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The Effectiveness of *Halymenia durvillei* Extract as Antibacterial Agent Against Fish Pathogenic Bacteria In Vitro

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ABSTRACT

The use of antibiotics in aquaculture has raised serious concerns regarding the emergence of bacterial resistance and adverse environmental impacts. Red seaweed (*Halymenia durvillei*) is known to contain bioactive secondary metabolites with potential antibacterial properties. This study aimed to evaluate the antibacterial efficacy of ethanol extract of *H. durvillei* against four fish pathogenic bacteria, namely *Edwardsiella tarda*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Vibrio parahaemolyticus*, using agar well diffusion and broth microdilution assays. The experiment was arranged in a completely randomized design consisting of five extract concentrations (100%, 75%, 50%, 25%, and 12.5%) with five replications. The results showed that *E. tarda* exhibited the largest inhibition zone (16.2 mm) at 100% extract concentration, followed by *A. salmonicida* (10.8 mm) and *A. hydrophila* (10.4 mm). In contrast, *V. parahaemolyticus* did not produce an inhibition zone in the agar diffusion assay. The broth microdilution test revealed minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 25% and 50%, respectively, for all tested bacteria. The red seaweed extract demonstrated both bacteriostatic and bactericidal activity, indicating its potential as a natural antibacterial alternative for disease control in aquaculture.

INTRODUCTION

The aquaculture sector in Indonesia has experienced a steady increase in production; however, bacterial diseases remain a major constraint, causing significant economic losses. Several pathogenic bacteria that commonly infect cultured fish include *Edwardsiella tarda*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Vibrio parahaemolyticus*. These bacteria are responsible for various diseases such as edwardsiellosis, motile *Aeromonas* septicemia, furunculosis, and vibriosis, all of which can lead to mass mortality in cultured fish (Austin & Austin, 2016; Fernández & Figueras, 2020).

Disease management in aquaculture has largely relied on the use of antibiotics. Nevertheless, the intensive and uncontrolled application of antibiotics has triggered the emergence of resistant bacterial strains and left residues in fishery products and aquatic environments (Elmahdi *et al*., 2016; Selim *et al*., 2025). This situation has driven the search for natural antibacterial alternatives that are environmentally friendly and sustainable. One promising source of natural compounds with antibacterial properties is marine organisms, particularly macroalgae.

The red seaweed *Halymenia durvillei* is a macroalgal species belonging to the class Rhodophyceae, distributed across tropical Indo-Pacific waters including Indonesia (Budak, 2015; Mon, 2018). Previous studies have reported that *H. durvillei* contains secondary metabolites such as alkaloids, flavonoids, saponins, tannins, and phenolic compounds, which are known to exhibit biological activities including antioxidant, antidiabetic, and antibacterial properties (Arguelles, 2022; Moniung *et al*., 2021; Singkoh *et al*., 2019). Kasmiati *et al*. (2022) reported that the methanol extract of *H. durvillei* collected from Kayangan Island waters, South Sulawesi, was capable of inhibiting the growth of *Salmonella typhi* and *Aeromonas hydrophila* with inhibition zones reaching 26.2 mm and 21.0 mm, respectively.

Although the antibacterial potential of *H. durvillei* has been investigated against certain bacteria, information regarding its activity against a broader range of fish pathogenic bacteria remains limited. Previous studies have generally tested only one or two bacterial species using a single assay method, and thus a comprehensive picture of the antibacterial spectrum of *H. durvillei* has not been obtained. Moreover, the differential response between agar diffusion and broth microdilution methods against Gram-negative fish pathogenic bacteria has not been extensively examined.

This study aimed to evaluate the effectiveness of ethanol extract of *H. durvillei* against four species of fish pathogenic bacteria *in vitro* using two assay methods, namely agar well diffusion and broth microdilution. The use of both methods was expected to provide more comprehensive information regarding the bacteriostatic (inhibitory) and bactericidal (killing) capacity of the extract.

METHODS

The study was conducted from November to December 2025 at the Laboratory of the Fish Quarantine, Quality Control, and Fishery Product Safety Agency (BKIPM) Surabaya II, located at Puspa Agro Modern Wholesale Market, Jl. Sawunggaling 177–183, Jemundo Village, Taman District, Sidoarjo Regency, East Java.

Equipment and Materials

The equipment used included an autoclave, laminar air flow (LAF) cabinet, incubator, hot plate with magnetic stirrer, rotary evaporator, sterile 96-well microplate, micropipette, vortex, and ruler. Research materials consisted of red seaweed obtained from Lombok waters; bacterial isolates of *V. parahaemolyticus*, *A. hydrophila*, *A. salmonicida*, and *E. tarda* (collection of BKIPM Surabaya II Laboratory); 96% ethanol; 10% DMSO; chloramphenicol; Tryptic Soy Agar (TSA); Tryptic Soy Broth (TSB); Mueller-Hinton Agar (MHA); Mueller-Hinton Broth (MHB); Buffered Peptone Water (BPW); NaCl; and sterile distilled water.

Experimental Design

The study employed a Completely Randomized Design (CRD) with five treatment concentrations of *H. durvillei* extract (100%, 75%, 50%, 25%, and 12.5%) (Rajakumar *et al*., 2022), a positive control (chloramphenicol), and a negative control (10% DMSO). Each treatment was replicated five times.

Extract Preparation

Fresh seaweed (1 kg) was washed under running water to remove debris and air-dried at room temperature (±27°C) for 3–5 days until approximately 100 g of initial dry weight was obtained. The samples were subsequently oven-dried at 40–45°C for 24–48 hours until a constant weight was reached, yielding approximately 50 g of oven-dried material.

The dried powder was then macerated in 500 mL of 96% ethanol for 24 hours at room temperature with occasional stirring. The macerate was filtered using vacuum filtration, and the filtrate was concentrated using a rotary evaporator at 50°C to obtain the crude extract (100%). The extract was subsequently diluted serially with 10% DMSO to obtain concentrations of 75%, 50%, 25%, and 12.5%.

Preparation of Bacterial Inoculation

Bacterial isolates were revived from −80°C deep freezer storage into TSB supplemented with 3% NaCl for *V. parahaemolyticus* (TSB + 3% NaCl) and without NaCl supplementation for the remaining bacteria (TSB, 0% NaCl). Cultures were incubated for 24 hours at 35°C for *V. parahaemolyticus* and at 28°C for *A. hydrophila*, *A. salmonicida*, and *E. tarda*. The bacterial cultures were then streaked onto TSA plates and confirmed through biochemical testing. Bacterial suspensions were standardized to a turbidity equivalent to 0.5 McFarland standard ($\approx 1 \times 10^8$ CFU/mL) and subsequently serially diluted to achieve a final concentration of 1×10^5 CFU/mL.

Agar Well Diffusion Assay

Bacterial suspensions were evenly inoculated onto the surface of Mueller-Hinton Agar (MHA) supplemented with 3% NaCl for *V. parahaemolyticus* (MHA + 3% NaCl) and without NaCl for the other bacteria (MHA, 0% NaCl) using sterile cotton swabs. Wells of approximately 6 mm in diameter were punched into the agar, and each well was filled with 75 µL of extract at the respective concentrations. Plates were incubated at the appropriate temperature for each bacterium for 24 hours. The diameter of the inhibition zone was measured using a ruler.

Determination of MIC and MBC

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by broth microdilution and disk diffusion methods, following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2023) with modifications. A volume of 75 µL of bacterial suspension (10^5 CFU/mL) was inoculated into 96-well microplates, followed by the addition of 75 µL of *H. durvillei* extract at each concentration. The positive control consisted of bacterial suspension without extract, and the negative control consisted of sterile MHB. Microplates were incubated for 24 hours at the appropriate temperature. Because the opacity of the extract made direct visual observation difficult, MIC and MBC values were determined by subculturing the suspension from each well onto the appropriate TSA medium. The MIC was defined as the lowest concentration at which bacterial growth was still observed upon subculture, whereas the MBC was defined as the lowest concentration at which no growth occurred.

Data Analysis

Inhibition zone diameter data were analyzed using one-way Analysis of Variance (ANOVA) at a 95% confidence level using GraphPad Prism version 10.6.1 software. Where significant differences were detected, Tukey's HSD multiple comparison test was performed as a post-hoc analysis. Inhibition data (agar diffusion and MIC) and bactericidal data (MBC) are expressed as mean ± standard deviation (SD).

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RESULTS

Antibacterial Activity by Agar Diffusion Method

The assay results demonstrated that *H. durvillei* extract exhibited antibacterial activity against three of the four tested bacteria using the agar diffusion method. The average of inhibition zone diameters at various extract concentrations are presented in Figure 1.

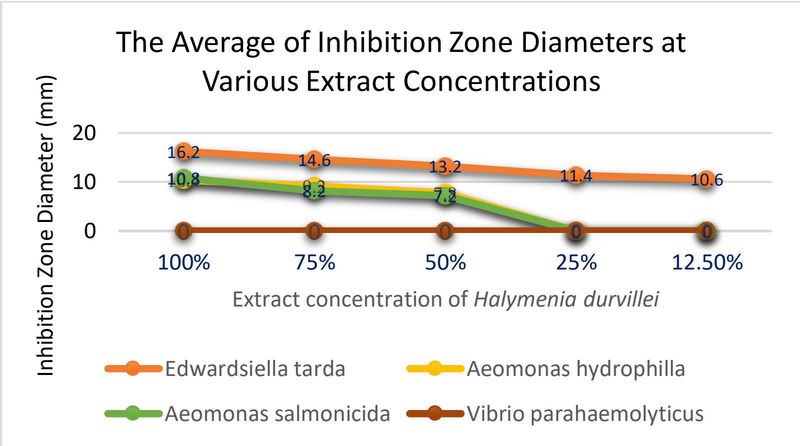

Figure 1. The Average of Inhibition Zone Diameter (mm) of *H. durvillei* Extract Against Four Species of Pathogenic Bacteria

As shown in Figure 1, demonstrate that the ethanolic extract possesses a discernible inhibitory effect on three out of the four tested pathogens: *Edwardsiella tarda*, *Aeromonas hydrophila*, and *Aeromonas salmonicida*. Otherwise, *Vibrio parahaemolyticus* exhibited complete resilience under the solid media conditions, with no detectable zones of inhibition (ZOI) recorded across all treatment groups. *E. tarda* displayed the highest sensitivity to *H. durvillei* extract. At 100% concentration, the inhibition zone reached 16.2 mm and remained detectable down to the lowest concentration (12.5%) at 10.6 mm. This response indicated that *E. tarda* was sensitive across all tested concentrations.

One-way ANOVA revealed that differences in extract concentration had a highly significant effect ($P < 0.0001$) on inhibition zone diameter for the three bacteria that showed a positive response. Tukey's multiple comparison test indicated that the 100% concentration was significantly different ($P < 0.05$) from the 75% concentration for all three bacterial species. These findings confirm that higher extract concentrations resulted in increased antibacterial potency.

Antibacterial Activity by Broth Microdilution Method

The broth microdilution assay provided a more complete picture of the antibacterial capacity of *H. durvillei* extract, particularly in quantitatively and qualitatively determining the inhibitory and bactericidal concentrations. A summary of the MIC and MBC values is presented in Table 1.

Table 1. MIC and MBC Values of *H. durvillei* Extract Against Four Species of Pathogenic Bacteria

Bacterial Isolate	MIC	MBC
E. tarda	25%	50%
A. hydrophila	25%	50%
A. salmonicida	25%	50%
V. parahaemolyticus	25%	50%

As shown in Table 1, all four tested bacteria exhibited uniform MIC values at 25% concentration and MBC values at 50% concentration. At 12.5%, all bacteria showed growth when subcultured on TSA, while bactericidal activity was first observed at 50% concentration. The uniformity of MIC and MBC values suggests that the mechanism of action of the bioactive compounds in the extract is relatively consistent against all four Gram-negative bacteria tested in the liquid medium.

DISCUSSION

Antibacterial Activity by Agar Diffusion Method

The response of *E. tarda* againt *H. durvillei* extract indicated that *E. tarda* was sensitive across all tested concentrations. This high sensitivity is presumably associated with the relatively more susceptible cell membrane structure of *E. tarda* to phenolic and terpenoid compounds, which readily disrupts membrane integrity and leads to cell death (Rahman *et al*., 2022). These findings are consistent with those reported by Putri *et al*. (2021), who documented strong antibacterial activity of *H. durvillei* extract from Sulawesi waters against *Edwardsiella* spp.

The antibacterial activity against *A. hydrophila* and *A. salmonicida* was classified as moderate. Inhibition zones for both bacteria were only observed at concentrations of 50% and above, with diameters ranging from 7.2 to 10.8 mm. At concentrations of 25% and 12.5%, no inhibition zones were detected, suggesting the existence of a minimum threshold concentration for the inhibition of both *Aeromonas* species. The capacity of *A. hydrophila* to form biofilms and its active efflux pump system likely contributed to its higher tolerance compared to *E. tarda* (Khatulistiani *et al*., 2023). Meanwhile, the denaturation of surface layer proteins (A-layer) of *A. salmonicida* by phenolic compounds in the extract is thought to be the primary inhibition mechanism (Vincent *et al*., 2020).

The three bacteria that showed a positive response. These findings confirm that higher extract concentrations resulted in increased antibacterial potency, which is consistent with the pharmacological principle of a positive dose-response relationship (Husni *et al*., 2021). A noteworthy finding was that *V. parahaemolyticus* showed no inhibition zone at any of the tested concentrations using the agar diffusion method. The absence of inhibition zones was presumably not due to a complete lack of antibacterial activity, but rather related to the limited diffusion of active compounds through the solid medium. *V. parahaemolyticus* is a halophilic bacterium with a lipopolysaccharide-rich outer membrane, which may restrict the penetration of antibacterial compounds in agar media (Nguyen *et al*., 2022). This hypothesis was confirmed by the broth microdilution results discussed in the following section.

The antibacterial activity observed in the agar diffusion assay aligns with the report by Kasmiati *et al*. (2022), who found that the methanol extract of *H. durvillei* from Kayangan Island waters, South Sulawesi, inhibited the growth of *A. hydrophila* with an inhibition zone reaching 21.0 mm. Physiologically, this activity is closely associated with the secondary metabolite content, including alkaloids, phenolics, saponins, tannins, and flavonoids, which have been identified through phytochemical screening of *H. durvillei* (Moniung *et al*., 2021; Hanani, 2021). These compounds are capable of disrupting bacterial cell membranes, interfering with protein synthesis, and inhibiting key enzymes involved in cellular metabolism. Alkaloids function by disrupting peptidoglycan components, resulting in incomplete cell wall formation and subsequent bacterial cell death (Hasnah, 2018). Padayao *et al*. (2025) further noted that fermentation of *H. durvillei* with indigenous epiphytic bacteria such as *Bacillus* *safensis* can enhance the diversity of bioactive compounds through biotransformation processes.

Antibacterial Activity by Broth Microdilution Method

The most notable finding from the broth microdilution assay was the ability of the extract to inhibit the growth of *V. parahaemolyticus* at a concentration of 25%, despite this bacterium not forming any inhibition zone in the agar diffusion assay. This discrepancy is presumably related to differences in the interaction between active compounds and bacteria in solid versus liquid media. In liquid media, the contact between active compounds and bacterial cells is more intensive due to more homogeneous distribution, enabling the detection of inhibitory effects at lower concentrations (AlKahlan *et al*., 2019). A similar phenomenon was reported by Nguyen *et al*. (2022), who demonstrated the antibacterial effect of red algal extracts from Vietnamese waters against *Vibrio* spp. compared to brown and green algae.

The difference between MIC and MBC values indicates that at lower concentrations, the *H. durvillei* extract is bacteriostatic, capable of inhibiting growth without completely killing the bacterial cells. At higher concentrations, the extract becomes bactericidal, completely disrupting cell viability. This transition pattern from bacteriostatic to bactericidal activity with increasing concentration is consistent with the findings of Zhang *et al*. (2021), who stated that antibacterial efficacy is highly influenced by concentration and duration of exposure.

The defense mechanisms of individual bacteria also play a role in determining the response to the extract. *A. hydrophila* is known to possess a surface layer protein (S-layer) and the ability to form biofilms, enhancing colony protection by 10- to 100-fold compared to planktonic cells (Rahman *et al*., 2022). Additionally, the efflux pump system in this bacterium actively expels bioactive compounds from the cell interior. Nonetheless, the MIC and MBC values for *A. hydrophila* in this study were comparable to those of the other bacteria, indicating that the intensity of contact in liquid media was sufficient to overcome these defense mechanisms.

Overall, the potential of red algae as a source of natural antibacterial agents is supported by their total phenolic content, which is presumably higher in samples from Lombok waters. Gazali *et al*. (2018) explained that phenolic compounds serve as the primary defense mechanism of red algae against solar radiation and salinity fluctuations, which subsequently enhances their bioactivity. The waters around Lombok, characterized by relatively stable light intensity and salinity, may stimulate more optimal production of secondary metabolites as an adaptive mechanism. The results of this study reinforce the findings of Akbar *et al*. (2025) regarding the phytochemical profile and antibacterial potential of red algae against fish pathogenic bacteria, while providing additional evidence that this red alga merits further development as a natural antibacterial alternative to support sustainable aquaculture practices.

CONCLUSION

The ethanol extract of red alga *Halymenia durvillei* was proven to possess antibacterial activity, both bacteriostatic and bactericidal, against fish pathogenic bacteria, namely *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella tarda*, and *Vibrio parahaemolyticus*. The findings demonstrate the potential of this red alga as a natural antibacterial source that can be developed to support fish health management in sustainable aquaculture. Toxicity testing and *in vivo* trials are recommended to evaluate the efficacy and safety of the extract prior to field-scale application.

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