Ethanolic extracts of *Moringa oleifera* leaves inhibit biofilm formation of *Vibrio alginolyticus* in vitro

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Abstract. Vibriosis caused by *Vibrio alginolyticus* infection is getting challenging to treat as the bacteria harbor the ability to form biofilm. One of the natural products that might be potentially applied to treat vibriosis through biofilm deformation is the leaves of *Moringa oleifera*. The objective of the study was to determine the effect of ethanol extracts of *M. oleifera* to inhibit *V. alginolyticus* biofilm formation in vitro. A microtiter plate biofilm assay (OD570nm) method was applied in this study. The treatments were the addition of ethanolic *Moringa* leaves extracts at different concentrations of 2%, 4%, 8% and 10%. The result of this study showed that *Moringa* leaves extract significantly inhibited the biofilm formation of *V. alginolyticus*. The optimal condition to inhibit biofilm formation was at 10% concentration for 60 hours incubation at room temperature signifying the potential application of *Moringa* leaf extracts to treat biofilm-mediated diseases in aquaculture.

1. Introduction

*Vibrio alginolyticus* are Gram negative opportunistic bacteria mainly causing secondary infections called vibriosis on marine and freshwater aquaculture for fish and shrimps. The infected fish and shrimps show clinical symptoms, such as the dullness and melanized body of adult or larval fish and shrimps; damages to the fins and gills; decreasing appetite and swimming activity; and glowing during the night [1]. In addition to the aquaculture infection, the vibriosis may also infect humans causing watery diarrhoea accompanied by septicaemia and inflammation in various tissues [2].

It is estimated that 80% of infections in the aquaculture are biofilm-mediated [3] allowing the pathogens to persist and survive within aquaculture system [4]. Biofilm-producing microorganism, including *Vibrio alginolyticus*, initially produce extracellular polymeric substances (EPS) followed by colonization of the surface and formation of biofilm. Once it is formed, the biofilm structure allows the bacteria to increase their infection and to be protected from the unfavourable environmental conditions, such as high salinity and antibiotics [5].

*Moringa oleifera* has been widely known as miracle tree and mother’s best friend due to their functional properties in food, medicine, and industries [6, 7]. Additionally, *Moringa* is also identified to be one of the bioactive compound producers, i.e. antimicrobials, that might be potentially applied to inhibit biofilm formation [8, 9]. However, studies of the antibiofilm potential of *M. oleifera*, specifically inhibit *V. alginolyticus* responsible for vibriosis in aquaculture is scarce. Therefore, the present study aimed to evaluate the effect and determine the phytochemical compounds and the optimum inhibition concentration of the ethanolic extract of *Moringa* leaves on biofilm formation of *V. alginolyticus* in vitro.
2. Methods
2.1. Bacterial isolates and plant extraction
The isolates *Vibrio alginolyticus* were recovered from the Freshwater Aquaculture Centre of Ujung Batee, Aceh, Indonesia. The isolates were regrown at Thiosulfate Citrate Bile Salt Sucrose Agar (TCBSA) (Merck, Germany) prior used in the assay.

A five kilograms of the *Moringa* leaves collected from Aceh Besar, Indonesia were cleaned and washed before the leaves were then air-dried for four weeks prior to grinding and sieving into powder. The simplicial powder was then extracted using maceration method in 1:10 ratio. Approximately 600 g of simplicial were mixed and homogenized in a flask containing six litters of ethanol; sealed and incubated for five days. The mixture was then filtered yielding macerates and lees and 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 ºC to obtain a 100% concentrate.

2.2. Phytochemical tests
Phytochemical tests of ethanolic extract of *Moringa* leaves were intended to examine various phytochemical contents found in the extracts. The extract samples were performed in the Research Laboratory of Chemistry Department, Faculty of Science, Syiah Kuala University to be qualitatively analysed, including the presence of alkaloid, flavonoid, terpenoid, as well as tannin compounds.

2.3. Determination condition and the ability of biofilm formation of *V. alginolyticus*
Quantification of biofilm formation was performed using Microtiter plate biofilm assay [11]. The standard curve of *Vibrio alginolyticus* suspension in 2% Tryptone Soy Broth (TSB) was calculated and approximate bacteria formed was ~3x10^5 cfu/mL (data not shown). Afterwards, a 10 μl/well of the bacterial suspension was inoculated into a microplate plate. Two types of TSB media; including that supplemented with 2% NaCl + 2% sucrose, as well as that supplemented with only 2% NaCl were prepared. A 100 μl/well medium was then added to the bacterial suspensions, inoculated in different wells and incubated for 12, 24, 36, 48, 60, 84, and 92 hours at room temperature.

After incubation, microplate was washed three times with sterile phosphate buffer saline (PBS). A 200 μL of 95% ethanol was gently poured in the microplate and left for 15 minutes prior to draining and drying. The staining process was conducted using 125 μL of 0.1% crystal violet for 10 - 15 minutes, 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 the 30% glacial acetic acid was added and the optical density (OD), which describes the quantity of biofilm formation, was measured at a wavelength of 570 nm using a microtiter plate reader (Bio-Rad, CA, USA). Determination of biofilm formation, i.e. Not found, low, moderate, or high, was determined using criteria based on OD values following previous study [10].

2.4. Determination for inhibition of biofilm formation
Inhibition of biofilm formation of ethanolic extract of *Moringa* leaves was evaluated using a microtiter plate biofilm assay method [10]. Various concentrations (2%, 4%, 8% and 10%) of ethanolic extracts, H_2O_2 3% (positive control), and the bacterial suspensions without additional ethanol extract (negative control), were used. The standard curve of *Vibrio alginolyticus* suspension grown in 2% TSB was adjusted so that the estimation of the bacteria formed was ~3x10^5 CFU/mL.

Ethanolic extract of *Moringa* leaves were serially diluted in TSB supplemented with 2% NaCl + 2% sucrose to obtain the concentrations of 2%, 4%, 8% and 10%. The extracts at all concentrations, along with H_2O_2 3% were then added to the bacterial suspensions, except to the negative control well, as much as a 100 μL/well. The treatments were then incubated at room temperature for 48 hours. After incubation, the microplates were washed 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 staining process. 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. The assay was performed in triplicate and the results of the biofilm formation inhibition were calculated using the following formula Sandasi et al [11]:

\[
\% \text{ of inhibition} = \frac{\text{OD of negative control} - \text{OD of experimental sample}}{\text{OD of Negative control}} \times 100\%
\]

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 by means of SPSS 20 (IBM, New York, USA). The significant level was set at \( p \leq 0.05 \) at two-tail.

3. Results and Discussion

The total extract and the total extraction yield obtained from a 600 g of simplicial Moringa leaves by 96% ethanol were 138.7 g and 23.11%, respectively. The phytochemical screening of ethanolic extract of Moringa leaves was carried out qualitatively. The extracts revealed the presence of flavonoids, phenols (tannins), terpenoids, steroids, and alkaloids; whereas saponins were absent (Table 1).

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

The phytochemical screening of ethanolic extract of Moringa leaves revealed the presence of flavonoids, phenols (tannins), terpenoids, steroids, and alkaloids; as well as the absence of saponins. This finding is in accordance with other study Putra et al [12] suggesting phytochemical screening of Moringa leaves showed positive results for all the mentioned compounds, except for saponins. The ethanolic extract of Moringa leaves contains active substances of tannins and flavonoids [13]. The presence of tannins in the extract was observed by the presence of dark green coloration in phytochemical assay of phenols using FeCl₃. The formation of dark green precipitated in the extract upon the addition of FeCl₃ is due to the formation of complex compounds by tannins and Fe³⁺ ions [14]. The presence of phenolic groups in the samples indicates the possibility of tannins’ occurrence in the extract since they are polyphenol compounds. As suggested, tannin compounds are potential for anti-biofilm agents [13]. The presence of flavonoids in the extract was confirmed by the formation of red-orange precipitate in the sample solutions after the addition of 0.5 Mg and HCl reagents. This red-orange coloration was observed as a result from the reduction of benzopyrone core contained in flavonoids by HCL as well as by reduction-oxidation reaction between Mg and flavonoids [15]. Flavonoids have been declared to possess anti-biofilm activities toward Staphylococcus aureus due to its ability to inhibit intercellular adhesion genes icaA and icaD [16].

In addition to flavonoids and tannins, the ethanolic extract of Moringa leaves in this study also contained terpenoids, steroids, and alkaloids. Terpenoids are defined as compounds whose carbon...
structures are composed of a complex cyclic structure of six isoprene units and are mostly found in the form of alcohols, aldehydes or carboxylic acids. Moreover, terpenoids are difficult to be characterized due to the absence of colours, appear in crystal form, and possess a high melting point. On the other hand, steroids are a group of compounds that possess a basic structure called cyclopentanoperhydrophenanthrene. It serves various biological functions in plants, such as growth regulators (sesquiterpenoids, abscisin, and gibberellin); dye (carotenoids) which play a role in photosynthesis; as well as insect repellents and attractants [14]. Steroids had been formerly considered as compounds found only in animals, but today, it turns out that the compounds are also widely discovered in plants in the form of phytosterols, including *Moringa* leaves which contained stigmasterol, sitosterol and kampesterol [17]. The presence of alkaloids in ethanolic extract of *Moringa* leaves was confirmed by the formation of brown precipitate upon the addition of Wagner’s reagent.

*Vibrio alginolyticus* isolates regrown on tryptone soya agar supplemented with 2% NaCl were tested its biofilm formation activities. Qualitative assay on biofilm formation of these bacteria on Congo red agar (CRA) showed positive results. In addition to biofilm formation test using CRA, biofilm of *V. alginolyticus* in this investigation was also directly observed in TSB supplemented with NaCl 2% + sucrose 2% in the form of cream-colored slime layers produced both in the bottom and on the surface of the suspension. This qualitative assay on biofilm formation of *V. alginolyticus* demonstrated the ability of the bacteria to form biofilm. Biofilm formation on CRA medium was induced by the addition of sucrose as the sucrose serves as raw materials in the formation of extracellular polymeric substance (EPS) that reacts with Congo red dye to produce black-coloured colonies [18]. In addition to sugar supplementation as a basic ingredient in preparing CRA medium, low-nutrient growth medium also effectively induced the formation of biofilm. Some Vibrio genera possess the ability to duplicate faster on low-nutrient medium since they have to compete with other bacteria in utilizing nutrients. Frequently- used media as basic ingredients in formulating CRA are Brain Heart Infusion Agar (BHIA) and Tryptone Soy Agar (TSA) [19]. Determination of biofilm formation using CRA allows the detection of exopolysaccharide occurrence by evaluating colour indicators formed in the colonies [20]. In addition to biofilm formation test using CRA, biofilm of *V. alginolyticus* in this investigation showed cream-colored slime layers produced both in the bottom and on the surface of the suspension in TSB supplemented with NaCl 2% + sucrose 2%.

![Figure 1](image.png)

**Figure 1.** Percentage Optical density of *V. alginolyticus* grown on two types of media, media A containing TSB+NaCl 2% and media B containing TSB+NaCl 2%+sucrose+2% incubated for various times of incubation (12, 24, 36, 48, 60, 84, and 92 hr)
Based on every-12-hour observations of biofilm formation at room temperature revealed that the optimum incubation period for biofilm formation by *V. alginolyticus* in this study was 60 hours with the OD values of 0.687 nm and 0.774 nm on media A (TSB + NaCl 2%) and B (TSB+NaCl 2%+sucrose 2%), respectively (Figure 1). The efficacy of this medium B is due to biofilm’s main constituents called exopolysaccharides which are composed of sucrose polymer [5]. Consequently, the addition of sucrose to the increased the ability of the bacteria to form thick biofilms in a shorter period of time.

Biofilm formation of *V. alginolyticus* began to decrease at 84 hours of incubation at room temperature with the OD values of 0.472 nm and 0.486 nm on A and B medium, respectively. The reduction of biofilm formation occurs after three days of incubation, assumingly, since biofilm has reached its final stage or also known as dispersion [5]. Dispersion is the reduction of biofilm due to the dispersal of cells from the colonies, followed by other matrix constituents to produce biofilms on new surfaces, since the former location has lacked nutrients and been accumulated with waste products [5]. Naturally occurring dispersion without the influence of anti-biofilm administration was induced by the presence of dispersion-causing enzymatic activity [21] or by a high cell density that triggers quorum sensing for *hapR* gene translation of exopolysaccharide [22].

After investigating both the optimum medium and incubation time for biofilm formation, determination of biofilm formation inhibition using Microtiter plate biofilm assay method was performed. The results revealed that ethanolic extracts of *Moringa* leaves were effectively inhibited the biofilm formation of *V. alginolyticus*. The highest average percentage of biofilm formation inhibition activities was obtained by the addition of *Moringa* leaf extracts at the concentration of 10% (Figure 2).

![Figure 2](image_url)

**Figure 2.** Percentage of inhibition of biofilm formation temperature after the addition of *Moringa* leaf extract at 2%, 4%, 8%, 10% and H2O2 3% (positive control) against *V. alginolyticus* incubated for 60 hours at room. The treatments followed by the same superscripts are not significantly different (P ≤ 0.05).

These results indicate that the higher the concentration of the extract applied was more likely to inhibit inhibition of *V. alginolyticus* obtained. This investigation is in accordance with other studies [13, 23] revealing that the higher the concentration of the administered extracts, the greater the ability of active compounds to penetrate into bacteria; leading to the interference of biofilm-inducing factors. The result of One-way ANOVA using SPSS revealed that there were statistically significant differences across various additions of *Moringa* extract concentrations, especially at the concentration of 10%. However, statistical differences between the treatment of positive control (H2O2 3%) and the addition of 10% concentration of ethanolic extract were not observed (P >0.05). The lack of these statistical differences might bring about the ethanolic extract of *Moringa* leaves to become a potential
alternative to the $\text{H}_2\text{O}_2$ 3% in overcoming vibriosis. Nonetheless, improper administration of $\text{H}_2\text{O}_2$ 3% might lead to the damage of seed and poisoning in fish [24].

Anti-biofilm potency of *Moringa* leaves extract against *V. alginolyticus* in this investigation was due to presence of various bioactive compounds, including flavonoids, tannins, terpenoids, steroids, and alkaloids. Flavonoids and tannins in *Moringa* leaves presumably play important roles inhibiting bacterial activity as well as biofilm formation by *V. alginolyticus* by means of blocking N-Acyl homoserine lactones (AHLs) signals [25, 26]. Additionally, flavonoids and tannins were also reported as potential Quorum sensing (QS) inhibitors in *Chromobacterium violaceum* [26], *Escherichia coli* O157:H7 and *Vibrio harveyi* [26]. Various flavonoids such as naringenin quertecin, sinensetin, and apigenin potentially inhibit the QS signal autoinducers (AHL) in *Vibrio harveyi*; resulting in the inhibition of biofilm formation [26].

4. Conclusion
It is concluded that the ethanolic extract of *Moringa oleifera* leaves in this study contained flavonoids, tannins, terpenoids, steroids, and alkaloids. The optimum time for biofilm formation of *V. alginolyticus* was 60-hour incubation at room temperature on TSA supplemented with NaCl 2%+sucrose 2%. Additionally, a significant inhibition of the ethanolic extract of *Moringa* leave, especially at concentration of 10%, on the biofilm formation of *V. alginolyticus* signifying the potential application of *Moringa* leaf extracts to treat biofilm-mediated diseases in aquaculture.

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