Antibacterial efficacy of *Clidemia hirta* leaf extract against pathogenic fish bacteria

Jarod Setiaji[1*], *Valentio* Febrian Prakoso[1], *Heriyanto*[2], *Hisra* Melati[1], *Handika* Azhari[1], *Anugrah* Nur Ramadhan[1], and *Saproni* Muhammad Samin[1]

[1]Department of Aquaculture, Faculty of Agriculture, Universitas Islam Riau, Pekanbaru 28284, Indonesia.
[2]Department of Agribusiness, Faculty of Agriculture, Universitas Islam Riau, Pekanbaru 28284, Indonesia.

Abstract. Bacterial diseases pose a serious threat to aquatic ecosystems, and the use of antibiotics to address these problems can have harmful effects on both fish and their environment. Therefore, there is an increasing demand for safe natural alternatives to treat fish diseases. This research investigates the antibacterial properties of an extract from *Clidemia hirta* against various bacterial strains, including *A. salmonicida, P. aeruginosa, E. ictaluri, A. hydrophila, V. alginolyticus*, and *E. tarda*. Phytochemical analysis of the extract identified the presence of phenolic compounds, terpenoids, saponins, and flavonoids. The antibacterial effectiveness of the *C. hirta* extract was assessed using the agar diffusion method at concentrations of 50, 100, 150, 200, and 250 mg/mL. The findings indicated that the extract significantly inhibited the growth of *A. salmonicida* (16.5 mm to 23.3 mm), *P. aeruginosa* (17.3 mm to 23.5 mm), *E. ictaluri* (15.3 mm to 23.5 mm), *A. hydrophila* (15.3 mm to 21.8 mm), *V. alginolyticus* (16.8 mm to 22.8 mm), and *E. tarda* (15.0 mm to 22.8 mm). In summary, the extract from *C. hirta* shows potential as a natural agent to control pathogenic bacteria affecting fish.

1. Introduction

Aquaculture is one of 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 and is expected to contribute as a source of protein, especially as wild fish catch production declines. Technological advancements in aquaculture are progressing rapidly, which greatly assists fish farmers in increasing production through intensive systems, leading to substantial profits.

The success of fish farming largely depends on disease control. Fish diseases can cause disruptions, both directly and indirectly, leading to fish mortality. Generally, fish diseases are caused by pathogenic microorganisms, particularly bacteria, such as *Aeromonas salmonicida*, *Pseudomonas aeruginosa*, *Edwardsiella ictaluri*, *A. hydrophila*, *Vibrio alginolyticus*, and *E. tarda*.

[*] Corresponding author: jr.setiaji@agr.uir.ac.id

Bacteria that can induce diseases in other organisms are referred to as pathogenic bacteria. These bacterial infections represent a significant challenge in aquaculture. For instance, *A. salmonicida* is known to inflict diseases on multiple fish species, particularly salmon [1][2]. Infections caused by this bacterium can result in symptoms such as bleeding, ulcers, and damage to blood vessels, which can ultimately lead to the death of the fish [3]. Similarly, *Pseudomonas aeruginosa* is responsible for a variety of diseases in cultured fish [4], causing conditions like gill necrosis, blood vessel damage, and injuries to the abdominal cavity, as well as harm to the kidneys and liver [5]. Additionally, *E. ictaluri* is a pathogen that affects catfish by causing several diseases, including Enteric Septicemia of Catfish (ESC) [6][7]. This bacterium can invade its host through the nose, gastrointestinal tract, and gills, subsequently disseminating to other organs via 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 areas of the chest, fins, and abdomen [10]. *Vibrio alginolyticus* bacteria is an opportunistic bacterium and a pathogen that poses a global threat to aquaculture development [11]. In fish, this bacterium can cause several diseases, including ulcers, septicemia, and blindness [12]. *E. tarda* bacteria is known as a pathogen that affects both freshwater and marine fish, causing significant losses in aquaculture [13], including in the cultivation of *Paralichthys olivaceus* [14]. 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, but excessive use can lead to bacterial resistance [15] and pose risks to fish, especially those consumed by humans [16]. As an alternative, natural compounds from plants, such as *C. hirta* (In Indonesia: senduduk bulu), 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].

C. hirta, which belongs to the Melastomataceae family, is commonly found in forest edges, in shrubs, and on cliff sides. This plant originates from South America and has been naturalized in several regions, including Sri Lanka, India, Australia, East Africa, and South Asia [18]. Plants in the Melastomataceae family typically grow in tropical areas and comprise 163 genera and 4,300 species. These plants are known to be rich in phenolic compounds and flavonoids. Phytochemicals in the Melastomataceae family include phenolics, flavonoids, tannins, terpenoids, saponins, steroids, glycosides, and alkaloids. Some species within this family exhibit antioxidant, antihypertensive, antihyperglycemic, homeostatic, antihepatitis, and antidiarrheal activities [19].

In Indonesia, *C. hirta* is known to contain chemicals such as tannins (catechols and pyrogallotannins), dioxanthraquinones, steroids, saponins, glycosides, and phenols, which function as antimicrobials [20]. Ethanol extracts of *C. hirta* leaves exhibit antioxidant activity with an IC_{50} value of 5 μg/mL using the DPPH method [21]. The plant also contains flavonoids, phenolics, and terpenoids, which are known for their antioxidant properties [22]. Additionally, *C. hirta* extracts are highly effective in inhibiting the growth of *Candida albicans* [23].

The leaves of *C. hirta* contain secondary metabolites with potential antimicrobial properties [24]. Secondary metabolites are compounds produced by plants, microbes, or animals through biosynthesis that support life, though they are not as essential as sugars, amino acids, and fatty acids. These compounds possess significant pharmacological and biological activities and are used as drug candidates or lead compounds for optimization into more potent substances with minimal toxicity. The secondary metabolites in *C. hirta* include flavonoids, phenolics, and saponins, which exhibit biological activity [25]. Additionally, *C. hirta* can be used as an environmentally friendly pesticide, effectively inhibiting the growth of the weed *Praxelis clematidea*, which can be problematic for crops

[26]. This study aims to investigate the antibacterial activity of *C. hirta* leaf extracts against pathogenic bacteria in fish.

2 Material and Method

2.1 Extraction of *C. hirta* leaf

The leaves of *C. hirta* were obtained from Desa Tungkurejo Ujung Batu Timur, Rokan Hulu, Indonesia. The leaves were dried in an oven at 40 °C for two days, then finely ground using a blender. The resulting *C. hirta* powder was macerated in 90% ethanol for three days. The filtrate was concentrated by evaporation using a rotary evaporator set to 50 °C at a speed of 50 rpm, yielding the final extract

2.2 Phytochemical test and FT-IR

A chemical analysis was carried out on the *C. hirta* extract to identify various bioactive compounds, such as phenolics, alkaloids, terpenoids, saponins, and flavonoids. For detecting phenolics, 10 mg of *C. hirta* extract was combined with 500 µL of 5% $FeCl_3$, and a change to a blue color confirmed the presence of phenolics. To test for alkaloids, 250 µL of Mayer's reagent was added to 10 mg of the extract, resulting in a white precipitate. Additionally, the introduction of Dragendorff's reagent caused the solution to turn orange, indicating the presence of alkaloids. For the terpenoid test, 10 mg of the extract was mixed with 10 drops of CH_3COOH and 3 drops of H_2SO_4, and the red color confirmed a positive result for terpenoids. Saponin presence was identified by shaking 10 mg of the extract with 5 mL of distilled water for one minute, followed by adding 150 µL of 1N HCl and shaking again, with the formation of foam indicating the presence of saponins. To detect flavonoids, 0.05 g of magnesium and 10 drops of 37% HCl were added to 10 mg of the extract, and a red color confirmed the presence of flavonoids.

To determine the functional group present in the *C. hirta* extract, Fourier-transform infrared (FT-IR) spectroscopy was used. Each sample was ground and mixed with KBr, and the infrared absorbance was measured across a wavelength range of 4500–450 cm^{-1} to identify the specific functional groups.

2.3 Inhibitory activity of *C. hirta* extract

The *C. hirta* extract obtained from the study was evaluated for its antibacterial properties against several pathogenic bacteria, including *A. salmonicida*, *P. aeruginosa*, *E. ictaluri*, *A. hydrophila*, *V. alginolyticus*, and *E. tarda*, using the agar diffusion technique with 6 mm paper discs. To begin the test, 1 mL of bacterial inoculum (with an optical density ranging between 0.08 and 0.1 at 600 nm) was mixed with 15 mL of liquid nutrient agar. This mixture was heated to 50 °C, thoroughly combined, and then poured into petri dishes. Once the medium solidified with the bacterial culture, Oxytetracycline antibiotic paper discs were utilized as the positive control, while a paper disc soaked in 30 mL of methanol acted as the negative control. The *C. hirta* extract was diluted with methanol to produce varying concentrations (50, 100, 150, 200, and 250 mg/mL). Subsequently, 30 µL of each concentration was placed on the paper discs, which were then incubated at 30 °C for 24 hours. The antibacterial activity of the extract was determined by measuring the diameter of the clear inhibition zones formed around each disc.

2.4 Data Analysis

The collected data was organized into a table and analyzed descriptively by assessing the diameter of the clear zones formed, which served as indicators of the antibacterial effectiveness of the *C. hirta* 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, it becomes easier to compare the effectiveness across different bacterial strains and extract concentrations, providing a clear visual representation of the results. This method of analysis not only highlights the potency of the extract but also allows for a straight forward interpretation of its potential as a natural antibacterial agent.

3 Results and Discussion

3.1 Phytochemical test and functional groups

A phytochemical analysis was conducted on the *C. hirta* leaf extract to detect the presence of key active compounds responsible for its antimicrobial effects [27]. This analysis aimed to identify specific categories of compounds by observing changes in the extract's color when reacting with particular reagents. The test confirmed the presence of phenolic compounds, as indicated by a colour change to blue-black upon reacting with $FeCl_3$. This reaction occurs due to the reduction of phosphomolybdic acid and phosphotungstate, forming a blue complex, with the intensity of the colour directly linked to the concentration of phenolic compounds. The blue-black hue also suggested the formation of bonds with sugars in the sample [28].

When Mayer and Dragendorff reagents were applied, no significant colour change or precipitate formation occurred, indicating the absence of alkaloids. In contrast, alkaloids typically cause a white or orange colour change in the presence of these reagents, as noted in prior studies [29].

The extract turned red when treated with the Lieber-Burchard reagent, a reaction characteristic of terpenoids. This colour change is due to acid oxidation facilitated by sulfuric acid, which causes the dissociation of hydrogen electrons and expands the compound’s conjugation, manifesting as red. The terpenoids detected are derivatives of dehydrogenation and oxygenation processes within terpene compounds [30].

The formation of foam, observed when the extract was mixed with HCl, confirmed the presence of saponins. The foam persisted for about 5 minutes, and its height (ranging from 1 to 5 cm) after adding 1 drop of 1N HCl further validated the presence of these compounds [31].

The colour change to pink in the sample, caused by the formation of benzopyrylium salt, indicated the presence of flavonoids. This red tint was attributed to the reduction of polyhydroxy groups in flavanols, triggered by magnesium in hydrochloric acid [32].

FT-IR analysis of the *C. hirta* leaf extract revealed the presence of functional groups such as O-H (alcohol), C-H (aliphatic), C=O (ester), C=C (alkene), and C-O. These functional groups, particularly hydroxyl (O-H), contributed to the antibacterial activity by facilitating bacterial cell death [33].

3.2 Inhibitory Activity

The inhibition test results demonstrated that *C. hirta* extract effectively inhibited the growth of *A. salmonicida* with inhibition zones ranging from 16.5 mm to 23.3 mm, *P. aeruginosa* from 17.3 mm to 23.5 mm, *E. ictaluri* from 15.3 mm to 23.3 mm, *A. hydrophila* from 15.3 mm to 21.8 mm, *V. alginolyticus* from 16.8 mm to 22.8 mm, and *E. tarda* from 15.0 mm to 22.8 mm (Table 1). The inhibitory activity of *C. hirta* extract against these pathogenic bacteria was classified as strong to very strong.

Table 1. Inhibition activity of *C. hirta* extract against pathogen bacteria

C. hirta **extract concentration (mg.mL^{-1})**	**Inhibition zone diameter (mm)**					
	A. salmonicida	***P. aeruginosa***	***E. ictaluri***	***A. hydrophila***	***V. alginolyticus***	***E. tarda***
50	16.5 ± 0.7	17.3 ± 0.4	15.3 ± 0.4	15.3 ± 0.4	16.8 ± 0.4	15.0 ± 0.7
100	19.8 ± 1.1	18.3 ± 1.1	19.3 ± 0.4	17.5 ± 0.7	18.0 ± 0.7	17.8 ± 0.4
150	21.3 ± 0.4	20.8 ± 0.4	20.5 ± 0.7	18.8 ± 0.4	20.0 ± 0.7	20.3 ± 0.4
200	22.8 ± 0.4	21.5 ± 0.7	21.8 ± 0.4	20.3 ± 1.1	21.3 ± 0.4	21.3 ± 0.4
250	23.3 ± 0.4	23.5 ± 0.7	23.3 ± 0.4	21.8 ± 1.1	22.8 ± 1.1	22.8 ± 0.4

The most significant finding from the inhibition tests of *C. hirta* extract was its potent effect on *P. aeruginosa* bacteria at the highest concentration. Differences in the size of the inhibition zones were likely due to the resistance mechanisms of the pathogenic bacteria against the antibacterial compounds found in the *C. hirta* extract. The effectiveness of an antibacterial substance in limiting microbial growth is influenced by both the concentration of the extract and the type of antimicrobial compounds present. As the extract's concentration increased, so did the quantity of active substances, which resulted in stronger antibacterial activity and larger inhibition zones [34]. The formation of these inhibition zones around the bacteria in culture suggests that *C. hirta* extract contains bioactive compounds such as phenolics, terpenoids, saponins, and flavonoids.

Phenolic compounds, which contain an aromatic ring and one or more hydroxyl groups, are known for their robust antimicrobial properties. These compounds work by altering the structure of proteins and preventing bacteria from absorbing necessary nutrients. Furthermore, phenolics disrupt bacterial cell membranes by dissolving lipids in the cell walls [35]. This disruption reduces the membrane's permeability, hindering the transport of essential organic ions into bacterial cells, eventually causing their death. In addition to their antimicrobial properties, phenolic compounds also serve other biological roles, such as anti-carcinogenic, anti-inflammatory, and antioxidant activities. Various studies have demonstrated the efficiency of phenolic compounds in suppressing the growth of numerous pathogenic bacteria [36]. These compounds also displace bacterial flagella, damage cell wall structures, and lead to the leakage of macromolecules from the bacterial cells [37].

Terpenoids are thought to function as antibacterial agents by damaging bacterial membranes, attributed to their lipophilic nature [38]. These compounds interact with porins, which are transmembrane proteins located in the bacterial cell membrane, forming strong bonds that compromise the integrity of the porins. This interaction reduces the cell wall's permeability, depriving bacteria of necessary nutrients, which hinders their growth. Terpenoids in the extract show antibacterial properties by inhibiting lipid biosynthesis and modifying the structure of bacterial membranes, particularly by disrupting the production of ergosterol [39]. Their antimicrobial effectiveness largely stems from their lipophilic properties, which enable terpenoids to disrupt the lipid components of bacterial plasma membranes, leading to imbalances in membrane permeability and disturbances in ion regulation within the bacterial cells [40].

Saponins exhibit both antibacterial and antifungal properties. Their antibacterial activity is facilitated by altering the permeability of bacterial cell membranes, causing the leakage of specific proteins and enzymes from bacterial cells [31]. Saponins interact with sterol-containing membranes, damaging the bacterial cell wall structure and resulting in the release of essential cellular components. The antibacterial mechanism of saponins involves reducing the surface tension of the bacterial cell wall. By binding to lipopolysaccharides within the bacterial cell wall, saponins increase permeability and reduce surface tension, rendering the cell wall more vulnerable to rupture. This allows antibacterial agents to penetrate the bacterial cell more easily, disrupt metabolic processes, and eventually lead to bacterial death [39, 41].

Flavonoids have been demonstrated to form complex compounds that cause damage to bacterial cell membranes. These compounds interact with extracellular proteins within bacterial cells, leading to the release of intracellular contents. Flavonoids are also effective at redirecting energy transfer within bacterial cells, which inhibits their mobility [42]. Flavonoid compounds inhibit bacterial growth by binding to adhesins, damaging bacterial membranes and cell walls, and deactivating essential enzymes. The structural elements believed to be responsible for their antibacterial action include beta rings and hydroxyl groups within the flavonoid structure. These compounds are capable of inhibiting nucleic acid synthesis, interfering with cytoplasmic membrane functions, disrupting biofilm formation, and altering porin activity and permeability, as well as interacting with key enzymes required for bacterial survival [43]. Flavonoids act as antibacterial agents through various mechanisms, including damage to bacterial cell walls and disruption of metabolic processes, which include inhibiting nucleic acid synthesis, disturbing cytoplasmic membrane function, and interfering with energy metabolism [44]. Additionally, flavonoids compromise the permeability of bacterial cell walls, which further enhances their antibacterial efficacy.

4 Conclusion

The *C. hirta* leaf extract was verified to contain key bioactive compounds, including phenolics, terpenoids, saponins, and flavonoids. Furthermore, the extract demonstrated notable antibacterial activity, effectively inhibiting the growth of several pathogenic bacteria such as *A. salmonicida*, *P. aeruginosa*, *E. ictaluri*, *A. hydrophila*, *V. alginolyticus*, and *E. tarda*.

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