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Antibacterial Activity of Ethyl Acetate Fraction of Butterfly Pea Flower (*Clitoria ternatea*) Against *Aeromonas hydrophila in vitro*

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ABSTRACT. *Aeromonas hydrophila is a pathogenic bacterium responsible for significant losses in freshwater aquaculture. In response to the growing issue of antibiotic resistance, this study examined the antibacterial activity of the ethyl acetate fraction of butterfly pea flowers (Clitoria ternatea) against A. hydrophila in vitro. Extraction was performed using ethyl acetate, followed by Thin Layer Chromatography (TLC) and column chromatography, which yielded nine fractions. Among them, fraction 5 showed the highest antibacterial activity. Phytochemical screening revealed the presence of alkaloids, tannins, flavonoids, and triterpenoids. The Minimum Inhibitory Concentration (MIC) test revealed that 250 ppm was the lowest effective concentration, while the disk diffusion assay demonstrated that 350 ppm produced the largest inhibition zone (10.03 ± 1.27 mm), which was statistically comparable to that of chloramphenicol ($F = 91.34$; $p < 0.01$). Scanning Electron Microscopy (SEM) revealed structural damage to A. hydrophila cells, including membrane disruption and lysis. This study introduces a new approach utilizing the ethyl acetate fraction of C. ternatea, combined with fractionation techniques and SEM imaging, to evaluate antibacterial activity. It offers potential for the development of environmentally friendly natural antibacterial agents for the aquaculture industry*

Keywords: *Aeromonas hydrophila, antibacterial activity, Clitoria ternatea, ethyl acetate fraction.*

INTRODUCTION

Bacterial infections pose a significant challenge in fish farming, particularly those caused by *Aeromonas hydrophila*. This opportunistic pathogen affects a wide range of freshwater fish species, causing symptoms such as hemorrhagic wounds, swelling of internal organs, and increased mortality, significantly impacting fisheries production [23]. Currently, the control of this disease relies on antibiotics and synthetic chemicals, which, while effective, pose risks such as bacterial resistance and environmental harm [30]. Therefore, exploring environmentally friendly and sustainable alternatives, such as antibacterial compounds derived from natural sources, is essential for combating *A. hydrophila* infections in aquaculture.

The use of natural ingredients as antibacterials in aquaculture is gaining attention, particularly in treating bacterial infections in freshwater fish [21]. Several herbal plants, including neem (*Azadirachta indica*), turmeric (*Curcuma longa*), garlic (*Allium sativum*), and sambiloto (*Andrographis paniculata*), have demonstrated antibacterial activity against *A. hydrophila*. However, each of these plants has limitations. Neem leaves can be toxic to fish at high doses [8]. Turmeric contains curcumin, which has low solubility in water, limiting its absorption and reducing its effectiveness against *A. hydrophila* [6]. Garlic contains allicin, which rapidly degrades under environmental conditions, thereby reducing its antibacterial activity [4]. Sambiloto contains andrographolide, which can decrease fish appetite and may lead to bacterial adaptation and resistance with prolonged use [13].

Compared to these natural ingredients, *C. ternatea* presents several advantages, including the stability of its active compounds, more substantial antibacterial efficacy, and improved safety for fish. These make it a promising candidate as a natural antibacterial agent against *A. hydrophila* in aquaculture [22]. This study specifically investigates the antibacterial fraction of *C. ternatea*, which refers to a specific portion of the extract obtained through a separation process (fractionation) to isolate compounds with the highest biological activity [11]. This approach is based on the presence of various bioactive compounds in *C. ternatea* that are known to contribute to its antibacterial properties.

Butterfly pea flower (*C. ternatea*) is known to contain various bioactive compounds, such as flavonoids, alkaloids, saponins, tannins, and triterpenoids, which contribute to its antibacterial properties against both Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus mutans*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae*, and *A. hydrophila*) [22]. Studies have suggested that *C. ternatea* has antimicrobial potential against pathogenic bacteria that affect aquatic species, such as tilapia (*Oreochromis niloticus*), catfish (*Clarias gariepinus*), and goldfish (*Cyprinus carpio*) [32]. Its antibacterial activity is believed to stem from its bioactive compounds, which disrupt *A. hydrophila* cell membranes. Flavonoids and triterpenoids increase membrane permeability, leading to ion leakage and cell lysis. Alkaloids inhibit essential protein synthesis needed for bacterial growth. At the same time, saponins and tannins interfere with cellular metabolism by interacting with enzymes and disrupting bacterial respiration, ultimately inhibiting growth and causing bacterial death [5]. However, the effectiveness of these compounds depends on the extraction and fractionation methods used. Therefore, further research is needed to identify the most effective antibacterial fractions from the *C. ternatea*.

Previous studies have predominantly focused on the antibacterial activity of *C. ternatea* using polar fractions, such as ethanol or aqueous extracts, without incorporating morphological analysis using Scanning Electron Microscopy (SEM) [14]. This study introduces a new approach by employing the semi-polar solvent ethyl acetate to selectively extract bioactive compounds. The semi-polar fraction, which also contains secondary metabolites with antibacterial properties, remains relatively underexplored. Ethyl acetate is a semi-polar solvent that is known to extract secondary metabolites insoluble in both polar and non-polar solvents, allowing for the more selective isolation of active components [12]. Bioactive compounds such as flavonoids, alkaloids, saponins, steroids, and triterpenoids which exhibit semi-polar characteristics can be effectively extracted using ethyl acetate, facilitating the selective isolation of antibacterial agents against *A. hydrophila* [10]. Furthermore, this study integrates fractionation techniques, including Thin Layer Chromatography (TLC) for compound profile monitoring and column chromatography for the separation and purification of the ethyl acetate fraction, combined with visual verification through Scanning Electron Microscopy (SEM) to observe morphological damage to *A. hydrophila* cells following treatment. This provides direct evidence of the antibacterial effect of the fraction. This integrated approach has not been extensively reported in previous literature and holds the potential to contribute significantly to the development of more targeted, plant-based antibacterial agents.

This study aims to identify the antibacterial potential of the ethyl acetate fraction of *C. ternatea* through the characterization of bioactive compounds and *in vitro* antibacterial testing against *A. hydrophila*. In addition, morphological analysis of *A. hydrophila* using Scanning Electron Microscopy (SEM) will be conducted to observe structural changes induced by the ethyl acetate fraction of *C. ternatea*. The findings of this study are expected to contribute to the development of a more effective and environmentally friendly alternative for controlling *A. hydrophila* infections in aquaculture

RESEARCH METHODS

Materials

Aeromonas hydrophila bacteria, with isolate code ATCC 7966, were obtained from the Microbiology Laboratory of the Center for Brackish Water Aquaculture (BBPBAP), Jepara, Central Java. This study used fresh butterfly pea flowers (*C. ternatea*) from the Agricultural Extension Center (BPP) Tembelang, Jombang Regency, East Java, with the nomenclature code BPP-JB001.

Extraction of Butterfly Pea Flower (*C. ternatea*)

This study used the maceration extraction method. A total of 2.500 g of wet *C. ternatea* flowers were dried under direct sunlight for 3 days at ambient temperature, and 513 g of the dried flowers were then ground into a fine powder. Soaking (maceration) was carried out in a ratio of 1:10 where 100 g of *C. ternatea* powder was macerated with 1000 ml of ethyl acetate solvent (Merck, Germany), after 3 days, the macerate was filtered from the pulp with a Buchner funnel which had been coated with Whatmann 40 filter paper. The formed macerate was evaporated using a rotary evaporator at 121 rpm and 45 °C until a thick extract in the form of a paste was obtained [26]. Phytochemical screening extract was performed to identify the presence of alkaloids, tannins, triterpenoids, saponins, and flavonoids.

Fractionation of Butterfly Pea Flower Extract (*C. ternatea*)

Fractionation of the *C. ternatea* extract with the Thin Layer Chromatography (TLC) method consists of a stationary phase in the form of a KLT plate measuring 8 cm x 2 cm and a mobile phase in the form of eluent, which is adjusted to the compounds in *C. ternatea* extract based on the

phytochemical test. A total of 10 mg of *C. ternatea* extract was dissolved in 1 mL of ethyl acetate. Then, approximately 5 µL was transferred with a capillary pipette and applied to the stationary phase. The TLC plate was inserted into the glass chamber with the eluent upright. The sample was eluted with a solvent and reagent system selected based on the compound classes identified in the phytochemical screening of the *C. ternatea* extract, using various solvent ratios. The reagent was sprayed on the eluted plate, namely glacial acetic acid: butanol: water (1: 4: 5) and ammonia reagent for flavonoids, methanol: water (6: 4) and 5% $FeCl_3$ reagent for tannins, chloroform: methanol (9:1) and Liebermann-Burchard reagent for saponins, chloroform: methanol (9:1) and Dragendorff reagent for alkaloids, and chloroform: methanol (9:1) and 10% H_2SO_4 reagent for triterpenoids [24]. The plate was dried until the color appeared, and the best-separated stain was visually observed under UV 254 nm (shortwave) and 366 nm (longwave) [3]. The TLC results were used as the basis for selecting the most suitable eluent for further separation in column chromatography. Eluent selection was based on the TLC profile that produced the clearest and most distinct spot separation, with Rf values appropriate for identifying the presence of various compounds in the extract [38].

Column chromatography was performed to separate the active compounds in the extract into several fractions, using silica gel 60 as the stationary phase and the best eluent from TLC results as the mobile phase. To achieve this, 20 g of silica gel 60 was dissolved in the selected eluent. The column was prepared by filling the bottom with cotton before the silica slurry was slowly inserted until it reached three-quarters of the column, and it was allowed to stand overnight. An extract of *C. ternatea*, weighing approximately 0.5 g, was dissolved in a sufficient amount of eluent, then slowly introduced into the column using a funnel to prevent damage to the silica gel 60 arrangement. The column tap was opened slowly, allowing various colors of *C. ternatea* extract fractions to be collected in vials according to the colors observed on silica gel 60 [19]. All collected fractions were subsequently tested for their antibacterial activity against *A. hydrophila* to determine the most active fraction.

Phytochemical Test of Ethyl Acetate Fraction of Butterfly Pea Flower (*C. ternatea*)

The phytochemical test is a qualitative analysis method used to identify the content of bioactive compounds in the ethyl acetate fraction of *C. ternatea* using specific color reagents. Phytochemical tests are conducted to detect the presence of various compounds, including tannins, using a 1% $FeCl_3$ reagent. The solution shows a dark blue or greenish-black color, indicating the presence of tannins. Alkaloids treated with the Mayer reagent form a white precipitate. In contrast, the Wegner reagent produces a brown precipitate, and the Dragendorff reagent yields an orange or red precipitate, all indicating a positive result for tannin. Flavonoids can be detected using Mg and HCl reagents, as evidenced by a positive result if an orange precipitate forms. Saponins can be detected by reacting with water and HCl reagents, as a permanent foam that persists for at least 7 minutes indicates the presence of saponins. Triterpenoids, when treated with Lieberman and Burchard reagents, will exhibit a brown color, indicating the presence of triterpenoids [1].

Antibacterial Activity Test

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antibacterial agent that can inhibit visible bacterial growth. The MIC test in this study was conducted as a single measurement without replicates or statistical analysis, as the aim was to determine the lowest concentration that inhibited bacterial growth based on both visual clarity and absorbance values measured by spectrophotometry [16]. The MIC testing method was carried out using the broth dilution method. Medium TSB (Tryptic Soy Broth, Merck, Germany) was prepared by dissolving 1.5 g in 49.5 mL of distilled water and homogenized using a hot plate. The medium was sterilized by autoclaving at 121°C for 15 minutes. The medium was then transferred to 12 test tubes, each 4 mL in volume. The ethyl acetate fraction of *C. ternatea* were added as much as 0.5 mL with a dose that refers to the research of [35], namely 1,000 ppm, 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 positive control used 30 ppm chloramphenicol antibiotic, while the negative control was not treated with any fraction. A total of 0.1 ml of *A. hydrophila* bacterial isolate (10^7 CFU/ml) was added, and the tubes were incubated for 24 hours at 32°C. Subsequently, the turbidity of the medium was measured using a spectrophotometer at a wavelength of 600 nm [18].

Disc Diffusion Assay

The disc diffusion assay was used to determine the inhibitory activity of the ethyl acetate fraction from *C. ternatea*, as indicated by the clear zone surrounding the paper disc. Medium MHA (Mueller Hinton Agar, Merck, Germany) was prepared by dissolving 3.8 g in 100 mL of distilled water and

homogenized using a hot plate. The medium was sterilized by autoclaving at 121°C for 15 minutes. Paper discs were soaked in the ethyl acetate fraction of *C. ternatea* for 15 minutes, using the following doses: A (250 ppm), B (300 ppm), and C (350 ppm). This dose is obtained from the MIC test of 250 ppm, which has inhibited the growth of *A. hydrophila* bacteria. The positive control used 30 ppm chloramphenicol antibiotic, while the negative control used distilled water. The sterile medium was poured into sterile Petri dishes, each with a volume of approximately 25 mL. Paper discs soaked in the ethyl acetate fraction of *C. ternatea* are placed on the surface of the agar medium. Bacteria were inoculated using a micropipette in a volume of 100 µL with a density of 10^7 CFU/mL, placed on the surface of the agar medium, and evenly spread using a sterile triangle. The treatment was carried out in LAF (Laminary Air Flow) and incubated for 24-48 hours at 32°C. The diameter of the clear zone formed around the disc paper was measured using a caliper and expressed in millimeters (mm) [17].

Scanning Electron Microscope (SEM)

Scanning Electron Microscope (SEM) observation was intended to determine the morphological damage of *A. hydrophila* bacterial cells caused by the administration of the ethyl acetate fraction of *C. ternatea* containing active compounds. *A. hydrophila* preparations involved 2 treatments. The first treatment is without additional treatment (control), and the second treatment is *A. hydrophila* bacteria given ethyl acetate fraction of *C. ternatea*. The step for the SEM test is that *A. hydrophila* bacteria are cultured in TSB liquid medium for 18-24 hours, then 1 mL of the culture was centrifuged (2000 g, 4°C, 10 min) and washed twice with physiological NaCl. Samples were incubated in 3% glutaraldehyde for 30 minutes, washed three times with PBS, and then centrifuged at 2000 g for 10 minutes at 4°C. After drying over a bunsen, the samples were dehydrated with a series of alcohols (30-96%), gold-plated, and observed using an SEM TM 3000 microscope (Hitachi, Japan) [12].

Data Analysis

This study used a Completely Randomized Design (CRD) with treatments A (250 ppm), B (300 ppm), C (350 ppm) of ethyl acetate fraction of *C. ternatea*, positive control K+ (chloramphenicol 30 ppm), and negative control K- (without treatment), each with 3 replicates. The inhibition zone data of the ethyl acetate fraction of *C. ternatea* to inhibit *A. hydrophila* were analyzed statistically using Microsoft Excel 2023. Analysis of variance (ANOVA) or the F-test was conducted to determine the effect of the treatment (independent variable) on the response of the tested parameters. Least Significant Difference (LSD) test was used to determine the difference between the most influential treatments if the results of the F test value were significantly or significantly different.

RESULT AND DISCUSSION

A total of 2.500 g of fresh *C. ternatea* yielded 513 g of dried flowers, with a dry yield of 20.52%. The obtained yield aligns with the typical characteristics of fresh *C. ternatea*, which have high moisture content (approximately 70–80%) [29], indicating that the drying process was effective. The extraction process, using 1000 mL of ethyl acetate solvent against 100 grams of dried simplisia, produced a thick extract weighing 10.38 grams, resulting in a yield of 10.38% for the *C. ternatea* extract. Organoleptically, the extract is dark green in color and viscous. *C. ternatea* extract was obtained, and then phytochemical tests were conducted to determine the content of secondary metabolite compounds that can be extracted with an ethyl acetate solvent. The results of the phytochemical test of *C. ternatea* extract are presented in Table 1.

Table 1. Phytochemical screening results of ethyl acetate extract of *C. ternate.*

Test Parameters	Results
Tannin	+
Alkaloid	+
Flavonoid	+
Saponin	+
Steroid	-
Triterpenoid	+

The results of the phytochemical test, shown in Table 1, indicate that the *C. ternatea* extract, when extracted with ethyl acetate solvent, contains several classes of active compounds, including tannins, alkaloids, flavonoids, saponins, and triterpenoids, as indicated by a positive sign (+). In contrast, steroid compounds were not detected, as indicated by a negative sign (-). The diversity of active compounds identified indicates that ethyl acetate, as a semi-polar solvent, is capable of extracting various bioactive compounds, most of which are semi-polar in *C. ternatea*. Compared to polar solvents such as ethanol, although ethanol can effectively extract polar compounds such as flavonoids and tannins, the solvent also tends to dissolve non-target compounds, resulting in lower extract purity [36]. Meanwhile, n-hexane, as a non-polar solvent, is generally only effective in extracting non-polar compounds, such as steroids or lipids, which are limited in *C. ternatea*. Hence, the yield and diversity of compounds obtained are

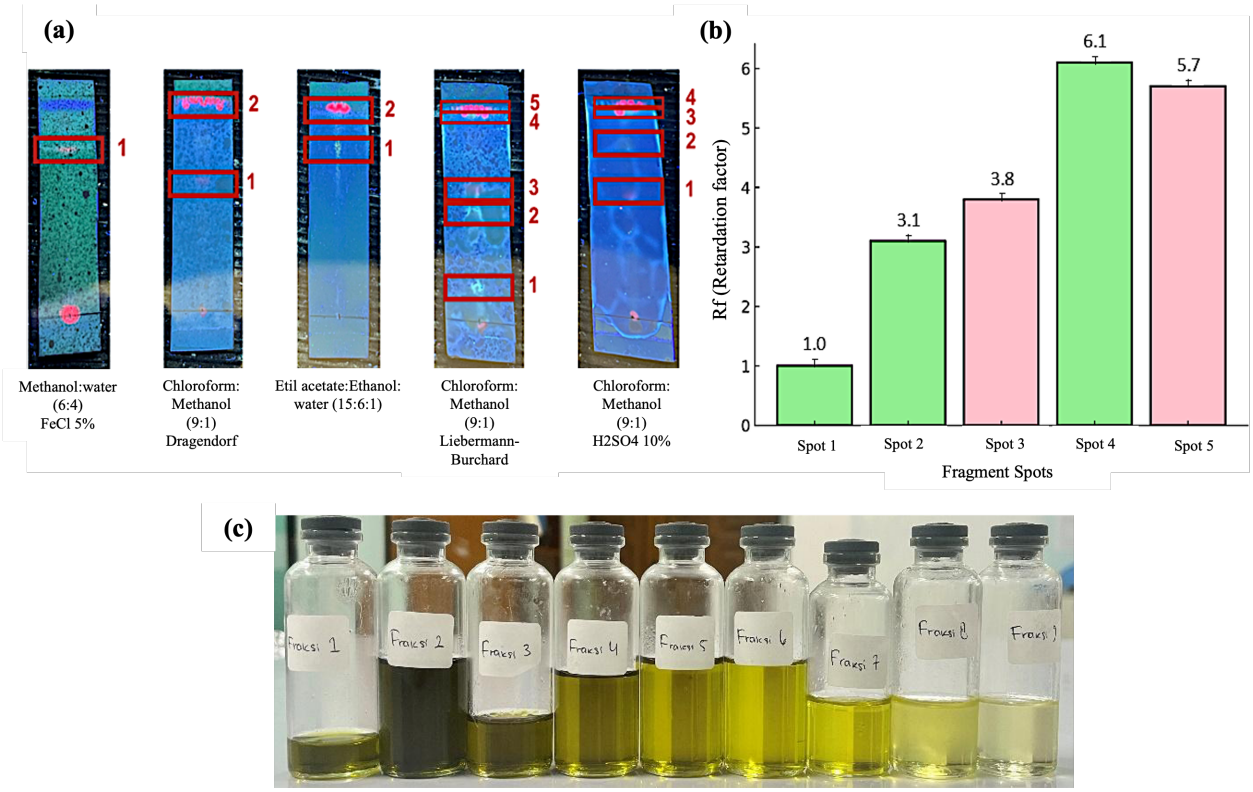


Figure 1. Fractionation Results of *C. ternatea* in the form of (a) Thin Layer Chromatography Spots, (b) Retardation factor (Rf) Value of the Best Eluent, and (c) Column Chromatography Fractions.

lower [14]. This characteristic of *C. ternatea* is dominated by semi-polar bioactive compounds, making the use of semi-polar solvents, such as ethyl acetate, more effective in extracting these compounds compared to polar and non-polar solvents [10].

Thin Layer Chromatography (TLC) is a method for separating compounds in a material based on differences in solubility. In this study, Thin Layer Chromatography (TLC) testing utilizes eluents and stain revealers adjusted to the results of the phytochemical test of *C. ternatea* extract. The analysis results are indicated by spots that appear due to variations in eluents and stains used, which are then observed under UV light at 366 nm and are presented in Figure 1(a).

Eluent chloroform: methanol (9:1) with Lieberman-Burchard reagent stain is the best eluent used during thin layer chromatography. This is because the eluent with this ratio has more spots than other eluents. The polarity match between the eluent and the target compound increases the efficiency of the compound's migration on the stationary phase, resulting in more clearly separated spots. The more spots that appear, the more complex the chemical components in the extract that can be detected [27]. The distance of each place from the lower limit line on the TLC plate is measured and expressed in the form of Rf (Retardation factor) values presented in Figure 1(b).

Thin Layer Chromatography (TLC) analysis yielded five distinct spots with varying Rf values and color differences, indicating the diversity of compounds present in the extract. The spot color that appears as a fluorescence band on the KLT plate reflects the characteristics of the separated compounds. In this study, the light green spot was identified as an indication of the presence of flavonoid compounds, while the pink spot indicated the presence of triterpenoid compounds. Compounds with high polarity tend to produce low Rf values (<3), while non-polar compounds show high Rf values (>7). The intermediate Rf values obtained in this study (around 3-6) indicate that the compounds in *C. ternatea* extract are semi-polar [15]. The choice of a chloroform:methanol (9:1) eluent as the mobile phase is considered optimal because it possesses semi-polar properties, which align with the polarity of the main bioactive compounds in *C. ternatea*. In addition, the use of Liebermann-Burchard reagent as a spotting reagent increases the visibility of triterpenoid spots on the TLC plate, as reported in a study by [20], which indicates that the reagent is highly selective for triterpenoid detection. This finding is consistent with the phytochemical characteristics of *C. ternatea*, which are primarily composed of semi-polar compounds, including flavonoids, alkaloids, saponins, and triterpenoids. Therefore, semi-polar

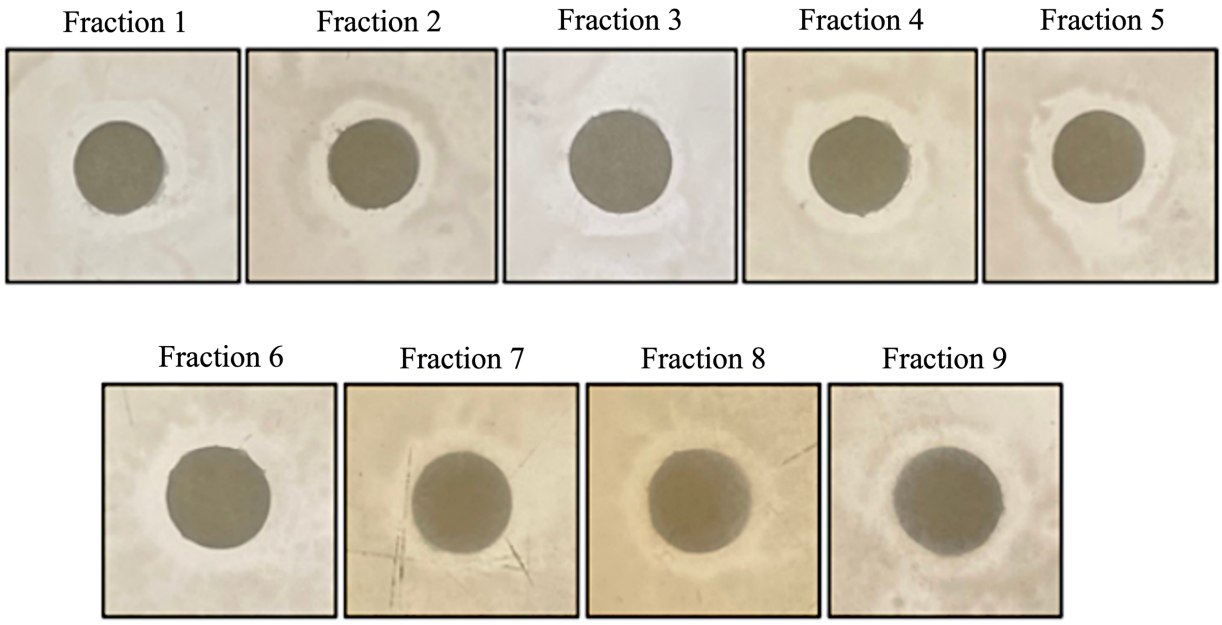


Figure 2. Inhibition Zone Observations of *C. ternatea* Fractions against *A. hydrophila*

solvents like ethyl acetate are more effective in separating these compounds compared to polar (ethanol) or non-polar (n-hexane) solvents [10].

The choice of eluent in this separation process is based on the optimal results of Thin Layer Chromatography (TLC) analysis, specifically using a chloroform:methanol eluent (9:1). The column chromatography process in this study produced nine different fractions, which were separated based on the color variations observed in the column tube. The first fraction is brown, the second fraction is blackish brown, the third fraction is dark green, the fourth fraction is green, the fifth fraction is bright green, the sixth fraction is yellowish green, the seventh fraction is yellow, the eighth fraction is bright yellow, and the ninth fraction is yellowish white which is presented in Figure 1(c).

The sequence of fractions obtained from the column chromatography process in this study reflects the differences in polarity among the compounds. Fractions that eluted earlier from the column, such as the first fraction, generally consist of non-polar compounds due to their low affinity for the stationary phase (silica gel), allowing them to be rapidly eluted by the mobile phase. In contrast, fractions that eluted at later stages, such as the ninth fraction, tend to contain more polar compounds. Polar compounds interact more strongly with the stationary phase, requiring either a more polar eluent or a longer elution time to be released from the column [16]. The purpose of fractionation is to isolate pure compounds or groups of compounds with similar chemical properties, which can subsequently be evaluated for antibacterial activity.

The fractions obtained through column chromatography were subsequently subjected to a disk diffusion assay to evaluate the antibacterial potency of each fraction. The inhibitory activity of each *C. ternatea* fraction against *A. hydrophila* is presented in Figure 2.

Based on the antibacterial activity test results of the *C. ternatea* fractions against *A. hydrophila*, the inhibition response can be determined, as shown in Figure 3. The classification of inhibition response refers to the standard interpretation of inhibition zone diameters commonly used in the disk diffusion method, as described by [9], in which a clear zone of 2–5 mm indicates weak activity, 5–10 mm indicates moderate activity, 10–22 mm indicates strong activity, and ≥ 20 mm indicates very strong activity.

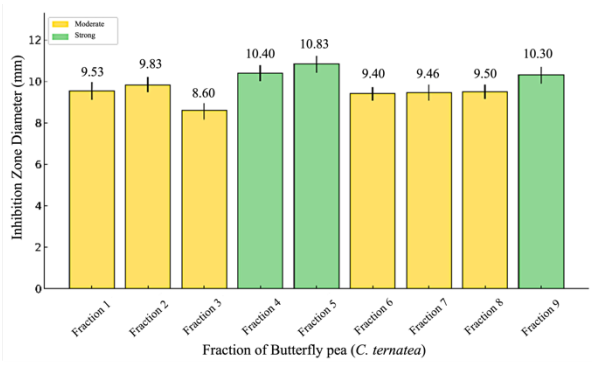


Figure 3. Inhibition Zone Diameters of *C. ternatea* Fractions against *A. hydrophila.*

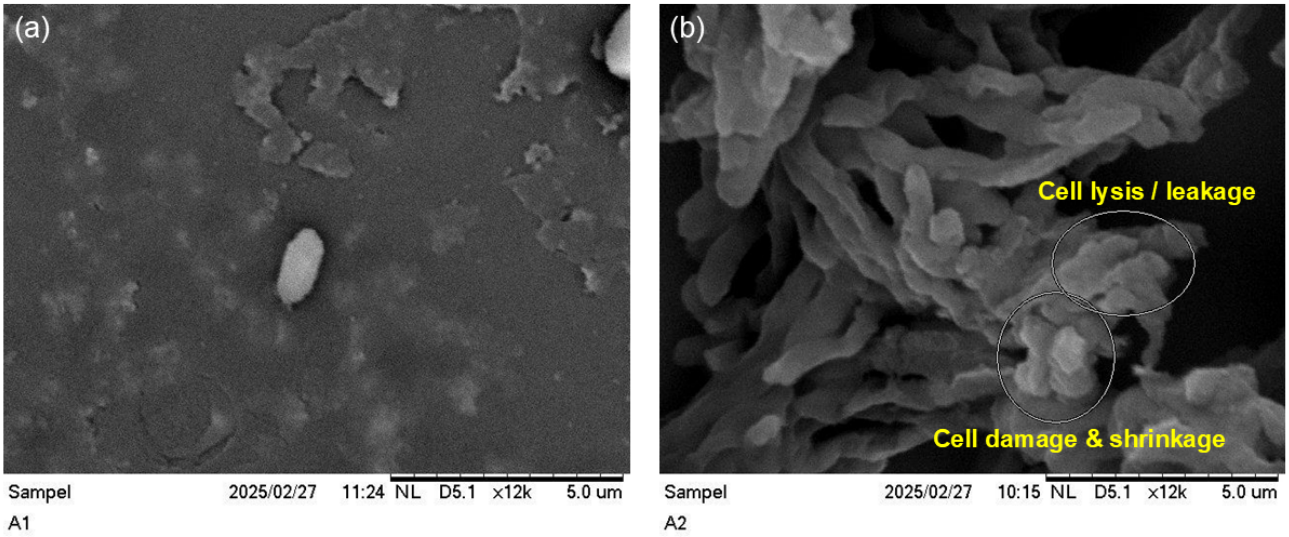


Figure 4. Morphology *of A. hydrophila* bacteria, (a) without treatment and (b) after application of ethyl acetate fraction of *C. ternatea* with SEM TM3000 microscope at 12.000x magnification

As shown in Figure 3. fraction 5 (indicated in bright green) exhibited the highest antibacterial activity, with an inhibition zone diameter of 10.83 mm, which falls under the strong category. This is presumably due to the presence of semi-polar compounds in this fraction. In column chromatography, fractions eluted in the intermediate phase are generally composed of semi-polar compounds with a balanced affinity between the mobile and stationary phases, allowing them to be eluted more easily with semi-polar eluents [16]. This fraction may have extracted a higher concentration of active compounds, as the dominant bioactive constituents in *C. ternatea* are known to be semi-polar, resulting in the most potent antibacterial activity among all tested fractions. To further investigate the antibacterial mechanism, Scanning Electron Microscopy (SEM) analysis was conducted before phytochemical screening. SEM observations revealed structural damage to *A. hydrophila* cells, indicating the presence of bioactive compounds. Following this, phytochemical screening was carried out to identify the specific active compounds present in fraction 5. The results of Scanning Electron Microscope (SEM) analysis showed that the treatment affected the structural changes of *A. hydrophila* bacterial cells, as presented in Figure 4.

The cell morphology of untreated *A. hydrophila* bacteria appeared intact, with a rod-shaped structure, and the cell surface appeared smooth, showing no visible structural damage. This indicates that the cell membrane of *A. hydrophila* is in normal condition and not disturbed. This finding is consistent with a study conducted by [28], that the morphological characteristics of Gram-negative bacteria *A. hydrophila* under normal physiological conditions generally have a rod shape with intact and organized cell walls. In contrast, *A. hydrophila* bacteria exhibited apparent morphological changes after being administered the ethyl acetate fraction of *C. ternatea*. The surface of the bacterial cells appeared disorganized, wrinkled, and had significant structural damage. Some cells collapsed and exhibited signs of cell lysis, indicating disruption of cell membrane integrity.

These morphological changes indicate that the ethyl acetate fraction of the *C. ternatea* has effective antibacterial activity, which is shown through severe structural damage to the cell surface of *A. hydrophila* due to exposure to the bioactive compounds in it. The antibacterial activity of alkaloid compounds works by inhibiting enzymes that play a role in bacterial DNA replication. Disruption of the replication process prevents bacteria from dividing, thereby halting their growth. Additionally, the alkaloids in the fraction can also interfere with the formation of the peptidoglycan structure in the bacterial cell wall by inhibiting the formation of cross-bridges between its components. As a result, the cell wall is not formed completely, which can cause cell death [7]. Triterpenoids are believed to act as antibacterial agents through mechanisms that involve damage to the cell membrane. These compounds are lipophilic and can interact with porin, a transmembrane protein on the outer membrane of bacteria, forming strong polymeric bonds. The bond damages the porin structure, decreases the permeability of the cell membrane, and inhibits the entry of essential nutrients. This condition disrupts cell metabolism, inhibiting bacterial growth and potentially leading to death [33]. Tannins exhibit antibacterial effects primarily by forming complexes with bacterial cell wall proteins and membrane-bound enzymes. This interaction causes disruption of the cell wall structure, increased

permeability, and leakage of intracellular contents, ultimately leading to cell lysis. Tannins may also chelate metal ions required for enzymatic activity, thereby inhibiting key metabolic processes within the bacterial cells [5]. Flavonoids, on the other hand, possess antibacterial activity through multiple mechanisms, including disruption of cytoplasmic membranes, inhibition of nucleic acid synthesis, and interaction with bacterial enzymes. They can integrate into bacterial membranes due to their lipophilicity, leading to altered membrane fluidity and leakage of cellular components. Some flavonoids are also known to inhibit DNA gyrase and topoisomerase, enzymes essential for bacterial replication [39]. The phytochemical screening results of *C. ternatea* fraction are presented in Table 2.

Table 2. Phytochemical screening results of ethyl acetate fraction of *C. ternatea*

Active Compund	Test Result	Indicator	Figure
Tannins	Positive	Greenish color	
Alkaloids	Positive	Brown color	
Flavonoids	Positive	Orange-tinged color	
Saponins	Negative	No foam	
Steroids	Negative	No bluish-green color	
Triterpenoids	Positive	Brown color	

Phytochemical screening revealed the presence of alkaloids, triterpenoids, tannins, and flavonoids in the ethyl acetate fraction of *C. ternatea*. However, Thin Layer Chromatography (TLC) analysis only detected a red spot indicative of triterpenoids and a green spot corresponding to flavonoids. The absence of visible alkaloid and tannin spots is likely due to their low concentrations, which were insufficient to produce characteristic colors on the TLC plate. Although these compounds were previously identified in the crude extract, differences in affinity toward ethyl acetate during the fractionation process may have affected their presence in this particular fraction. Ethyl acetate is known to be more selective in extracting certain semi-polar compounds, particularly triterpenoids and flavonoids [20]. This fraction holds promise as a potential candidate for developing active phytopharmaceutical ingredients, especially those targeting antibacterial activity. Triterpenoids have been widely reported to possess various pharmacological activities, including antibacterial, anti-inflammatory, antioxidant, and anticancer properties. Flavonoids are also known for their antimicrobial and anti-inflammatory capabilities including enzyme inhibition, disruption of microbial protein synthesis, and stabilization of cell membranes. Alkaloids contribute to antimicrobial activity by disrupting bacterial cell membrane integrity, inhibiting DNA or RNA synthesis, and interfering with essential enzymatic functions required for microbial survival. Meanwhile, tannins exhibit antibacterial activity by precipitating proteins and disrupting the permeability of microbial cell membranes [11]. The presence of these four active compounds in the ethyl acetate fraction of *C. ternatea* enhances its potential for bioactivity.

Table 3. MIC Test Observation Results

Concentration (ppm)	Absorbance Value	Media Color
1,9	0,317	Turbid
3,9	0,388	Turbid
7,8	0,344	Turbid
15,6	0,243	Slightly Turbid
31,3	0,257	Slightly Turbid
62,5	0,250	Slightly Clear
125	0,244	Slightly Clear
250	0,191	Clear
500	0,158	Clear
1000	0,132	Clear
Control (+)	0,188	Clear
Control (-)	0,504	Turbid

Minimum Inhibitory Concentration (MIC) test was conducted on the best fraction of *C. ternatea* using DMSO solvent with different doses to determine the smallest dose that can inhibit *A. hydrophila* bacteria. The results of the MIC test observations, based on absorbance levels measured using a spectrophotometer at a wavelength of 600 nm, are presented in Table 3.

Incubation for 24 hours produces a color change in the sample, indicating that the various MIC test treatments show different results.

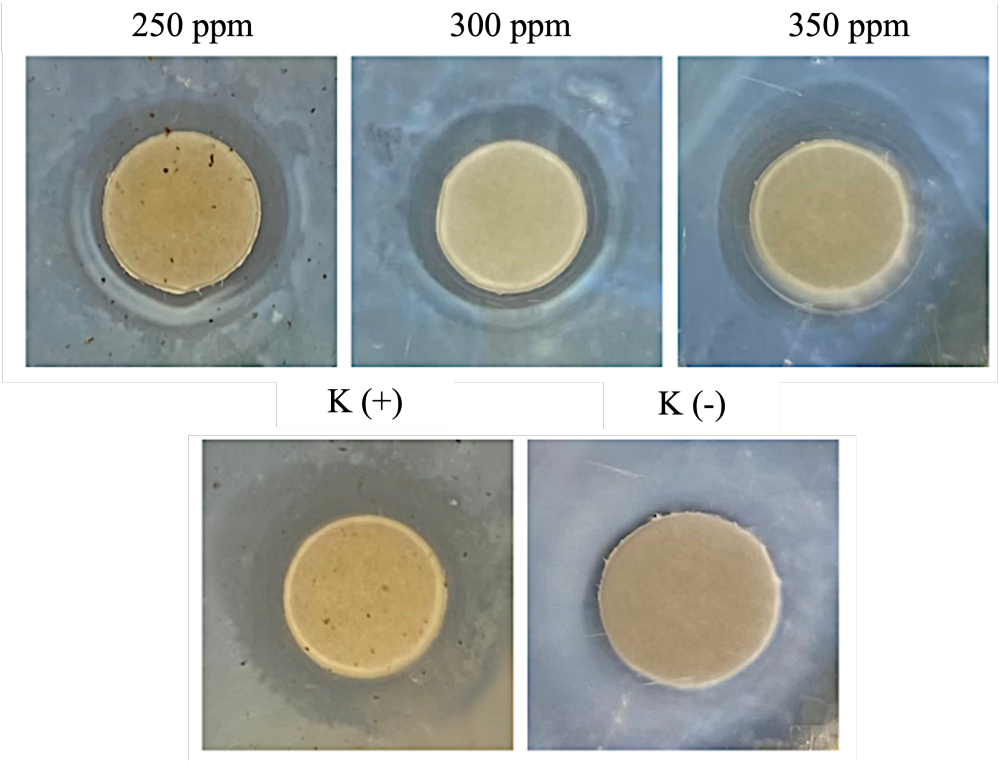


Figure 5. Visual Appearance Inhibition Zone of *C. ternatea* Fraction against *A. hydrophila*

Changes in color or turbidity of the sample were also observed with a spectrophotometer. According to a study by [31], the MIC test determination is based on the level of turbidity and clarity of the sample. The sample was then compared with the control treatment's turbidity level in the study. Test results using a spectrophotometer, which is close to the positive control value, indicate that the fraction can inhibit bacterial development.

The MIC test in this study employed 10 different concentrations, along with two control treatments: control (+), which used 30 ppm of chloramphenicol antibiotic, and control (-), which was untreated. The MIC test observations showed that a concentration of 250 ppm inhibited the growth of *A. hydrophila* bacteria. This concentration has an absorbance value that is closest to the positive control value and produces a transparent color for the first time, so it can already have an effect [34]; it can be concluded that the fraction of *C. ternatea* at a concentration of 250 ppm can inhibit the growth of *A. hydrophila* bacteria and can be continued in the disc test.

Compared to other studies that also utilized *C. ternatea*, the MIC results in this study demonstrate promising effectiveness against *A. hydrophila*. A study by [30] using ethanol extract of *C. ternatea* reported that a concentration of 6.250 ppm was required to inhibit the growth of *Streptococcus mutans* and *Staphylococcus aureus*. Although the target bacteria differ, this highlights that antibacterial effectiveness is strongly influenced by both the type of solvent and the microbial target. With an MIC of 250 ppm, the ethyl acetate fraction in this study exhibits high potential, attributed to the semi-polar solvent's ability to selectively extract bioactive compounds that are more effective against *A. hydrophila*.

The dose treatment in the disc test used concentrations of 250 ppm, 300 ppm, and 350 ppm, with a positive control of 30 ppm chloramphenicol antibiotics and a negative control without treatment. This dose was obtained from the MIC test, which showed an inhibition of 250 ppm against the *A. hydrophila* bacteria. The results of the inhibition test image of *A. hydrophila* bacteria after giving the fraction of *C. ternatea* are presented in Figure 5.

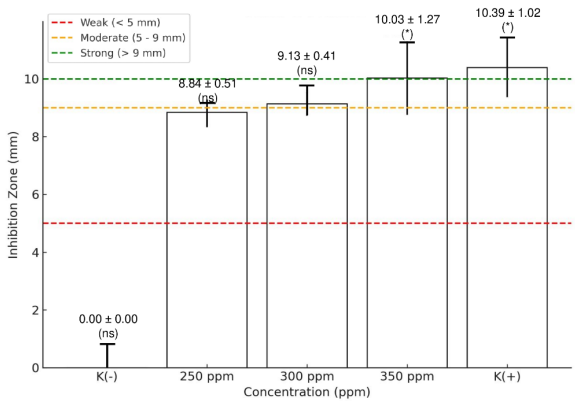


Figure 6. Inhibition Zone Diameters of *C. ternatea* Fraction against *A. hydrophila.* Note: * =

significantly different; ns = not significantly different

The results of the inhibition test image of *A. hydrophila* bacteria after giving the fraction of *C. ternatea* are presented in Figure 5. The results of the disc test on the *C. ternatea* with three concentration treatments and three replicates were obtained. The diameter of the clear zone was obtained after 24 hours of observation, as presented in Figure 6. The values shown are the mean ± standard deviation.

The treatment with a concentration of ethyl acetate fraction of *C. ternatea* of 350 ppm produced the highest inhibition zone diameter, 10.03 ± 1.27 mm, with a strong sensitivity category. Based on the results of the analysis of variance (ANOVA), differences between treatments proved to be highly statistically significant ($F = 91.34$; $p < 0.01$). Further tests of the Least Significant Difference (LSD) revealed that treatment with various concentrations of the ethyl acetate fraction of *C. ternatea* resulted in significant differences in the growth inhibition of *A. hydrophila*. Treatment C (350 ppm), indicated by (*), exhibits a notable difference compared to the other treatments. In contrast, treatments A (250 ppm) and B (300 ppm) showed no significant difference (ns). This indicates that increasing the concentration from 250 ppm to 300 ppm did not give a significantly different inhibitory effect on bacterial growth. The control (+), which showed a mean inhibition zone of 10.39 mm, was also noted (*), indicating that the effectiveness of the 350-ppm fraction was equivalent to that of the positive control in inhibiting the growth of *A. hydrophila*. The 350-ppm concentration was the most effective treatment, producing statistically significant inhibition zones that were biologically equivalent to the positive control. The difference in the size of the inhibition zone produced at each concentration, according to [2], can be caused by variations in concentration levels or the amount of minimal antibacterial active substances contained therein; the greater the concentration applied, the wider the diameter of the clear zone formed. On the other hand, the lower the concentration used, the narrower the transparent area. This is in line with the theory stated by [25] that the higher the concentration of an antibacterial agent, the more active compound content interacts with the bacterial cell wall, thereby disrupting cell function and inhibiting its growth. The treatment with the highest concentration (350 ppm) demonstrated effectiveness comparable to that of the positive control, indicating the potential of using the ethyl acetate fraction of *C. ternatea* as a natural antibacterial agent.

The control (-), which showed no inhibition zone (0 mm), strengthened the evidence that without the administration of ethyl acetate fraction of *C. ternatea*, there was no inhibition of *A. hydrophila* bacterial growth. This suggests that the inhibition zones observed in the other treatments were indeed caused by the antibacterial activity of the bioactive compounds in the ethyl acetate fraction of *C. ternatea*, specifically alkaloids, triterpenoids, tannins, and flavonoids. The antibacterial mechanism of alkaloid compounds involves intercalating bacterial DNA, thereby disrupting the replication and transcription processes essential for protein synthesis and cell division. In addition, alkaloids can also increase cell membrane permeability, leading to leakage of cell contents and ultimately causing lysis and death of bacterial cells [7]. The antibacterial mechanism of lipophilic triterpenoid compounds involves their easy interaction with membrane lipids, thereby increasing membrane permeability and causing the leakage of ions and essential metabolites from the cell. Not only that, triterpenoids also inhibit the biosynthesis of peptidoglycan, the main component of the bacterial cell wall, leading to weakness of the cell wall structure and increasing the risk of cell lysis. In addition, triterpenoids can induce oxidative stress by forming Reactive Oxygen Species (ROS) that damage vital components such as proteins, lipids, and nucleic acids [33]. The antibacterial mechanism of tannin compounds involves protein precipitation and enzyme inhibition, which interfere with vital functions within bacterial cells. Tannins can also disrupt the permeability of microbial cell membranes, leading to ionic transport dysfunction and structural integrity changes in the membrane, ultimately resulting in cell death [5]. The antibacterial mechanism of flavonoid compounds involves the inhibition of essential enzymes, disruption of microbial protein synthesis, and stabilization of the cell membrane. Flavonoids can also form complexes with cell wall proteins and nucleic acids, as well as induce oxidative damage, ultimately resulting in bacterial cell dysfunction [33].

However, several limitations should be considered before the ethyl acetate fraction of *C. ternatea* can be widely applied in aquaculture environments. The stability of bioactive compounds such as flavonoids, tannins, alkaloids, and triterpenoids may be affected by environmental conditions, including temperature, light, and pH, which can potentially reduce their antibacterial effectiveness over time during storage or field application. In addition, the scalability of producing the ethyl acetate fraction at an industrial level requires optimization of extraction and

fractionation processes to ensure consistent quality and cost efficiency. Equally important, the potential toxicity to non-target organisms, including cultured fish species and beneficial aquatic microorganisms, must be further investigated to ensure the safe and sustainable use of this fraction in aquaculture systems.

Despite these limitations, the ethyl acetate fraction of *C. ternatea* shows strong practical potential as a natural antibacterial agent in aquaculture. Its relatively low effective concentration (350 ppm), comparable inhibition to conventional antibiotics, and plant-based origin make it a promising alternative to synthetic antimicrobial agents. Moreover, the use of locally available plant materials enhances its economic feasibility, especially in tropical regions. With appropriate formulation and safety validation, this fraction could be integrated into aquaculture practices as a feed additive or immersion treatment, contributing to sustainable disease management strategies.

CONCLUSION

The ethyl acetate fraction of butterfly pea flower (*C. ternatea*) was shown to have significant antibacterial activity against *A. hydrophila in vitro*, supported by the results of phytochemical screening, which showed the presence of active compounds in the form of alkaloids, triterpenoids, tannins, and flavonoids. The concentration of 350 ppm is the most effective treatment, with an inhibition zone diameter of 10.03 ± 1.27 mm, which falls within the strong sensitivity category and is equivalent to the positive control. Scanning Electron Microscope (SEM) observations showed damage to the morphology of *A. hydrophila* bacterial cells, such as wrinkled surfaces and cell lysis, indicating membrane damage due to treatment. These findings suggest that the ethyl acetate fraction of *C. ternatea* has the potential to serve as a natural antibacterial agent that can inhibit the growth of *A. hydrophila*, making it a relevant candidate for the development of environmentally friendly strategies to control bacterial diseases in the fisheries and aquaculture sectors. The practical implications of these findings include the development of active compounds for bioadditives or alternative antibacterial formulations in aquaculture, which could reduce reliance on synthetic antibiotics. In addition, the bioactive properties of the ethyl acetate *C. ternatea* fraction also indicate broad potential applications in other sectors such as the food industry, veterinary medicine, and natural cosmetics, where plant based antimicrobial agents are in demand. Further studies are recommended to evaluate the *in vivo* efficacy of this fraction and to conduct field scale aquaculture trials to validate its commercial potential in the fisheries sector and other environmentally friendly bioproduct industries.

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CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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