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Antimicrobial Activity of *Averrhoa bilimbi* (Lin) Against *Vibrio parahaemolyticus* in the Culture of *Penaeus monodon* Post Larvae: An *In Vitro* and *In Vivo* Study

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ABSTRACT

Vibriosis, caused by *Vibrio parahaemolyticus*, poses a significant challenge in the cultivation of *Penaeus monodon*. This study evaluated the antimicrobial activity of *Averrhoa bilimbi* L. leaf extract against *V. parahaemolyticus* using both *in vitro* (disk diffusion assay) and *in vivo* (shrimp immersion and challenge test) methods. Phytochemical screening revealed the presence of flavonoids, tannins, saponins, alkaloids, steroids, and phenols in the extract. The highest inhibition zone (15.33 mm) was observed at a concentration of 64 mg/mL, which also corresponded to the minimum inhibitory concentration (MIC). In the *in vivo* experiment, *P. monodon* larvae treated with 40 mg/L of *A. bilimbi* extract exhibited the highest survival rate (96%) following bacterial challenge. Clinical observations showed that untreated shrimp displayed symptoms such as body reddening and high mortality, whereas treated groups demonstrated enhanced resistance to infection. These findings suggest that *A. bilimbi* L. leaf extract has strong potential as a natural antibacterial agent for controlling *V. parahaemolyticus* infection in shrimp aquaculture systems.

INTRODUCTION

Global shrimp aquaculture production accounted for 8.9% of the total global fishery output in 2021, with Indonesia contributing approximately 7%, ranking as the fifth-largest shrimp producer worldwide **(FAO, 2020)**. *Penaeus monodon* (tiger prawn) dominated shrimp cultivation from 1990 to 1995, with North Kalimantan emerging as a key production area. Although the cultivation of *P. monodon* remains important, traditional farming methods have experienced a significant decline. While the total farming area spans around 140,000 hectares, it yields only 10,000 tons per year **(DKP,**

2021). This drop in productivity has been attributed to several factors, including environmental degradation, reduced pond carrying capacity, poor pond management, and an increasing incidence of uncontrollable disease outbreaks **(Rukisah *et al.*, 2019)**.

Among various infectious diseases, vibriosis continues to pose one of the most serious threats to shrimp farming. Caused by different species of *Vibrio*, vibriosis leads to high mortality rates and substantial economic losses in aquaculture operations. *Vibrio parahaemolyticus*—a Gram-negative, halophilic bacterium naturally present in marine and estuarine waters—is among the most virulent species and is frequently associated with severe infections in shrimp **(Letchumanan *et al.*, 2014)**. It has been identified as the causative agent of red disease in *P. monodon* and is often co-detected with white spot syndrome virus (WSSV) infections **(Babu *et al.*, 2021)**. Clinical symptoms of *V. parahaemolyticus* infection in shrimp include necrosis, muscle opacity, anorexia, growth retardation, and high mortality during both the nursery and grow-out phases **(Manchanayake *et al.*, 2023)**. Numerous studies have also reported that *V. parahaemolyticus* causes mass mortality in other crustaceans, including crabs and lobsters **(de Souza Valente *et al.*, 2021)**, reinforcing its status as a major concern in aquaculture.

Historically, antibiotics have been widely used in aquaculture to manage bacterial infections. However, inappropriate usage, insufficient farmer education, and prolonged application have led to the emergence of antibiotic-resistant bacteria, ecological disruption, and residue concerns in seafood products **(Bondad-Reantaso *et al.*, 2019; Vincent *et al.*, 2023)**. These issues have had serious implications for seafood safety and international trade, resulting in increased restrictions on marine product imports **(Chen *et al.*, 2020)**. To ensure the continued viability of aquaculture and the safety of its products, there is a growing need to adopt sustainable practices—especially in the search for environmentally friendly and effective alternatives to synthetic antibiotics **(Aich *et al.*, 2018)**.

One promising alternative is the use of plant-derived bioactive compounds, which have demonstrated antimicrobial, antioxidant, immunostimulatory, and growth-promoting properties, without the harmful effects typically associated with synthetic chemicals **(Elgendy *et al.*, 2024)**.

Averrhoa bilimbi L., commonly known as bilimbi or cucumber tree, is a tropical plant whose leaves and fruits have been traditionally used in folk medicine to treat infections, inflammation, and other ailments. Phytochemical analyses have revealed that *A. bilimbi* contains a wide array of bioactive compounds, including tannins, triterpenoids, flavonoids, saponins, alkaloids, and phenolic acids **(Iwansyah *et al.*, 2021)**, which exhibit antimicrobial activity against a broad spectrum of pathogens. Previous studies have demonstrated that both the fruit and leaf extracts of *A. bilimbi* possess inhibitory effects against *V. parahaemolyticus*, with the minimum inhibitory concentration (MIC) of the fruit extract reported at 128 µg/mL **(Das *et al.*, 2011)**. However, comprehensive studies

focusing specifically on the antibacterial potential of leaf extracts—especially under both in vitro and in vivo conditions—remain limited.

Therefore, this study aimed to evaluate the antibacterial activity of *A. bilimbi* L. leaf extract against *V. parahaemolyticus* using *in vitro* disk diffusion and MIC assays, as well as *in vivo* shrimp immersion and challenge tests. The findings are expected to contribute to the development of natural and sustainable alternatives for managing bacterial infections in shrimp aquaculture, while supporting broader efforts toward eco-friendly and safe aquaculture practices.

MATERIALS AND METHODS

Preparation and extraction of plant material

Fresh leaves of *Averrhoa bilimbi* were obtained from a local farmer in Tarakan City, Indonesia. The leaves were first rinsed with distilled water and then sun-dried for three days. Once dried, they were crushed into a fine powder. A total of 200 grams of the powdered leaves was soaked in 800 mL of 95% ethanol (Merck, Germany) in a sealed container for three days. After the soaking period, the mixture was stirred continuously for three hours to maximize extraction efficiency. The resulting solution was filtered through Whatman 1002-125 filter paper, and the ethanol was subsequently removed using a rotary evaporator, leaving behind the crude extract **(Ingle *et al.*, 2017)**.

Determination of active compounds

A phytochemical assessment was conducted to identify the presence of alkaloids, steroids, triterpenoids, tannins, saponins, flavonoids, and phenolic compounds in the *A. bilimbi* leaf extract. The specific methods used for detecting each compound are summarized in Table (1) **(Ingle *et al.*, 2017)**.

Table 1. Determination method of active compounds

Active Compounds	Method
Tannins	0.5 g of extract mixed with 10 ml distilled water, filtered; 2 ml of filtrate mixed with 1% $FeCl_3$ (Merck, Germany). A blue-black or green colour indicates positive tannins.
Alkaloids	0.01 g of extract dissolved in 2 N of H_2SO_4 (Merck, Germany); tested with Dragendorff's, Mayer's, and Wagner's reagents. Coloured precipitates indicate positive tests. Positive test results are determined if Dragendorff's reagent produces a reddish-orange precipitate, Meyer's reagent reveals a yellowish-white precipitate, and Wagner's reagent reveals a brown precipitate.
Steroids	0.01 g of extract dissolved in chloroform (Merck, Germany), then reacted with 10 drops of acetic anhydride (Merck, Germany) and 3 drops of sulfuric acid. A colour change to red, blue, or green indicates positive steroids.
Saponins	0.5 ml extract diluted with 20 ml distilled water and shaken for 15 minutes. Foam formation indicates the presence of saponins.
Phenol Hydroquinone	0.01 g of extract diluted in 70% ethanol, 1 ml of solution was treated with 5% $FeCl_3$ (2 drops). A green or bluish-black colour confirms phenols.

Flavonoids	NaOH test: Dissolving 1 ml of the extract in 1 ml of distilled water and filtering the mixture. 2 ml of the extract is mixed with 10% Naoh (Merck, Germany); a yellow colour that becomes colourless after adding HCl indicates flavonoids. Lead acetate test: A yellow-colored precipitate was observed after a few drops of lead acetate solution were added to a test tube that contained 50 mg of the extract. The tube was shaken for a few minutes.

Bacterial culture preparation

Vibrio parahaemolyticus ATCC 17802 was provided by the Fish Quarantine and Quality Control Center (BKIPM) of Tarakan, Indonesia. The bacteria were streaked onto thiosulfate citrate bile salt sucrose (TCBS) agar (Difco, USA) and incubated at 30°C for 24 h. A single colony was introduced into 50 ml of Tryptic Soy Broth (TSB; Difco, USA) and incubated under identical conditions. The bacterial growth was observed using a spectrophotometer at 600 nm until an absorbance of 0.500 was reached, indicating an appropriate density for subsequent experiments.

Inhibitory activity assay

The antibacterial activity of *A. bilimbi* leaf extract against *V. parahaemolyticus* was assessed using the disk diffusion method (Kirby-Bauer method). Sterile paper disks were soaked in various extract concentrations (2, 4, 7, 10, 16, 32, 48, and 64 mg/ml) for 15 minutes. Tetracycline (10 µg/ml) was used as the positive control and dimethyl sulfoxide (DMSO; Merck, Germany) was used as the negative control. The disks were placed on Mueller-Hinton Agar (MHA) plates previously inoculated with the bacterial suspension and incubated at 30°C for 24 h. The size of the inhibition zones surrounding each disk was evaluated in millimeters.

Determination of the Minimum Inhibitory Concentration (MIC).

The MIC of *A. bilimbi* extract was determined using the broth dilution method **(Elisha *et al*. 2017)**. Sterile tubes containing 9-ml of Nutrient Broth (NB) were prepared. The tubes were filled with the bacterial suspension (0.5 ml of A. bilimbi extract (0.5 mL) at concentrations of 2-64 mg/ml. The positive control tube contained bacteria without the extract, and the negative control tube contained only sterile NB. Optical density (OD) was assessed at 600 nm before and after 24-h of incubation at 30°C. The minimum inhibitory concentration (MIC) was identified as the lowest concentration of the extract, and no increase in OD was observed after incubation.

***In vivo* disease resistance experiment**

Shrimp preparation

Penaeus monodon post-larvae (PL-10) were obtained from a hatchery in Tarakan City and acclimated in aerated tanks for three days. Shrimp were fasted for 24 h prior to the experiment. Throughout the acclimation period, shrimp were fed a standard control diet twice daily (08:00 and 17:00). The water quality parameters were maintained at 25 ± 2 °C, pH 8.2, and salinity of 27 ppt.

Administration of A. billimbi L leaf extract

The *A. billimbi* leaf extract was administered via immersion. The shrimp were divided into four treatment groups: EBW0 (0 mg/ml extract), EBW1 (20 mg/L extract), EBW2 (30 mg/L extract), and EBW3 (40 mg/L extract); this concentration refers to **Handayani (2020)**. The immersion treatment was conducted for 12 days.

Challenge test with Vibrio parahaemolyticus

After treatment, shrimp were challenged by immersion in water containing *V. parahaemolyticus* at a concentration of 10^3 CFU/ml **(Fierro-Coronado *et al.* 2019)**. The tanks were covered with sterile plastic to prevent external contamination. Mortality and clinical symptoms were monitored for 48 h post-challenge.

Data analysis

All data were analyzed using one-way analysis of variance (ANOVA) using SPSS version 26. Differences among treatments were considered significant at $P < 0.05$. Post hoc tests were conducted to identify specific differences between the groups.

Vibrio parahaemolyticus ATCC 17802 was provided by the Fish Quarantine and Quality Control Center (BKIPM) of Tarakan, Indonesia. The bacterium was streaked onto thiosulfate citrate bile salt sucrose (TCBS) agar (Difco, USA) and incubated at 30 °C for 24 hours. A single colony was then inoculated into 50 mL of Tryptic Soy Broth (TSB) (Difco, USA) and incubated under the same conditions. Bacterial growth was monitored using a spectrophotometer at 600 nm until an absorbance of 0.500 was achieved, indicating an appropriate density for subsequent experimentation.

Inhibitory activity assay

The antibacterial activity of *Averrhoa bilimbi* leaf extract against *V. parahaemolyticus* was assessed using the disk diffusion method (Kirby–Bauer technique). Sterile paper disks were soaked in varying concentrations of the extract (2, 4, 7, 10, 16, 32, 48, and 64 mg/mL) for 15 minutes. Tetracycline (10 µg/mL) served as the positive control, while dimethyl sulfoxide (DMSO; Merck, Germany) was used as the negative control. The disks were then placed on Mueller–Hinton Agar (MHA) plates pre-inoculated with the bacterial suspension and incubated at 30 °C for 24 hours. Inhibition zones were measured in millimeters to determine antibacterial effectiveness.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of *A. bilimbi* leaf extract was determined using the broth dilution method **(Elisha *et al.*, 2017)**. Sterile tubes containing 9 mL of Nutrient Broth (NB) were prepared and inoculated with 0.5 mL of the bacterial suspension and 0.5 mL of the extract at concentrations ranging from 2 to 64 mg/mL. The positive control consisted of bacteria without the extract, while the negative control contained only sterile NB. Optical density (OD) was measured at 600 nm before and after 24 hours of incubation at 30 °C. The MIC was defined as the lowest concentration at which no increase in OD was observed after incubation, indicating inhibition of bacterial growth.

***In vivo* disease resistance experiment**

Shrimp preparation

Penaeus monodon post-larvae (PL-10) were obtained from a hatchery in Tarakan City and acclimated in aerated tanks for three days. Shrimp were fasted for 24 hours prior to the experiment. During acclimation, they were fed a standard commercial diet twice daily (08:00 and 17:00). Water quality was maintained at 25 ± 2 °C, pH 8.2, and salinity 27 ppt.

Administration of *A. bilimbi* leaf extract

The *A. bilimbi* leaf extract was administered via immersion treatment. Shrimp were divided into four treatment groups:

- **EBW0**: 0 mg/L (control)
- **EBW1**: 20 mg/L
- **EBW2**: 30 mg/L
- **EBW3**: 40 mg/L

The concentration levels were based on **Handayani (2020)**. Immersion treatments were carried out daily for 12 days.

Challenge test with *Vibrio parahaemolyticus*

After the immersion treatment period, shrimp were challenged by immersion in water containing *V. parahaemolyticus* at a concentration of 10^3 CFU/mL, following the method of **Fierro-Coronado *et al.* (2019)**. Tanks were sealed with sterile plastic to prevent external contamination. Mortality and clinical symptoms were monitored for 48 hours post-challenge.

Data analysis

All data were analyzed using one-way analysis of variance (ANOVA) with SPSS version 26. Differences among treatments were considered statistically significant at $P < 0.05$. Post hoc tests were conducted to identify specific differences between treatment groups.

RESULTS

1. Phytochemical analysis

Phytochemical analysis of *the A. bilimbi* L. leaf extract indicated the existence of multiple categories of secondary metabolites known for their biological activity. Specifically, this analysis confirmed the presence of flavonoids, alkaloids, steroids, tannins, saponins, and phenols (Table 2). Among these, flavonoids and phenolic compounds were observed at notably high intensities, indicating strong antioxidant and antimicrobial profiles.

Table 2. Phytochemical screening of *Averrhoa bilimbi* L. extract

Phytochemicals Compounds	Current Result	Valsan et al	Ashrafudoulla et al
flavonoids	+	+	+
Alkaloids	+	+	+
Steroids	+	+	NT
Tannins	+	+	+
Saponins	+	-	+
Phenols	+	+	NT

NT: Not tested

The qualitative results demonstrated that flavonoids and saponins produced deep coloration during reagent tests, indicating their high abundance in the extract. Tannins and phenols were identified by the formation of characteristic precipitates, while alkaloids were detected at moderate levels, based on the intensity of turbidity observed after treatment with Dragendorff's and Mayer's reagents. The presence of steroids was confirmed through a positive Liebermann–Burchard reaction.

The consistent detection of these phytochemical groups across three independent extractions demonstrates the reproducibility and chemical stability of the *Averrhoa bilimbi* leaf extract. These findings provide a solid biochemical foundation for understanding its antimicrobial efficacy against *Vibrio parahaemolyticus* and offer insight into the mechanisms underlying the inhibitory effects observed in subsequent antibacterial assays.

2. Antibacterial activity: Inhibition zone and MIC

The antibacterial activity of *Averrhoa bilimbi* L. leaf extract against *Vibrio parahaemolyticus* was found to be concentration-dependent. At a concentration of 16 mg/mL, the extract produced an inhibition zone of 8.83 ± 1.03 mm, which significantly increased to 11.67 ± 0.57 mm at 32 mg/mL (Table 3). Further increases in extract concentration yielded inhibition zones of 13.17 ± 0.28 mm at 48 mg/mL and 15.33 ± 0.84 mm at 64 mg/mL. These results indicate a positive correlation between extract concentration and antibacterial activity ($P < 0.05$).

In comparison, tetracycline (10 µg/mL) exhibited a significantly larger inhibition zone of 25.75 ± 7.96 mm ($P < 0.05$), indicating that although *A. bilimbi* leaf extract has notable antibacterial properties, its efficacy remains lower than that of standard antibiotics.

The broth dilution technique, a widely accepted method for assessing antimicrobial susceptibility, was used to determine the minimum inhibitory concentration (MIC) of the extract **(Elisha *et al.*, 2017)**. In this assay, bacterial growth was measured by changes in optical density (ΔOD) in broth media containing serially diluted extract concentrations ranging from 2 to 64 mg/mL.

Extract concentrations between 2 and 48 mg/mL did not inhibit bacterial growth, as indicated by ΔOD values exceeding 0.00. However, at 64 mg/mL, the ΔOD value was 0.00, indicating complete inhibition of bacterial growth. Therefore, the MIC of *A. bilimbi* leaf extract against *V. parahaemolyticus* was determined to be 64 mg/mL.

Table 3. Result of inhibition zone of *Averrhoa bilimbi* L extract against *Vibrio Parahaemolyticus* bacteria

Concentration extract	Inhibition Zone (mm) ± SD
Tetracycline	25.75 ± 7.96^{e}
16 mg/mL	8.83 ± 1.03^{a}
32 mg/mL	11.67 ± 0.57^{b}
48 mg/mL	13.17 ± 0.28^{c}
64 mg/mL	15.33 ± 0.84^{d}

Values were expressed as mean SD (N=3). Those with various letters are statistically distinct from those with the same letter (P<0.05), whereas those with the same letter are not significantly different (P>0.05).

2. *In vivo* antibacterial activity (Immersion method)

Penaeus monodon post-larvae were immersed in rearing media containing various concentrations of *Averrhoa bilimbi* L. leaf extract for a period of 12 days. Following the treatment, the shrimp were challenged with Vibrio parahaemolyticus by adding the bacteria to the culture water at a final concentration of 10^3 CFU/mL. Clinical symptoms (Picture 1) and survival rates were monitored for 48 hours post-challenge.

The survival data are presented in Table (4). The highest survival rate was observed in the group treated with 40 mg/L extract ($96.00 \pm 6.93\%$), while the control group (0 mg/L) exhibited the lowest survival ($57.00 \pm 8.89\%$). The differences in survival rates among treatments were statistically significant ($P < 0.05$), indicating a dose-dependent protective effect of the *A. bilimbi* extract. These results suggest that higher concentrations of the extract enhanced shrimp resistance to *V. parahaemolyticus* infection

DISCUSSION

These findings are consistent with previous research on the phytochemical composition of *Averrhoa bilimbi* (Table 2). **Suluvoy and Grace (2017)** reported a similar spectrum of compounds in *A. bilimbi* fruit extracts, emphasizing their roles in free radical scavenging and antimicrobial activity. Similarly, **Seebaluck-Sandoram *et al.* (2019)** demonstrated that these phytochemicals confer broad-spectrum antibacterial effects, particularly against Gram-negative pathogens, and elucidated structure–activity relationships for key flavonoid and phenolic constituents. More recently, **Iwansyah *et al.* (2021)** utilized LC-MS metabolomic profiling to identify additional bioactive

components such as quercetin-3-O-glucoside and kaempferol derivatives in *A. bilimbi* leaves, which correlated with enhanced antibacterial potency.

Mechanistically, flavonoids disrupt bacterial membranes and denature essential proteins, leading to cell death **(Farhadi *et al.*, 2019)**. Alkaloids inhibit peptidoglycan biosynthesis, compromising the bacterial cell wall and inducing lysis **(Chusnie *et al.*, 2014)**. Tannins exert antimicrobial action by precipitating membrane proteins and chelating metal ions, thereby weakening membrane integrity. Saponins, on the other hand, promote pore formation in the cytoplasmic membrane, causing leakage of intracellular contents and eventual cell collapse **(Farha *et al.*, 2020)**. These complementary modes of action suggest that the *A. bilimbi* leaf extract has a potent, multi-targeted antibacterial effect against *Vibrio parahaemolyticus*, supporting its potential development as a natural prophylactic or therapeutic agent in shrimp aquaculture.

Our study demonstrated that the *A. bilimbi* leaf extract inhibited the growth of *V. parahaemolyticus* (Table 3), consistent with the findings of **Siddique *et al.* (2013)**, who reported a 9-mm inhibition zone using a 400 µg/disc bark extract. According to standardized interpretations **(NCCLS, 2012)**, inhibition zones are categorized as: ≤16 mm (resistant), 17–20 mm (intermediate), and ≥21 mm (susceptible). Additionally, **Bubonja-Šonje *et al.* (2020)** further classified inhibition zones of plant extracts as weak (≤5 mm), moderate (6–10 mm), strong (11–20 mm), and very strong (≥21 mm).

In our study, *A. bilimbi* extract showed potent antibacterial activity at 32, 48, and 64 mg/mL (inhibition zones of 11.67–15.33 mm) and weaker activity at 16 mg/mL (8.83 mm). Significant differences in inhibition zones ($P< 0.05$) indicate that higher concentrations yielded greater antibacterial effects. Numerous variables can influence inhibition zone size, including:

- Concentration and growth rate of the test organism **(van den Bijllaardt *et al.*, 2017; Jonasson *et al.*, 2020; CLSI, 2022)**
- Amount and diffusion rate of the extract **(Klancnik *et al.*, 2010; Ibrahim & Kebede, 2020; Hossain, 2024)**
- Susceptibility of the organism **(Seebaluck-Sandoram *et al.*, 2019; Jubair *et al.*, 2021)**
- Type, pH, and composition of the agar medium **(Bubonja-Šonje *et al.*, 2020; CLSI, 2022; EUCAST, 2025)**
- Incubation conditions and cell wall structure of the bacterium **(Verma & Balekar, 2023; Youssef *et al.*, 2024)**

Generally, both disk and agar diffusion methods are accepted as preliminary screening tools for assessing antibacterial activity **(Bubonja-Šonje *et al.*, 2020)**. In our study, the inoculum density was 9.02×10^6 CFU/mL, and extract concentrations ranged from 16 to 64 mg/mL, yielding inhibition zones of 8.83–15.33 mm against *V.*

parahaemolyticus. These results justify future studies using higher concentrations and advanced isolation techniques to fully explore the antibacterial potential of *A. bilimbi*.

Phytochemical profiling confirmed the presence of alkaloids, saponins, and flavonoids, while previous reports also identified triterpenoids, cyanidin-3-O-β-D-glucoside, phenolic acids, vitamin A, amino acids, potassium, and citric acid in *A. bilimbi* extracts **(Valsan & Raphael, 2016; Suluvoy & Grace, 2017)**.

Research on the use of plant-based antimicrobials in aquaculture—particularly those from mangrove ecosystems—has shown promising outcomes. For example:

- *Geloina coaxans* and *Centella asiatica* showed inhibition against *V. parahaemolyticus* **(Weliyadi *et al.*, 2018; Rukisah *et al.*, 2019)**
- *Nephrolepis biserrata* and *Moringa oleifera* demonstrated strong inhibitory effects **(Maulianawati *et al.*, 2020; Jannat & Sultana, 2024)**
- *Avicennia marina* and *Rhizophora apiculata* extracts yielded 17.3% and 15.85% inhibition, respectively **(Salim *et al.*, 2022; Supono & Elisdiana, 2022)**
- *Eleutherine bulbosa* exhibited a 91.32% inhibition rate with an MIC of 0.156 mg/mL **(Munaeni *et al.*, 2019)**
- Garlic (*Allium sativum*) showed 11.9 mm zones at high concentrations **(Indrawati & Prasetyo, 2023)**
- *Dunaliella salina*, a microalgae species, showed 27% inhibition **(Farag *et al.*, 2025)**

In comparison, *A. bilimbi* extract produced a 15.33 mm inhibition zone at 64 mg/mL, which is more than a threefold increase compared to earlier reports using lower concentrations. This underscores the effectiveness of optimizing extraction procedures and expanding concentration ranges for enhancing antibacterial outcomes.

Despite showing significant antibacterial activity, the MIC value in this study **(64 mg/mL)** was higher than the 250 µg/mL MIC previously reported for the methanolic fruit extract of *A. bilimbi* **(Seebaluck-Sandoram *et al.*, 2019)**, suggesting lower potency of the leaf extract. Differences in MIC can result from several factors, including plant part used, solvent system, culture media, and incubation conditions **(Van de Vel *et al.*, 2019)**. In this case, the use of leaves instead of fruits likely contributed to the higher MIC observed.

Nevertheless, various parts of *A. bilimbi*—including leaves, flowers, and stems—have been found to contain bioactive phytochemicals with broad-spectrum antimicrobial properties. These extracts have shown inhibitory effects against pathogens such as:

- *Bacillus cereus* ATCC 14579 **(Hassan Cheong *et al.*, 2022)**
- *Escherichia coli* ATCC 25922 **(Hassan Cheong *et al.*, 2022)**
- *Salmonella typhimurium* ATCC 13311 **(Hassan Cheong *et al.*, 2022)**
- *Staphylococcus aureus* ATCC 25923 (**Seebaluck-Sandoram *et al.*, 2019)**
- *Vibrio parahamolyticus* ATCC 17802 (**Seebaluck-Sandoram *et al.*, 2019)**
- *Aeromonas hydrophila* (**Garg *et al.*, 2022)**

- *Pseudomonas aeruginosa* ATCC 27853 (**Seebaluck-Sandoram *et al.*, 2019)**
- *Streptococcus pyogenes* **(Dewi *et al.*, 2019)**

Taken together, our findings support the antibacterial potential of *A. bilimbi* leaf extract as a sustainable, natural alternative to conventional antibiotics for controlling *Vibrio* infections in shrimp aquaculture.

Table 4. The survival rate of *Penaeus monodon* for two days after infected with 10^3 CFU/mL of *Vibrio parahaemolyticus*

Treatment	Survival Rate (%) ± SD
EBW0 (Extract 0 mg/L)	57.00 ± 8.89^a
EBW1 (Extract 20 mg/L)	67.00 ± 5.77^a
EBW2 (Extract 30 mg/L)	83.67 ± 6.11^b
EBW3 (Extract 40 mg/L)	96.00 ± 6.93^c

Values were expressed as mean SD (N=3). Those with various letters are statistically distinct from those with the same letter (P<0.05), whereas those with the same letter are not significantly different (P>0.05).

Shrimps that were not treated with the *Averrhoa bilimbi* L. extract died at a rate of approximately 50% and showed clear signs of illness, indicating a weakened immune response to *Vibrio parahaemolyticus* (Table 4). In contrast, shrimps treated with the extract exhibited higher survival rates, suggesting an improvement in disease resistance. Medicinal plants are known to contain diverse beneficial phytochemicals (phenolics, flavonoids, alkaloids, saponins, anthraquinones, and proteins) **(Guo *et al.*, 2019)** that can stimulate immune responses and exert protective effects. Extraction techniques can significantly affect the yield and bioactivity of phytochemicals **(Ingle *et al.*, 2017)**.

The immune system of shrimp, including *Penaeus monodon*, is dominated by innate immune mechanisms involving hemocytes and antimicrobial molecules such as phenetidines, defensins, and lectins **(Rajendran *et al.*, 2022)**. Although this study did not directly measure the number or activity of hemocytes, the increased survival observed in shrimp treated with *A. bilimbi* L. extract is thought to be related to the stimulation of the shrimp's innate immune system. Hemocytes are the primary immune cells in shrimp and play a key role in fighting bacterial infections through phagocytosis, encapsulation, and the production of antimicrobial enzymes **(Nhnhkorn *et al.*, 2019)**.

Several previous studies have shown that administering natural immunostimulants can increase hemocyte counts and enhance shrimp resistance to pathogens **(Dai *et al.*, 2022; Azhar *et al.*, 2023; Elshopakey *et al.*, 2024)**. Therefore, the protective effect of *A. bilimbi* L. extract observed in this study may also involve hemocyte-mediated immune activation, although this needs to be confirmed through measurements of specific immune parameters in future studies.

Based on our findings, it is reasonable to conclude that *A. bilimbi* L. extract enhances the immunity of *P. monodon* and offers protection against *V. parahaemolyticus* infection. Previous studies support this conclusion. For example, **Handayani *et al.* (2020)** reported that adding starfruit extract to the rearing water of *V. parahaemolyticus*-challenged *Litopenaeus vannamei* significantly reduced *Vibrio* counts both in the environment and in shrimp tissues by day 9 of culture. Antibacterial compounds in *A. bilimbi* (flavonoids, saponins, and tannins) are likely responsible for this immune-enhancing effect.

Similarly, **AftabUddin *et al.* (2021)** reported that *P. monodon* infected with *V. parahaemolyticus* and treated with a seaweed extract achieved a 61–82% survival rate, compared to a 68% survival rate in untreated shrimp. This finding suggests that natural compounds can significantly enhance shrimp immunity and survival following infection. The use of *A. bilimbi* L. extract could thus serve as an effective immunoprophylactic strategy in shrimp aquaculture to improve disease resistance and reduce dependence on antibiotics.

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium typically found in marine and brackish environments. It is recognized as a major pathogen in shrimp aquaculture, causing severe economic losses due to disease outbreaks and high mortality rates **(Kohli *et al.*, 2021; Haifa-Haryani *et al.*, 2023)**. Shrimps exposed to *V. parahaemolyticus* often exhibit a range of clinical symptoms, including dysfunctions in the absorptive, storage, and secretory activities of the hepatopancreas, as well as disruptions in osmoregulatory, respiratory, and other physiological systems **(Deris *et al.*, 2020; Haifa-Haryani *et al.*, 2023)**. Early diagnosis of these clinical signs is essential for timely intervention and successful disease management **(Ali *et al.*, 2018)**.

In this study, clinical observations of *P. monodon* post-larvae infected with *V. parahaemolyticus* revealed passive movement, prolonged bottom-dwelling, irregular swimming, and diminished reflex responses to *Artemia salina* as prey. Additional symptoms included shell discoloration, damaged antennae, skin melanization, tail necrosis, and reddened walking and swimming legs. Shrimps displaying these signs typically died within 24 hours. These findings are consistent with previous reports describing similar clinical manifestations—such as a flushed body, melanized skin, necrotic tail, and darkened hepatopancreas—in shrimp affected by vibriosis **(Haifa-Haryani *et al.*, 2023; Huang *et al.*, 2025)**.

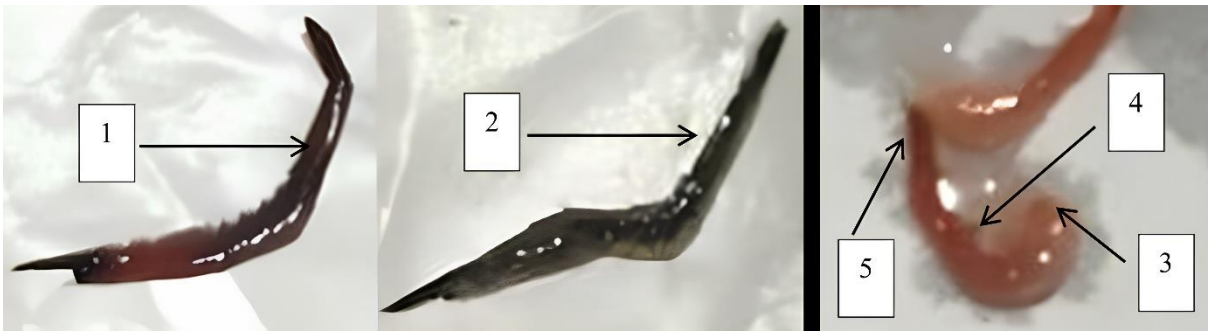

Fig. 1. Clinical Symptoms of *Penaeus monodon* post larva after infected with *Vibrio parahaemolyticus*. (1) Infected *P. monodon*; (2) uninfected *P. monodon*; (3) Red rostrum; (4) Red carapace; (5) Red tail

The pathogenesis of *Vibrio parahaemolyticus* in shrimp involves the expression of multiple virulence factors, including adhesins, thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), and type III secretion systems (T3SS1 and T3SS2) **(Ghenem *et al.*, 2017)**. These factors facilitate bacterial attachment, invasion, and the release of toxins that disrupt cellular functions and induce apoptosis, leading to rapid tissue damage and death **(Ghenem *et al.*, 2017; Haifa-Haryani *et al.*, 2023)**. Histopathological studies have revealed sloughing of epithelial cells in the hepatopancreatic tubules, hemocytic infiltration, extensive vacuolation, and loss of tubule structure in infected shrimp **(Raja *et al.*, 2017)**. The rapid onset of mortality following the appearance of clinical symptoms suggests that the infection induces severe physiological stress and organ failure.

Similar clinical symptoms have been reported in other shrimp species, such as *Litopenaeus vannamei*, indicating a common pathogenic mechanism among *Vibrio* infections in penaeid shrimps **(Kohli *et al.*, 2021; Zhang *et al.*, 2021)**. The high incidence and virulence of *V. parahaemolyticus* in shrimp farms underscore the importance of ongoing surveillance and early detection of clinical signs to prevent disease outbreaks and minimize economic losses **(Hossain *et al.*, 2020)**.

In summary, the clinical symptoms observed in *P. monodon* post-larvae infected with *V. parahaemolyticus* indicate systemic infection and significant tissue destruction. Early recognition of these signs is critical for implementing timely interventions, including water quality management, biosecurity measures, and the application of immunostimulants, to reduce disease incidence and improve shrimp survival in aquaculture systems.

CONCLUSION

This study evaluated the antibacterial effects of *Averrhoa bilimbi* L. extract against *Vibrio parahaemolyticus* both *in vitro* and *in vivo*. The findings demonstrated that *A.*

bilimbi L. extract exhibited significant antibacterial activity against *V. parahaemolyticus*, indicating its potential as a natural antimicrobial agent. These results contribute to the growing body of knowledge on the use of plant-based compounds in shrimp aquaculture, particularly for the control of *V. parahaemolyticus* infections. However, the present study was limited by the absence of histopathological analysis and the relatively short experimental duration. Further research is necessary to evaluate the therapeutic efficacy, safety, and practical applications of *A. bilimbi* L. in aquaculture settings, especially under pond culture conditions.

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