The Activity of *Melicope ptelefolia* Leaf Extract Against Pathogenic Bacteria in Fish

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Abstract. The application of antibiotics to treat bacterial infections poses risks to fish and the ecosystem, highlighting the need for safe, natural alternatives for fish disease treatment. This research focuses on evaluating the antibacterial properties of *Melicope ptelefolia* extract against various pathogens, including *Aeromonas salmonicida*, Edwarsiella *tarda, Edwarsiella ictaluri*, Aeromonas hydrophila, and Pseudomonas aeruginosa. The extract underwent phytochemical analysis, which confirmed the presence of phenolic compounds, flavonoids, saponins, and terpenoids. Antibacterial effectiveness was assessed using the agar diffusion method at 100, 200, 300, 400, and 500 mg/mL concentrations. The results showed that *M. ptelefolia* extract significantly inhibited the growth of A. salmonicida (13.5 mm to 14.8 mm), E. tarda (12.0 mm to 13.1 mm), E. ictaluri (13.9 mm to 15.8 mm), A. hydrophila (13.5 mm to 14.1 mm), and P. aeruginosa (13.1 mm to 14.0 mm), compared to a control (+) group with inhibition zones of 25.5 mm to 26.0 mm. Overall, the extract exhibited strong antibacterial activity against these pathogenic bacteria, indicating its potential as a natural antibacterial agent for fish.

1 Introduction

Aquaculture is one of the sector with significant potential for improving the economy of society. The potential of aquatic resources for fish farming development in our country is still quite broad. It is expected to contribute as a source of protein, especially as wild fish catch production declines. Technological advancements in aquaculture are progressing rapidly, greatly assisting fish farmers in increasing production through intensive systems, leading to substantial profits.

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The success of fish farming largely depends on disease control. Fish diseases can cause direct and indirect disruptions, leading to fish mortality. Generally, fish diseases are caused by pathogenic microorganisms, mainly bacteria, such as *Aeromonas salmonicida*, *Edwardsiella tarda, Edwardsiella ictaluri, Aeromonas hydrophila* and *Pseudomonas aeruginosa*.

Pathogenic bacteria are types of bacteria that can cause diseases in other organisms. Bacterial diseases are a serious issue in fish farming. *Aeromonas salmonicida* bacteria are known to cause diseases in various types of fish [1], particularly in salmon [2]. Infections by this bacterium can lead to bleeding, ulcers, and damage to blood vessels, ultimately resulting in fish death [3]. *Pseudomonas aeruginosa* bacteria causes various diseases in cultured fish [4]. It can lead to gill necrosis, damage to blood vessels, injury to the abdominal cavity, and damage to the kidneys and liver of the fish [5]. Meanwhile, *Edwardsiella ictaluri* bacteria is a pathogen that causes various diseases in catfish [6], including Enteric Septicemia of Catfish (ESC) [7]. This bacterium can infect its host through the nose, gastrointestinal tract, and gills, and then spread to other organs through acute bacteremia [8].

Aeromonas hydrophila bacteria, a Gram-negative pathogenic bacterium, can cause Motile Aeromonas Septicemia (MAS) [9]. Infections by *A. hydrophila* lead to bleeding in the fish's body, particularly in the chest, fins, and abdomen [10]. *E. tarda* bacteria is a pathogen that affects freshwater and marine fish, causing significant losses in aquaculture [11], including in the cultivation of *Paralichthys olivaceus* [12]. This bacterium causes severe diseases in fish, often leading to high mortality rates.

The use of synthetic antibiotics is commonly applied to combat bacterial infections in fish. Still, excessive use can lead to bacterial resistance [13] and pose risks to fish, especially those consumed by humans [14]. Escherichia coli bacteria isolated from the digestive organs of catfish showed high resistance levels towards tetracycline, ampicillin, and chloramphenicol [15]. Over the past few decades, the antibiotic group Fluoroquinolones (FQ) has been detected in aquatic ecosystems and has become a major pollutant in the environment. These antibiotics can cause ecotoxicological effects on various species of animals and plants [16] As an alternative, natural compounds from plants, such as *Melicope ptelefolia* (tenggek burung), which possess antibacterial properties, can be used to treat fish diseases. Various plants growing around us can be utilized to inhibit the growth of pathogenic bacteria [17].

One of the plants suspected to have pharmacological benefits is *M. ptelefolia*, which belongs to the Rutaceae family. *M. ptelefolia* contains antioxidants, phenolic compounds, and flavonoids [18]. In Indonesia, particularly in Riau, *M. ptelefolia* is consumed as a salad. This plant can also be found in Malaysia, China, and Vietnam [19-20]. *M. ptelefolia* is a plant commonly found in the forests of Riau. It is a herbaceous plant belonging to the Rutaceae family, typically a shrub or small tree, and can grow to a height of 1-14 meters. The main branches have a diameter of 2.5-4 mm at the third node, and the leaves are oval or elliptical, with a width that varies from 6-22 mm at the terminal [21, 19].

The leaves of *M. ptelefolia* contain flavonoids, alkaloids, and saponins, which function as antibacterial agents. The presence of these secondary metabolites indicates that the leaves of *M. ptelefolia* play a significant medical role [22]. The phytochemicals present in *M. ptelefolia* include phenolic compounds, flavonoids, tannins, alkaloids, and saponins [23]. Specific chemical compounds found in *M. ptelefolia* include three selected candidates: kaempferol 3-robinobioside, kaempferol 3-o-β-D-glucopyranosyl (1-2)-α-D-xylopyranoside, and tamarixetin 3-robinobioside, which have been shown to exhibit cytopathic effects induced by the H1N1 virus [24]. This study aims to investigate the antibacterial activity of *M. ptelefolia* leaf extracts against pathogenic bacteria in fish.

2 Material and Method

2.1 Extraction of *M. ptelefolia* leaf

M. ptelefolia leaves were collected from Desa Teluk Mesjid, Sungai Apit Siak, Riau Indonesia. The leaves were then dried in an oven at 40°C for 2 days. After drying, they were finely ground using a blender. The resulting *M. ptelefolia* powder underwent maceration in 90% ethanol for 3 days, because ethanol is a universal solvent that can extract compounds soluble in non-polar and polar solvents. The filtrate was then concentrated by evaporation using a rotary evaporator set at 50°C with a speed of 50 rpm, resulting in the final extract.

2.2 Phytochemical test

A chemical analysis of the *M. ptelefolia* extract was conducted to detect various compounds, such as phenolics, alkaloids, terpenoids, saponins, and flavonoids. In the phenolic test, 10 mg of the extract was combined with 500 µL of 5% FeCl3, and a color change to blue indicated a positive result. For alkaloids, 250 µL of Mayer's reagent was added to 10 mg of the extract, forming a white precipitate. The addition of Dragendorff's reagent produced an orange color, confirming the presence of alkaloids. The terpenoid test involved mixing 10 mg of the extract with 10 drops of acetic acid and 3 drops of sulfuric acid, which yielded a red color, indicating a positive reaction. To detect saponins, 10 mg of the extract was agitated with 5 mL of distilled water for one minute, followed by adding 150 µL of 1N hydrochloric acid and shaking again for another minute; foam formation signified a positive result. Lastly, in the flavonoid test, mixing 0.05 g of magnesium with 10 drops of 37% hydrochloric acid and 10 mg of *M. ptelefolia* extract resulted in a red color change, confirming the presence of flavonoids.

2.3 Inhibitory activity of *M. ptelefolia* extract

The *M. ptelefolia* extract from the study was evaluated for its antibacterial effects against pathogens such as *A. salmonicida, E. tarda, E. ictaluri*, *A. hydrophila*, and *P. aeruginosa* using the agar diffusion method with 6 mm paper discs. Initially, 1 mL of the bacterial inoculum (with an optical density between 0.08-0.1 at 600 nm) was combined with 15 mL of liquid nutrient agar. This mixture was heated to 50°C, mixed well, and poured into a petri dish. After the medium solidified, Oxytetracycline antibiotic paper was utilized as a positive control. In contrast, a paper disc soaked in 30 mL of methanol served as a negative control due to Oxytetracycline's broad-spectrum efficacy. The *M. ptelefolia* extract was diluted with methanol to create 100, 200, 300, 400, and 500 mg/mL concentrations. A volume of 30 µL from each concentration was placed on the paper discs, which were then incubated at 30°C for 24 hours. The antibacterial activity of the *M. ptelefolia* extract was determined by measuring the diameter of the clear inhibition zone surrounding each disc.

2.4 Data Analysis

The collected data was organized into a table and analyzed statistics (one-way analysis of variance) by assessing the diameter of the clear zones formed, which served as indicators of the antibacterial effectiveness of the *M. ptelefolia* extract. The size of these inhibition zones reflects the ability of the extract to prevent bacterial growth, with larger zones indicating greater antibacterial activity. By presenting the data in a table format, comparing the

effectiveness across different bacterial strains and extract concentrations becomes easier, providing a clear visual representation of the results. This method of analysis highlights the extract's potency and allows for a straightforward interpretation of its potential as a natural antibacterial agent.

3 Results and Discussion

3.1 Phytochemical test and functional groups

Phytochemical analysis was carried out on M. ptelefolia leaf extract to identify the active compounds responsible for the antimicrobial effects observed in *M. ptelefolia* leaf extract. This identification process was carried out by monitoring changes in the extract's color upon adding specific reagents. The phytochemical testing of the *M. ptelefolia* extract was designed to confirm these active compounds' presence and determine the specific group of compounds that contribute to its antimicrobial properties.

Phenolic compounds were confirmed in the *M. ptelefolia* extract by the appearance of a blue-black color when mixed with iron chloride (FeCl3) [25]. The blue-black color results from a chemical reaction where phenolic compounds reduce phosphomolybdic and phosphotungstic acids, creating a blue complex. The intensity of this color indicates the amount of phenols present [25]. Phenolic ions reduce the heteropoly acid, forming a molybdenum-tungsten complex that causes the blue color. The presence of sugars in the sample may also contribute to the color change [25]. Tests with Mayer and Dragendorff reagents showed no signs of alkaloids in the *M. ptelefolia* extract. Alkaloids are usually detected by color changes or precipitates, as reported in previous studies [26]

The categorization of compound groups present in the *M. ptelefolia* extract relied on the alteration in color observed in the sample, which transformed into a pink hue. This shift in color to pink was a consequence of the formation of benzopyrylium salt, leading to a red tint. This red coloration was attributed to the reduction of polyhydroxy groups from flavanols induced by the presence of magnesium in hydrochloric acid [27].

Finally, when the *M. ptelefolia* extract was mixed with HCl, foam formation was observed. The persistence of foam for approximately 5 minutes after the reaction confirmed the presence of saponins in the sample. The height of the foam produced, which ranged from 1 to 5 cm after adding 1 drop of 1N HCl, further validated the presence of saponins [28].

The *M. ptelefolia* extract turned red when treated with the Lieber-Burchard reagent, a reaction indicative of the presence of terpenoids. This color shift to red is due to acid oxidation facilitated by sulfuric acid, where the dissociation of hydrogen group electrons within the compound leads to an expansion of the compound's conjugation, visually manifesting as a red color [29]. The presence of terpenoids in the sample, which are derivatives formed through the dehydrogenation and oxygenation of terpene compounds, accounts for this reaction. Terpenes are hydrocarbon groups commonly synthesized by plants.

3.2 Inhibitory Activity

The results of the inhibition test showed that *M. ptelefolia* extract was able to inhibit the growth of *A. salmonicida* by 13.5 mm to 14.8 mm. On *E. tarda* by 12.0 mm to 13.1 mm. On *E. ictaluri* by 13.9 mm to 15.8 mm. On *A. hydrophila* 13.5 mm to 14.1 mm. On *P. aeruginosa* by 13.1 mm to 14.0 mm (Table 1). The inhibitory ability of *M. ptelefolia* extract on pathogenic bacteria is classified as strong. The results of the inhibitory effect of M.

ptelefolia leaf extract on pathogenic bacteria (12.0 mm - 15.8 mm) are higher compared to the inhibitory effect of Muntingia calabura leaf extract (8.1 mm - 14.2 mm) [30]

Table 1. Inhibition activity of *M. ptelefolia* extract against pathogen bacteria

M. ptelefolia extrac concentration ($mg.mL^{-1}$)	Inhibition zone diameter (mm)				
	Aeromonas salmonicida	*Edwarsiella tarda*	*Edwarsiella ictaluri*	*Aeromonas hydrophila*	*Pseudomonas aeruginosa*
100	13.5 ± 0.8^{a}	12.0 ± 0.8^{a}	13.9 ± 1.4^{a}	13.5 ± 1.6^{a}	13.1 ± 0.3^{c}
200	14.0 ± 1.3^{a}	12.3 ± 1.0^{a}	14.1 ± 1.4^{a}	13.7 ± 1.5^{a}	13.4 ± 0.1^{bc}
300	14.1 ± 1.3^{a}	12.7 ± 0.7^{a}	14.8 ± 2.1^{a}	13.8 ± 1.6^{a}	13.5 ± 0.0^{b}
400	14.4 ± 1.7^{a}	12.9 ± 0.4^{a}	14.9 ± 2.1^{a}	13.9 ± 1.4^{a}	13.8 ± 0.1^{ab}
500	14.8 ± 1.9^{a}	13.1 ± 0.4^{a}	15.8 ± 1.4^{a}	14.1 ± 1.5^{a}	14.0 ± 0.0^{a}

Note: Mean values with different superscripts in the same columns were significantly different ($p < 0.05$)

The most notable finding from evaluating the *M. ptelefolia* extract's inhibitory activity was its pronounced effect against the *E. ictaluri* bacterium. The differences in the sizes of the inhibition zones could be linked to the ability of the pathogenic bacteria to withstand the antibacterial compounds present in the extract. The efficacy of an antibacterial agent in curbing microbial growth is affected by its concentration and the specific types of antimicrobial compounds it contains. As the concentration of the extract increased, so did the quantity of active substances, which enhanced its antibacterial effectiveness, resulting in larger inhibition zones [31]. These inhibition zones on the culture medium with the pathogenic bacteria indicate that the *M. ptelefolia* extract contains antibacterial compounds such as phenolics, flavonoids, saponins, and terpenoids.

Phenolic compounds, defined by their aromatic rings containing one or two hydroxyl groups, demonstrate significant antimicrobial properties. They operate by modifying protein structures and preventing bacterial nutrient absorption. Additionally, phenolics disrupt bacterial cell membranes by dissolving the lipids in the cell walls [32]. These compounds reduce the permeability of the cytoplasmic membrane system, obstructing the transport of vital organic ions into the cells and ultimately resulting in bacterial cell death. Beyond their antimicrobial effects, phenolic compounds exhibit various biological functions, including anti-carcinogenic, anti-inflammatory, and antioxidant properties. Numerous studies have shown the ability of phenolic compounds to inhibit a wide array of pathogenic bacteria [33]. They induce the release of bacterial flagella, damage cell walls and membrane structures, and lead to the leakage of macromolecules from the cells [34].

Terpenoid compounds are thought to function as antibacterial agents by inducing membrane damage due to their lipophilic nature [35]. They can interact with porins, transmembrane proteins found in the outer layer of bacterial cell membranes, creating strong polymer bonds that compromise the porins. This interaction significantly reduces the permeability of the bacterial cell wall, depriving the cells of essential nutrients and ultimately inhibiting or stopping their growth. The terpenoids in this extract display antibacterial properties by inhibiting lipid production and modifying cell membrane structure, mainly by interfering with ergosterol synthesis [36]. Their antimicrobial effectiveness is primarily attributed to their lipophilic characteristics, which enable terpenoids to break down and disrupt the lipid components of bacterial plasma membranes. This leads to imbalances in cell membrane permeability and disturbances in ion homeostasis within the bacterial cells [37].

Saponins possess antibacterial and antifungal properties, with their antibacterial activity achieved by disrupting membrane permeability. This disruption causes an increase in permeability, leading to the leakage of specific proteins and enzymes from bacterial cells

[38]. When saponins interact with sterol membranes, they can damage the cell wall structure, releasing essential components from within the bacterial cells. The antibacterial mechanism of saponins involves reducing the surface tension of cell walls. By binding to lipopolysaccharides in the bacterial cell wall, saponins increase cell wall permeability and decrease surface tension, making the cell wall more susceptible to breakage or lysis [36]. This allows antibacterial substances to penetrate the cell more easily, disrupting its metabolism and leading to bacterial death [39].

Flavonoids have been shown to form complex compounds that can damage bacterial cell membranes. These compounds interact with extracellular proteins within the bacterial structure, causing the release of intracellular components. Additionally, flavonoids effectively redirect energy transfer within bacterial cells, inhibiting bacterial mobility [40]. Flavonoid compounds inhibit bacterial growth by binding to adhesins, damaging membranes and cell walls, and deactivating enzymes. The structural elements believed to be involved in their antibacterial action are the beta rings and hydroxyl groups within flavonoids. Flavonoids can inhibit nucleic acid synthesis, disrupt cytoplasmic membrane functions, interfere with biofilm formation, impact porin activity, and permeability, and interact with various essential enzymes [41]. When acting as antibacterial agents, flavonoid compounds employ several mechanisms to disrupt bacterial functions, including damaging cell walls and interfering with metabolic processes. These mechanisms include three primary actions: inhibiting nucleic acid synthesis, disrupting cytoplasmic membrane function, and interfering with energy metabolism [42]. Furthermore, flavonoid compounds also damage the permeability of bacterial cell walls.

4 Conclusion

The *M. ptelefolia* leaf extract was confirmed to contain phenolics, flavonoids, saponins and terpenoids. Additionally, the extract exhibited significant inhibitory effects on the growth of bacteria including *A. salmonicida*, *E. tarda, E. ictaluri*, *A. hydrophila* and *P. aeruginosa*. For the future research will focus on the use of Melicope ptelefolia leaf extract for the treatment of bacterial diseases in fish.

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