Ethanolic leaf extracts of Waru (Hibiscus tiliaceus) inhibit biofilm formation of Vibrio alginolyticus in vitro

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Abstract. Vibriosis is still a major threat in aquaculture generating significant implications in ecology and economy. One of the ethologic agents of vibriosis is Vibrio alginolyticus that infects shrimp and fish aquaculture through biofilm-mediated. The objective of the study was to determine the biopotency of ethanolic extract of waru (Hibiscus tiliaceus) leaves to inhibit biofilm formation of V. alginolyticus in vitro. A microtiter plate biofilm assay (OD 570) method was applied in this study. The treatments were the addition of ethanolic leaves extracts of waru (H. tiliaceus) at different concentrations of 2%, 4%, 8%, and 10%. The values of biofilm inhibition activity were measured as optical density data that were statistically analyzed using One Way ANOVA, followed by Tukey’s test and the tests were considered statistically significant at a $P \leq 0.05$ on two-tailed. The result of this study showed that Waru leaves extract significantly inhibited the biofilm formation of V. alginolyticus. The best inhibition was shown at 10% concentration of extract signifying the potential application of waru to treat biofilm-mediated diseases in aquaculture.

1. Introduction

The increasing fish and crustacean aquaculture require integrated management to guarantee its sustainability. One of the main challenges in aquaculture management is the occurrences of diseases infecting the fish and shrimps. The infectious diseases involve various factors, including the decreasing fish or crustacean immunity as well as the persistence of the infectious agents by means of biofilm formation [1]. Biofilm is an association within bacterial communities secreting extra-polysaccharide matrix to assist their attachment in biotic or abiotic surface [2]. For the aquatic pathogenic bacteria, the existence of biofilm offers some beneficial features, such as the protection from the environmental stresses and increasing resistance to desiccations, predation, and antibiotics [3].

Vibriosis is one of the most prevalent infectious diseases in aquaculture possessing biofilm-mediated infection with high mortality rates in cultured fish and shrimps. The disease is etiologically caused by the Gram-negative bacteria from the genus Vibrio, including V.- alginolyticus. The fish suffering from vibriosis show typical symptoms internal bleeding, anemia, hemorrhages, and watery air bladder, whereas the shrimps shows opacity of the abdomens, brown spots on the gills and lymphoid organs (juveniles), and pale hepatopancreases (post larvae) [4].
A variety of means have been employed to control the infectious diseases, including the application of antibiotics and disinfectant agents. However, the extensive use of the antibiotics and disinfectant may lead to the resistant pathogenic bacteria, the polluted environment, and residual accumulation within cultured fish and shrimps [5,6]. As a result, it is essential to find natural-based alternatives, especially from the plants, including waru (H. tiliaceus) to control the infections in aquaculture. Therefore, the objective of the study was to determine ethanolic extract of waru (H. tiliaceus) leaves to inhibit biofilm formation of V. alginolyticus in vitro.

2. Methods
2.1. Bacterial isolates and Hibiscus leaf extraction
Initial isolates of V. alginolyticus were recovered from the freshwater aquaculture center of Ujong Batee, Aceh, Indonesia. The isolates were regrown at on Thiosulfate Citrate Bile Salt Sucrose Agar (TCBSA) (Merck, Germany) prior used in the assay.

Five kilograms of the Hibiscus leaves collected from Aceh Besar, Indonesia. The leaves were then cleaned and washed before they were air-dried for four weeks prior to grinding and sieving into powder. The simplicial powder was subsequently extracted using a maceration method in 1:10 ratio. A 600 g of simplicial were mixed and homogenized in a flask containing six liters of ethanol followed by sealing and incubation for five days. The mixture was then filtered yielding macerates and lees. The resulting macerates were stored in a place protected from light; while the lees underwent the same maceration process for five days using 1.5 L ethanol. All the obtained macerates were concentrated using a rotary evaporator at temperatures below 50 °C to obtain a 100% concentrate. The concentrates were then aliquoted into desired concentrations of 2%, 4%, 8%, and 10%.

2.2. Phytochemical assay
Phytochemical screening assay of ethanolic extract of Hibiscus leaves was intended to qualitatively analyze phytochemical contents found in the extracts, including the presence of alkaloid, flavonoid, terpenoid, as well as tannin compounds.

2.3. Antibiofilm activity assay
The assay to measure the antibiofilm activity of ethanolic extract of Hibiscus leaves was evaluated using a microtiter plate biofilm assay method [7]. Various concentrations (2%, 4%, 8% and 10%) of ethanolic extracts, H\textsubscript{2}O\textsubscript{2} 3% (positive control), as well as the bacterial suspensions without additional ethanol extract (negative control), were used. The standard curved of V. alginolyticus suspension grown in 2% TSB was adjusted so that the estimation of the bacteria formed was ~3x10\textsuperscript{5} CFU/mL.

As much as a 100 μl/well of the extracts at all concentrations, along with H\textsubscript{2}O\textsubscript{2} 3% were added to the bacterial suspensions, except to microtiter plate wells containing the negative control. The plate was then incubated at room temperature for 48 hours followed by washing with sterile PBS three times. A 200 μL of 95% ethanol was gently added to the microplate and allowed to settle for 15 minutes before it was drained and dried. A 125 μL of 0.1% crystal violet was added for dying. After 10 -15 minutes, the microplate was rinsed off using sterile distilled water and dried for several hours. The dried microplate was then filled with 125 μL of 30% glacial acetic acid and incubated for 15 minutes. A total 125 μL of 30% glacial acetic acid was added and the quantity of biofilm formation was calculated by measuring the absorbance values at 570 nm using a microtiter plate reader.

2.4. Antibiofilm activity assay
The data obtained from this study were both qualitative (illustrated qualitatively in the form of tables and figures) and quantitative (values of Optical Density – OD). The values of OD data were statistically analyzed using One Way ANOVA, followed by Tukey’s test using XLStat 2016 (Addinsoft, New York, USA) and the tests were considered statistically significant at a P ≤ 0.05 on two-tailed.
3. Results and Discussion

The phytochemical screening of ethanolic extract of *Hibiscus* leaves was carried out qualitatively. The extract obtained revealed the presence of flavonoids, terpenoids, steroids, tannin, and alkaloids; whereas alkaloids were absent (Table 1). This result is in parallel with the previous study demonstrating the occurrence of phenol, flavonoids, and tannin of ethanolic *Hibiscus* extracts suggesting the potential application of the leaves as antioxidants as well as antimicrobials [8]. Additionally, the result in the current study is also in line with others [9,10] signifying the occurrence of tannin and flavonoid in the *Hibiscus* leaf extracts.

| Phytochemical Assays | Reagents/ Treatments | Observations         | Results |
|----------------------|----------------------|----------------------|---------|
| Alkaloids            | Mayer’s              | No white precipitate | Negative|
|                      | Wagner’s             | No brown precipitate | Negative|
|                      | Dragendorff’s        | No red precipitate   | Negative|
| Steroids             | Liebermann-Burchard test | Green precipitate | Positive|
| Terpenoids           | Liebermann-Burchard test | A small amount of purple precipitate | Positive|
| Saponins             | Shaking              | Foamy                | Positive|
| Flavonoid            | 0.5 Mg and HCl       | Orange precipitate   | Positive|
| Phenolics/Tannins    | FeCl₃                | Green precipitate    | Positive|

**Figure 1.** Percentage of inhibition of biofilm formation temperature after the addition of *Hibiscus* extract at 2%, 4%, 8%, 10% and H₂O₂ 3% (positive control) against *V. alginolyticus* incubated for 60 hours at room temperature. The treatments followed by the same letters above each column are not significantly different (P ≤ 0.05)
The results revealed that ethanolic extract of *Hibiscus* leaves was significantly inhibited the biofilm formation of *V. alginolyticus*. The optimum percentage of biofilm formation inhibition activities was obtained by the addition of *Hibiscus* leaves extract at the concentration of 10% (Figure 1). These results demonstrate the higher the concentration of the extract applied was more likely to have higher inhibition of *V. alginolyticus* biofilm formation. This investigation is in accordance with other studies [11] revealing that the higher the concentration of the administered extracts, the higher the ability of active compounds to penetrate into bacteria; leading to the interference of biofilm-inducing factors. Moreover, another study also found the addition of *Hibiscus* extract showed significant inhibition of bacterial growth and biofilm formation on *Pseudomonas aeruginosa* highlighting the potential application of *Hibiscus* as antibacterial and antibiofilm [9]. The phytochemical compounds of tannin and flavonoid are linked to the antibacterial and antibiofilm activity of *Hibiscus* as those compounds trigger hydroxylation and form a combined complex within the bacterial cell wall [8] or they might disrupt the matrix of extracellular polysaccharides (EPS) or quorum sensing mechanism as an integral part for biofilm formation [9].

4. Conclusion

*Hibiscus* leaves extract significantly inhibited the biofilm formation of *V. alginolyticus*. The most optimum inhibition was shown at 10% concentration of the extract signifying the potential application of *Hibiscus* as a biocontrol agent to treat biofilm-mediated vibriosis in aquaculture

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