Vibriocidal Activity of Ethanol Extract of Moringa Leaves and Its Effect on the Growth of Pacific White Shrimp

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Abstract

This study aimed to evaluate the vibriocidal potency of Moringa leaf ethanol extract (MLEE) and assess the effectiveness of dietary supplementation of MLEE on Pacific white shrimp growth performance. A vibriocidal activity was performed using disk diffusion and tube dilution methods. The results showed Vibrio parahaemolyticus was more susceptible than *Vibrio harveyii* with an inhibitory zone of 11.30 to 22.90 mm. The minimum inhibitory concentration (MIC) of MLEE against *V. parahaemolyticus* was recorded at 12.5%, similar to *V. harveyii*. The effectiveness of MLEE on diet was monitored by the growth of the shrimps for 40 days of culture. Shrimps with an average initial weight of 2.50±0.05 g were acclimatized in 10 days. The MLEE was administered to the shrimps at different concentrations i.e., 10%, 15%, 20%, and 25%. The control diet was prepared without MLEE supplementation. After 40 days of culture, the shrimps fed with 10% of MLEE had the best growth. This study reported that MLEE potentially become vibriocidal agents. However, the administration on shrimp had no beneficial effect on its growth performance.

Keywords: moringa, extract, growth, vibriocidal, Pacific white shrimp

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INTRODUCTION

Indonesia has become one of the most important shrimp suppliers in the world, especially in the United States, Japan, and the European Union (Wati, 2018). The increasing population and human protein needs have demanded higher shrimp production through intensification. On the other hand, the intensification of shrimp production can cause the risk of disease as the emerging loss of shrimp production (Awad and Awaad, 2017). One of the common diseases in the shrimp-intensive culture is vibriosis caused by Vibrio, especially *Vibrio harveyii* and *Vibrio parahaemolyticus* (Patel *et al*., 2018).

Various technologies, e.g., the utilization of antibiotics and vaccines, have been applied in shrimp culture to manage shrimp health and reduce the spread of pathogens. Nowadays, some antibiotics have been banned in aquaculture because of adverse effects such as the emergence of bacterial resistance, residual accumulation in shrimp body tissue, and the environment. Meanwhile, the use of vaccines is considered less effective because vaccines are expensive and only work against one type of pathogen (Aoki *et al*., 2008; Reverter *et al*., 2014). Consequently, using medicinal plants to prevent shrimp diseases can be an alternative method. Medicinal plants in aquaculture are not only therapeutic agents but also feed additives that can stimulate growth, immune system, and anti-stress (Chakraborty and Hancz, 2011).

Moringa oleifera belongs to the Moringaceae family which is indigenous to India. This plant has a high tolerance to the environment and thus can grow in many tropical and subtropical regions, such as Egypt, Cuba, Nigeria, Philippines, Thailand, Malaysia, and Indonesia (Muhammad *et al*., 2016). Moringa trees are known to have many beneficial properties due to their high nutritive content. Every part of the tree has been widely used for culinary purposes, bioremediation, and medicinal treatment (Gupta *et al*., 2018).

Leaves are the most potent part of the Moringa tree for use. Leaves grow more sustainably than other parts, such as flowers, fruits, and seeds. Moringa leaves are rich in essential amino acids, starch, iron, calcium, and vitamins A, B, and C (Ganatra *et al*., 2012). A hundred grams of Moringa leaves contain around 28.65% of crude protein, 7.09% of crude lipids, 44.36% of carbohydrates, and 10.90% of ash. Moreover, Moringa leaves contain phytochemical compounds such as tannins, triterpenoids, alkaloids, polyphenols, flavonoids, and carotenoids (Teixeira *et al*., 2014). Numerous phytochemical compounds in Moringa leaves provide various potential therapeutic properties, for example, being antioxidant, anticancer, antidiabetic, antifungal, antimicrobial, analgesic, anti-inflammatory, and immune-system stimulant (Martínez-González *et al*., 2017; Punia and Singh, 2018).

Technically, one of the medicinal plant administrations in aquaculture can be done through dietary supplementation (Immanuel *et al*., 2009). The utilization of Moringa leaf extract as an in vitro vibriocidal agent has been done by Peixoto *et al*. (2011). However, there is a lack of information on the effectiveness of dietary supplementation of Moringa leaf ethanol extract (MLEE) on Pacific white shrimp (*Litopenaeus vannamei*) for developing natural alternative drugs in aquaculture. Therefore, this study aimed to evaluate the vibriocidal potency and assess the effectiveness of dietary supplementation of MLEE on Pacific white shrimps as seen from their growth.

MATERIALS AND METHODS

Moringa Leaves Extract Preparation

Moringa leaves were collected from the plantation area in Banyuwangi and were air-dried for 3 days and ground. About 500 grams of dried leaves were soaked in ethanol 96% solvent for 24 hours. Then, the mixture was filtered, and the filtrate was concentrated using a rotary vacuum evaporator (IKA, RV10, Malaysia). The concentrated ethanol extract was stored in a refrigerator. A phytochemical test was performed to determine total flavonoid content, total alkaloid content, total tannin, and total saponin using a spectrophotometer UV-Visible (Shimadzu, UV-1800, Japan). These tests were carried out in the Faculty of Pharmacy, Universitas Airlangga, Indonesia.

Antibacterial Assay

Antibacterial activity of MLEE was performed against *V. harveyii* and *V. parahaemolyticus*. Vibrio cultures were obtained from the culture collection of Brackishwater Aquaculture Development Center Situbondo. The test was carried out using disc diffusion and tube dilution methods with slight modification as reported by Genovese *et al*. (2012). The microbes were suspended in a physiologic solution and counted using the total plate count until they reached the same turbidity as the Mc Farland standard ($\pm 10^6$-10^8 CFU/mL). Then, the suspension was cultured using the Tryptone Soya Agar (TSA) (Merck, Germany) with 3% NaCl. The extracts with different concentrations were injected into a paper disc (6 mm) and placed on a medium containing the tested microbes. The medium was incubated at 32ºC for 24 h and the inhibition diameter zone was then measured. An oxytetracycline disc (Oxoid, CT0041B, United Kingdom) was used as a positive control, while dimethylsulfoxide (DMSO) was used as a negative control.

A minimum inhibitory concentration (MIC) was determined by the serial dilution assay. Serial dilution of extracts at 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78% of concentrations were prepared in Nutrient Broth (Merck, Germany) tubes. Microbe suspension (0.2 mL) was added to each tube and incubated at 32ºC for 24 h. The inhibition activity of MLEE was confirmed by growing the suspension onto a TSA medium containing 3% NaCl plate after overnight incubation at 32ºC.

Experimental Diet Preparation

Experimental diets were prepared using a slightly altered method as reported by Yunis-Aguinaga *et al*. (2016). A commercial shrimp feed (Kaiohji produced by PT Matahari Sakti,

Indonesia) contains 35% crude protein used as the basal experimental diet. The experimental diets were prepared by being supplemented with MLEE with different concentrations, i.e.,10%, 15%, 20%, and 25%. The MLEE was weighed according to the determined concentration, i.e., 10, 15, 20, and 25 g. The MLEE was added with 1 mL egg yolk and 10 mL aquadest. The mixture was homogenized using a vortex and sprayed into 100 g of commercial feed. Then, the experimental diet was dried using an oven at 60°C overnight. The control diet did not contain MLEE.

Study Design

This study used juvenile Pacific white shrimps collected from PT Surya Windu Kartika, Banyuwangi. This study was conducted without ethical approval due to no harmful/abusive actions were performed on the shrimps. The shrimps have an average weight of 2.50±0.05 g. They were acclimatized for 2 weeks before the treatment and fed four times daily as much as 4% of body weight. This current study used 5 treatments and 4 replications. The shrimps were fed for 40 days using the control and experimental diets. All treatments were performed in twenty tanks with a capacity of 36 liters. Water quality parameters such as temperature, pH, dissolved oxygen, and ammonia were measured during the maintenance. The temperature range was 25-26°C, pH at 7-8, salinity at 30-32‰, dissolved oxygen at 5-8 mgL^{-1}, and ammonia nitrogen at 0-0.8 mgL^{-1}. All of these data were collected during the experiment period. Removal of residual feed and fecal matter was performed by siphon once a week.

Growth Performance

During the maintenance period, the body weight of shrimps was measured at intervals of 10 days. The quality of diets was observed from the biological parameters such as average daily growth (ADG), specific growth rate (SGR), and feed conversion ratio (FCR).

Statistical Analysis

Statistical analysis was performed with SPSS v13. All of the data were analyzed using a one-way analysis of variance and Duncan's multiple range tests. Differences were considered significant if a significance level was less than 0.05.

RESULTS AND DISCUSSION

In this study, MLEE was obtained from maceration. The obtained extract is blackish green and the yield is around 25.74-31.38%. Based on its phytochemical screening test, the extract contained flavonoids, polyphenols, terpenoids or steroids, and saponin. The total flavonoid, total alkaloid, total tannin, and total saponin content of MLEE were displayed in Table 1.

The MLEE was screened for its antibacterial activity against *V. harveyii* and *V. parahaemolyticus*. The antibacterial activity of MLEE against both bacteria was attributed to the appearance of a clear zone around the disc. The result of the antibacterial activity test was presented in Table 2.

The inhibition activity increased along with higher concentrations of MLEE given. Based on the statistical analysis, there were significant differences ($p<0.05$) in the antibacterial activity between all treatment and control groups. Oxytetracycline as positive control showed the strongest inhibition activity towards *V. harveyii* and *V. parahaemolyticus*. The inhibition activity of 100% MLEE towards V. parahaemolticus did not differ significantly from oxytetracycline. However, the inhibition activity of 100% MLEE towards *V. harveyii* was significantly lower than oxytetracycline. The MIC of MLEE against *V. harveyii* was recorded at 12.5%, similar to *V. parahaemolyticus*.

The results revealed that MLEE inhibited the growth of both bacteria where V. parahaemoliticus was more susceptible than *V. harveyii*. This result is consistent with an earlier study by Peixoto *et al*. (2011), which stated that MLEE has vibriocidal compounds which inhibit *V. parahaemolyticus*. The antibacterial activity of MLEE was identified from various phytochemicals such as alkaloids, flavonoids, polyphenols, terpenoids, saponin, and tannin (Ravikumar *et al*., 2010; Sharma *et al*., 2009).

Table 1. Phytochemical content of MLEE

Analysis	Total (mg.g^{-1})
Total flavonoid in QE	71.9
Total alkaloid in quinine equivalent	3
Total tannin in tannic acid equivalent	24.7
Saponin	44.4

Table 2. Antibacterial activity of MLEE

Treatments	Inhibition zone (mm)	
	V. parahaemoliticus	***V. harveyii***
DMSO	0.00^{d}±0.00	0.00^{d}±0.00
Oxytetracycline	22.30^{a}±1.70	27.50^{a}±0.84
25% MLEE	11.35^{c}±1.38	6.32^{c}±0.70
50% MLEE	17.62^{b}±2.25	9.27^{bc}±3.17
75% MLEE	20.02^{ab}±2.90	12.55^{b}±3.90
100% MLEE	22.92^{a}±2.81	8.27^{c}±2.29
p-value	<0.001	<0.001

Means with different superscripts in the same column are significantly different (p<0.05).

Table 3. The growth of *L. vannamei* fed with different experimental diets

Treatment	Initial Weight (g)	Final Weight (g)	ADG (g.day^{-1})	SGR (%)	FCR
Control	3.81±0.35	6.73±1.88	0.07±0.04	1.35±0.53	4.09±2.59
10% MLEE	3.74±0.17	6.62±1.21	0.07±0.03	1.40±0.39	4.09±1.93
15% MLEE	3.76±0.21	6.33±1.02	0.06±0.02	1.28±0.35	4.13±0.60
20% MLEE	3.71±0.25	6.11±0.83	0.06±0.02	1.24±0.3	4.33±0.97
25% MLEE	3.68±0.40	5.87±0.56	0.05±0.01	1.17±0.1	4.74±0.45
p-value			0.821	0.901	0.970

This current study has also reveal that the extracts contained flavonoids, polyphenols, terpenoids, steroids, and saponins. The screening results confirmed the possible use of MLEE as an antibacterial agent in shrimp disease management.

L. vannamei fed with MLEE with different concentrations demonstrated growth results as shown in Table 3. Generally, *L. vannamei* given all treatments had improved growth during the maintenance period. However, supplementation of MLEE on diet gave no significant differences in growth (p>0.05). The shrimps fed with 10% MLEE showed the highest ADG and SGR (0.07±0.03 and 1.40±0.39, respectively), while the lowest ADG and SGR were found in the shrimps fed with 25% MLEE. The highest FCR was observed in the shrimps fed with 25% MLEE. The high value of FCR indicated the poor quality of feed.

The diet containing 10% MLEE yielded the best values of ADG, SGR, and FCR. Meanwhile, a higher concentration of MLEE in the shrimp diet tended to decrease the shrimp's growth. Mansour *et al*. (2018) reported a similar result that supplementation of more than 10% MLEE declined fish growth. Puycha *et al*. (2017) also reported that the growth of Pangasius bocourti reduced significantly due to the supplementation of Moringa leaf in the diet. The reduction of shrimp growth may be related to phytochemicals in MLEE. Some phytochemicals in plants can be anti-nutritional factors. Anti-nutritional factors are substances or chemicals produced by a plant that interferes with nutrient absorption and affects the health of organisms (Kumar *et al*., 2012). Based on the phytochemical screening test in this current study, MLEE contained flavonoids, polyphenols, terpenoids, and saponins. At optimal doses, these phytochemicals will benefit the organism. For example, it can enhance the shrimp's immune system (Kamble *et al*., 2014). However, the excessive dose will induce deleterious effects e.g., being poisonous to animals, adverse physiological effects, decreased growth, and health conditions (Hajra *et al*., 2013).

Some previous studies explained that Moringa leaf has anti-nutritional factors that can lead to the reduction of protein digestion and vitamin-mineral absorption (Falowo *et al*., 2018). In addition to the appearance of anti-nutritional factors, almost all protein in Moringa leaves is insoluble and has low digestibility. Teixeira *et al*. (2014) claimed that a Moringa leaf has poor quality protein, proven by a protein hydrolysis test with the result of its digestibility of only about 33%, which is lower than casein. Furthermore, the presence of tannins may increase resistance against enzymatic hydrolysis.

Nutrition is one of the factors related to shrimp's immune response. Aja *et al*. (2014) explained that Moringa leaf methanol extract contains around 16 compounds such as ascorbic acid or vitamin C. Ascorbic acid, phenolic compounds, flavonoids, and carotenoids in diet serve as an antioxidant for improving the shrimp's immune system (Lotaka and Piyatiratitivorakul, 2012). However, the appearance of anti-nutritional factors in MLEE causes hard absorption of protein, essential vitamins, and minerals in the shrimp's body tissues (Gilani *et al*., 2012; Gopan *et al*., 2020). Consequently, the utilization of nutrients in the diet becomes less optimal and it revealed that in poor growth performance.

CONCLUSION

The presence of phytochemicals such as flavonoids, terpenoids, polyphenols, and saponins in MLEE improved its vibriocidal activity against *V. harveyii* and *V. parahaemolyticus*. However, the supplementation of MLEE on the shrimp diet negatively impacted the shrimps. The phytochemicals e.g., saponin could be anti-nutritional factors that depressed the nutrient intake by the shrimps. Therefore, the growth of shrimps would be poor. Further, it is important to explore the most proper supplementation of MLEE as an antibacterial agent in the shrimp culture which is useful to improve the added value of Moringa in aquaculture.

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