacterial growth.

4 Conclusion

The M. calabura leaf extract tested positive for the presence of flavonoids, saponins phenolics, and terpenoids. Furthermore, the M. calabura extract demonstrated medium to strong inhibitory effects on the growth of bacteria such as A. salmonicida, P. aeruginosa, E. ictaluri, A. hydrophila, E. tarda, and V. alginolyticus. M. calabura leaf extract has the potential to be used as an antibacterial agent.

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