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Effectiveness of Meniran Leaf Extract (*Phyllanthus niruri* L.) as Immunostimulant in Vannamei Shrimp (*Litopenaeus vannamei)* Against Vibriosis Disease

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Abstract

Vibrio harveyi infection was an obstacle in shrimp culture. Meanwhile, the use of antibiotics in disease control was no longer effective, so the use of immunostimulants such as meniran leaves was the safest alternative. The purpose of this study was to determine the effect of adding different doses of meniran leaf extract (*Phyllanthus niruri* L.) to feed on the immune system of vannamei shrimp (*Litopenaeus vannamei*) which was challenged with *Vibrio harveyi*. This research was an experimental study using completely randomized design (CRD) consist of 5 treatments and 3 replications, namely, P1 (positive control): commercial feed + *V. harveyi* infection; P2 (negative control): commercial feed + 0.9% NaCl; P3: commercial feed + 0.5% Meniran leaf extract + *V. harveyi* infection; P4: commercial feed + 1% Meniran leaf extract + *V. harveyi* infection; and P5: commercial feed + 2% Meniran leaf extract + *V. harveyi* infection. The parameters observed were survival and immune response. Immune response analysis was carried out by counting hemocytes, phagocytic activation and total bacteria present in the shrimp intestines at the end of the study. The results of this study indicate that the addition of meniran leaf extract affects the immune system and the viability of vannamei shrimp. In conclusion, the addition of meniran extract to vannamei shrimp feed with a dose of 1% can improve the immune system and viability of vannamei shrimp against vibriosis.

INTRODUCTION

Vannamei shrimp (*Litopenaeus vannamei*) is a mainstay commodity in the Indonesian fisheries sector which is included in the list of fisheries revitalization programs other than seaweed and tuna. According to data from the Central Statistics Agency (2017), the export value of vannamei shrimp in 2015 reached 145,007.9 tons. However, behind these achievements, the obstacles that are still experienced by the farmers are diseases caused by *Vibrio harveyi infection*, such as eye lesions in shrimp, vasculitis (inflammation of the blood vessels) and fluorescent vibriosis. According to Huang *et al.* (2013), fluorescent vibriosis is the main cause of death in shrimp farming, causing a lot of losses for shrimp farmers up to 1 US$ per year in the world. Chandrakala and Menaka (2017), revealed that the presence of *Vibrio* sp. is a normal flora in brackish waters that has

a maximum limit so if it is present in large quantities it can cause disease problems for shrimp and consumers.

The use of antibiotics is common for controlling shrimp diseases. However, if the application does not match the prescribed dose it can cause pathogen resistance, chemical residues, and antibiotic residues in shrimp meat that can harm consumers. So, their use is no longer effective in controlling shrimp disease.

Immunostimulants are one of the safest alternatives to replace antibiotics in overcoming disease attacks in shrimp. Immunostimulant is a material derived from living things such as synthetic biological compounds that can increase non-specific immune responses (Johnny *et al.*, 2008). According to Tacchi *et al.* (2011), the increase in phagocytic activity can be assisted by the addition of immunostimulants derived from phytochemicals.

Phytochemicals that can be used as immunostimulants that are quite effective in preventing bacterial infections and can improve the shrimp immune system are meniran plants (*Phyllanthus niruri* L). Pratiwi and Rivai (2015) revealed that meniran contains secondary metabolites namely flavonoids, terpenoids, alkaloids and steroids. According to Febryantono *et al.* (2020), the flavonoid in meniran leaves can improve or enhance the immune system. In addition, Perdana (2021) revealed that meniran contains chemical flavonoids such as quercetin, quercitrin, isoquercitrin, astragalin, rutin, and kaempferol-1-4-rhamnopyranoside, eridiktol-7-rhamnopyranoside, nirurine, niruriside, philanthine, hypophyllanthin, triterpene which function as natural immunomodulators. Flavonoid compounds will work on hemocyte cells by sending intracellular signals to cell receptors so that cells work optimally.

However, the use of meniran in enhancing the immune system against white vannamei shrimp infected with *V. harveyi* has not been widely reported. Therefore, it is necessary to research the administration of meniran extract on white shrimp which was challenge tested with *V. harveyi.*

METHODOLOGY

Place and Time

This research was conducted for 60 days in March-May 2021 at the Aquaculture Laboratory, Department of Fisheries and Marine Sciences, Faculty of Agriculture, University of Mataram.

Research Materials

The tools used are containers (28 cm x 30 cm x 40 cm), aerator, petri dish, loop needle, Bunsen, autoclave, Erlenmeyer, analytical balance, micropipette, syringe, hemocytometer, microscope, drigalski, glass preparations, hot plate, test tubes, and serological pipettes. The ingredients used were PL-20 shrimp, commercial pellets, seawater, meniran leaf extract, TCBS media, liquid SWC media, TSA media, 75% alcohol, pure isolate of *V. harveyi,* Giemsa dye, 10% EDTA, 0.9% NaCl solution, trisodium citrate, and methanol.

Research Design

The research method used was an experimental method using a completely randomized design (CRD) consisting of 5 treatments with different extract doses added to the feed. Each treatment was replicated three times to obtain 15 experimental units as follows:

P1 = Commercial feed + *V. harveyi* infection (positive control)
P2 = Commercial feed + 0.9% NaCl (negative control)
P3 = Commercial feed + 0.5% Meniran leaf extract + *V. harveyi* infection
P4 = Commercial feed + 1% Meniran leaf extract + *V. harveyi* infection
P5 = Commercial feed + 2% Meniran leaf extract + *V. harveyi* infection

Work Procedure

Making Meniran Leaf Extract

The preparation of simplicia is done by collecting the fresh meniran leaves, then sorting and washing them. The leaves

were then air-dried for about 1 week, then in the oven at 60 °C for approximately 2 hours. After being baked, the meniran leaves went through a pollination process using a blender and sieved to obtain a fine powder of meniran leaves. Furthermore, the manufacture of meniran leaf extract was made by maceration of dry powder of meniran leaves with 90% ethanol in a ratio of 1:5. After the maceration process for 48 hours, the material was filtered using filter paper to obtain meniran extract without pulp. Furthermore, the filter results are concentrated in a rotary vacuum evaporator at a temperature of 50 °C to form a thick extract.

Test Feed Preparation

In maintenance activities, commercial pellet feed (IRAWAN) is used with a protein content of 40%. The pellet feed was added to a solution of meniran leaf extract and then stirred until well mixed. Shrimp are fed 5%-7% of the weight of the biomass per container. Feeding is done 4 times a day, namely at 07.00 WITA; 12.00 WITA; 17.00 WITA and 22.00 WITA with doses of 30%, 20%, 20% and 30%.

Preparation of Test Biota

At the beginning of the study, the vannamei shrimp that were kept were in the PL-20 phase. Vannamei shrimp fry came from PT Bibit Unggul, Gangga Village, North Lombok Regency. Prior to the study, shrimp were acclimatized for 7 days. Each container was filled with as many as 20 test animals with a container size of 28 cm x 30 cm x 40 cm as many as 15 boxes were cleaned using an antiseptic and then filled with seawater that had been deposited for 7 days as much as 20 liters/container. Each container is equipped with an aeration device, 3 shelters from paralon pipes, and a plastic container cover so that the shrimp don't jump out. Measurements of salinity, DO and pH in the media water were carried out before use and the water change system was carried out once a day periodically as much as 80% of the total volume of the aquarium.

Maintenance of Seeds

Giving meniran leaf extract to shrimp fry through mixing with feed begins with sampling the weight of shrimp in each treatment and calculating the amount of feed to be given and calculating the amount of extract to be added from the amount of each feed. The results of the calculation are converted from milliliters to microliters by multiplying by 1000 (1 ml = 1000 microliters) because the amount of extract added is too small for the milliliter size so the microliter size is used. Meniran leaf extract was taken using a micropipette based on the results of calculations with each treatment dose to be given the extract. The amount of extract was changed every 10 days along with the sampling process of biomass and the amount of shrimp feed.

Survival Rate

The survival rate is calculated using Azhar (2014) formula, as follows:

$$SR = \frac{Nt}{No} \times 100\%$$

Where:
SR = survival rate (%)
Nt = final number of fish
No = initial number of fish

Relative Percent Survival

Relative Percent Survival (RPS) can be calculated by the formula (Marbun *et al.*, 2019):

$$RPS = \left(1 - \frac{\%\ \text{infected shrimp mortality}}{\%\ \text{control shrimp mortality}}\right) \times 100\ \%$$

Vibrio harveyi Challenge Test

The bacteria used for the challenge test was obtained from the collection of the Aquaculture Laboratory, Mataram University. The *V. harveyi* isolates were recultured and purified to obtain younger and more virulent bacteria. Then re-characterization was carried out. The isolates were cultured in 25 ml of liquid SWC media for approximately 18 hours in a water shaker at 29 °C. Then, the dilution stage was carried out with a bacterial

density of 10^6 cfu/ml (Oktaviana *et al.*, 2014). The process of injecting bacteria was carried out intramuscularly in the shrimp body as much as 0.1 ml/head, except for the negative control injected with 0.1 ml NaCl 0.9%/head because it was related to its role as a comparison with other post-infection treatments. Furthermore, the shrimp were reared again for 10 days to determine the survival rate of the shrimp.

Total Haemocyte Count

Sampling of hemolymph in each treatment was carried out after 10 days of maintenance using a 1 ml syringe. A syringe filled with anticoagulant solution as much as 0.6 ml. Furthermore, 0.1 ml hemolymph samples were taken from the 3rd leg of 3 shrimps and placed in a microtube. Hemolymph that has been taken is used for the measurement of immune response parameters. The total haemocyte count (THC) was calculated by dripping the sample of hemolymph that had been taken on the haemocytometer. The hemolymph samples were then observed under a microscope with a magnification of 400 times. Then it is calculated by the following formula (Oktaviana *et al.*, 2014):

$$\text{THC} = \text{ACC} \times \frac{1}{\text{HV}} \times \text{DF}$$

Where:
THC = total haemocyte count
ACC = average cell count
HV = haemocytometer volume
DF = diluent factor

Differential Haemocyte Count

The slides were immersed in methanol for 5 minutes and fixed. After that, a single drop of hemolymph was added to the slide and spread over the entire slide using another glass slide and dried. The preparations were then re-fixed and immersed in methanol for 10-15 minutes. After drying, the glass slides were put into a staining jar containing Giemsa for about 10-15 minutes and then dried. The Giemsa was then cleaned and observed under a microscope. Differential haemocyte count (DHC) of hyaline is calculated using the following formula (Jannah *et al.*, 2018):

$$\text{DHC (\%)} = \frac{\Sigma \text{ hyaline cell}}{\Sigma \text{ haemocyte observed}} \times 100$$

Phagocytic Activity

Measurement of phagocytic activity (PA) was carried out by mixing 100 µL hemolymph samples with 25 µL *Staphylococcus* in a microplate and incubated for 20 minutes. The incubation results were dripped on a glass slide that had been previously soaked with methanol and thoroughly smeared. Then the smear preparations were dried and soaked in methanol for 10-15 minutes. Then put into a staining jar containing Giemsa solution for 10-15 minutes, the slides are then observed. Phagocytosis activity was calculated based on the percentage of cells that carry out phagocytes (Jannah *et al.*, 2018). The calculation of the phagocytic activity value is as follows:

$$\text{PA} = \frac{\Sigma \text{ phagocytic cell}}{\Sigma \text{ haemocyte}} \times 100\%$$

Total Bacteria in the Intestine

Calculation of the total bacteria present in the shrimp intestine was carried out at the end of the rearing period. The calculation consists of 2 parameters, namely the Total Viable Bacterial Count (TBC) and the calculation of the Total Presumptive Vibrio Count (TVC). The process of calculating the bacterial population in shrimp intestines by collecting 0.1 g shrimp intestine samples. In the next process, shrimp intestines were homogenized in 0.9 ml phosphate buffer saline (PBS) solution in microtubes. The bacterial population calculation process was carried out using the cup count method on Sea Water Complete (SWC) media for the calculation of TB and specific media TCBS Thiosulfate Citrate Bile Salt Sucrose (TCBS) for TVC calculations (Oktaviana, 2014).

Data Analysis

The data obtained were analyzed using Analysis of Variance (ANOVA) with SPSS at a significant level of 5% to determine the effect of the treatment in the study. If the data shows a significant difference, further analysis is carried out with Duncan's further test.

RESULTS AND DISCUSSION

Survival Rate

The survival rate of vannamei shrimp after the rearing period is presented in Figure 1.

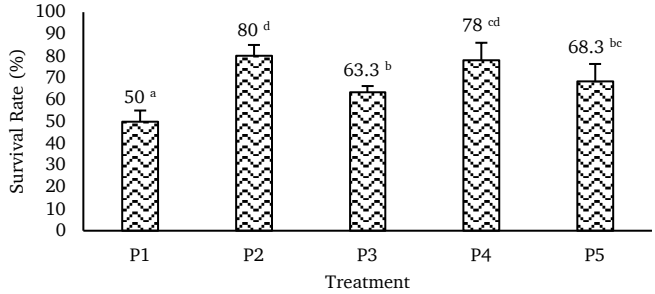

Figure 1. Survival rate.

Mathematically, the addition of meniran leaf extract to the feed had a significant effect ($P < 0.05$) on the survival rate of white vannamei shrimp infected with *V. harveyi*. P1 (positive control) showed a 50% survival rate, this value was lower than P2 (negative control) which was 80%. The higher survival rate in P2 was suspected because the treatment was not infected with *V. harveyi*. The highest survival rate happened in P4 (78%), followed by P5 (68.3 %), and P3 (63.33%) (Figure 1). The lower survival rate at P5 was suspected due to 1% dose being the optimum dose that could be tolerated by vannamei shrimp. It can be indicated that an increase in total hemocytes after the addition of meniran leaf extract provided protective protection for white vannamei shrimp after being infected with *V. harveyi*.

The results of phytochemical tests indicate that the compounds contained in the extract of meniran leaf were alkaloids, tannins, steroids, phenolics and flavonoids. Aldi *et al.* (2014) revealed that alkaloid compounds, tannins, flavonoids, saponins, and phenolic components have an immunomodulating effect. Serang (2019), also revealed that *phenolic* compounds contain antioxidants that are closely related to the immune system. Meniran extract that enters the shrimp body will stimulate an increase in the activity of haemocyte cells in shrimp, to fight pathogens that enter. In addition, Sijuade (2016) explained that the alkaloid compounds can help hemocyte cells in reducing inflammation, treating infections, and healing wounds. Increased body immunity and wound healing in the area of infection is a way for shrimp to respond to pathogens so that the survival rate even after the infection is higher. In general, the survival rate showed good results, this was supported by Widigdo (2013), which stated that the SR value>70% after bacterial infection was still categorized as good. This means that the administration of 1% meniran leaf extract (P4) can be applied to increase the survival rate of vannamei shrimp.

Relative Percent Survival

The relative percent survival of vannamei shrimp after the rearing period is presented in Figure 2.

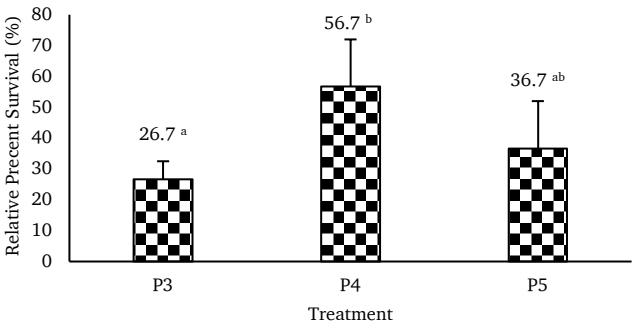

Figure 2. Relative percent survival.

Relative percent survival (RPS) calculation is based on shrimp mortality that occurred after the challenge test. The challenge test was carried out for 10 days after giving meniran leaf extract for 50 days. The administration of meniran leaf extract to white shrimp infected with *V. harveyi* was not significantly different (P>0.05). The highest RPS value was found at P4 (56.7 %), followed by P5 (36.7 %), and the lowest was at P3 (26.7%) (Figure 2).

According to Wijayanti *et al.* (2018), an ingredient can be categorized as effective as an immunostimulant if the RPS > 50%, the higher RPS value indicates the effectiveness of the meniran leaf extract overcome *V. harveyi* infection.

Total Haemocyte Count

Total haemocyte count (THC) of shrimp after rearing ranged from 17.14 × 10^6 -29.04 × 10^6 cells /ml (Figure. 3).

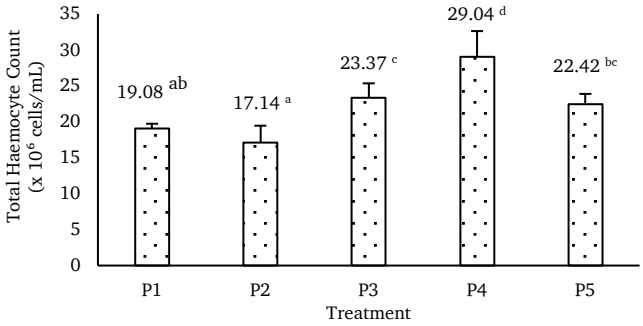

Figure 3. Total haemocyte count.

From the observations on day 60 (post-challenge test), it was shown that the addition of meniran leaf extract to the feed had a significant effect (P< 0.05) on the THC of infected white shrimp. The range of THC at P4 was 29.04×10^6 cells/ml, followed by P3 23.37×10^6 cells/ml, and P5 22.42×10^6 cells/ml. In contrast, the amount of THC in P1 is 16.29×10^6 cells/ml and in P2 17.14×10^6 cells/ml (Figure 3).

Based on the statement of Jantan *et al.* (2014), which revealed that among the abundant compounds containing immunostimulants in meniran, it was identified as catechin compounds contained in flavonoids. According to Febryantono *et al.* (2020), flavonoid compounds in meniran can quickly send intracellular signals to hemocytic cells when foreign objects or pathogens attack shrimp. When the antigen (flavonoid compounds) is inserted into the shrimp body, it will help activate lectin or agglutinin in the shrimp hemocytes which will associate themselves with the carbohydrates found in the bacterial cell wall and the agglutination process occurred. This process will be followed by

the elimination of bacteria through phagocytosis (Ekawati *et al.*, 2012).

Differential Haemocyte Count

The differential haemocyte count (DHC) of vannamei shrimp after 60 days of rearing with commercial feed in various treatments and added meniran leaf extract is presented in Figure 4.

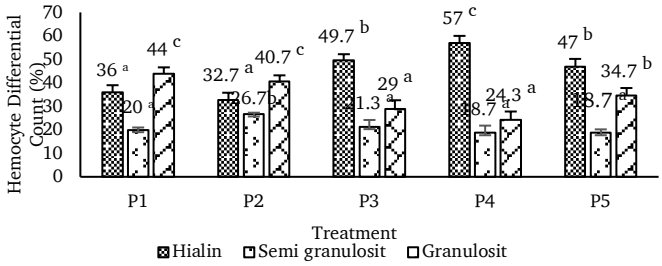

Figure 4. Differential haemocyte count.

An indicator of an increased immune response in addition to the total number of hemocytes is the differential number of hemocytes which also shows a tendency to increase in infected vannamei shrimp. The addition of meniran leaf extract to the feed had a significant effect ($P < 0.05$) on the DHC of vannamei shrimp infected with *V. harveyi*. Hyaline cell observation showed significantly different results, at P1 (36%), P2 (32.7%), P3 (49.7%), P5 (47%), and the highest value was at P4 (57%) (Figure 4).

The difference in hyaline cells between the control and the treatment given meniran leaf extract indicated that there was an attempt to resist the test shrimp against *V.* harveyi previously infected with shrimp. Darwantin and Sidik (2016) stated that the shrimp immune system does not have immunoglobulins like other fish, the role of immunoglobulins in shrimp is carried out by prophenoloxidase activating enzyme (PPA) which is located in the granular cells of shrimp hemocytes. In this study, PPA was activated by an immunostimulant, namely meniran leaf extract which could stimulate hyaline cells to increase their activity in the phagocytosis process.

Semi-granulocyte values showed significantly different results ($P < 0.05$). At P1 the results were significantly different from P2, P4 and P5 but not significantly different from P3. Mathematically, the value of semi-granulocytes showed that there was a difference between treatments, namely P1 (20%), P2 (26.7%), P3 (21.3%), P4 (18.7%), and P5 (18.7%). The low total semi-granulocytes in the treatment added with meniran leaf extract was due to the low production of semi-granulocyte cells which are the maturation of hyaline cells. According to Munaeni *et al*. (2014), semi-granulocyte cells are characterized by the presence of granules in the cytoplasm. Semi granulocytes can carry out the encapsulation process and do not play many roles in the phagocytosis process.

Meanwhile, the granulocyte values in this study showed significantly different results ($P < 0.05$). P1 was not significantly different from P2, but the results were significantly different from P3, P4, and P5. Mathematically, the granulocyte values showed significantly different results, namely P1 (44%), P2 (40.7%), P3 (29%), P4 (24.3%) and P5 (34.7%). Putri *et al.* (2013) reported that the percentage of granulocyte cells in healthy shrimp ranged from 17% to 40% of the total hemocytes. This indicates that the range of granulocyte cells in this study is still in the normal range. Ekawati *et al.* (2012) stated that granulocyte cells play a role in producing the phenoloxidase (PO)

enzyme which plays an important role in the non-specific immune system.

Phagocytic Activity

The phagocytic activity of vannamei shrimp after being reared for 60 days with commercial feed given to various treatments and added meniran leaf extract was 70.3% -78.4 % (Figure 5).

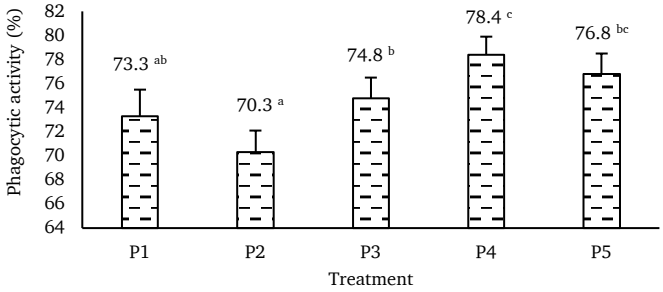

Figure 5. Phagocytic activity.

Observations on day 60 showed that there was a significant effect (P< 0.05) from the addition of meniran leaf extract to the feed on the phagocytic activity of vannamei shrimp hemocytes infected with *V. harveyi*. P1 showed significantly different from P4 but not significantly different from P2, P3, and P5. Increased phagocytic activity may indicate the effect of adding meniran extract to feed can increase the shrimp's immune system.

Putri *et al.* (2013) revealed that the system of immunostimulants is to stimulate increased activity of phagocytic cells. Immunostimulatory activity in meniran is known to be found in phenolic extracts, flavonoids, and methanol contained in meniran extract (Suparman and Saptarini, 2019). Based on research by Luhurningtyas *et al.* (2021), the components of phenolic compounds can increase the ability of phagocytic cells to eliminate pathogens that enter the body. The highest phagocytic activity in this study was observed in P4.

Total Bacterial Count

The total gut bacteria of vannamei shrimp after 60 days of rearing was 52 × 10^8 cfu/ml - 168 × 10^8 cfu/ml (Table 1).

Table 1. Total bacteria count.

Treatment	Total Bacteria (cfu/ml)	Information
P1 (K+)	168 × 10^8 d	Significantly different from P2
P2 (K-)	61 × 10^6 a	Significantly different from P3
P3 (0.5 %)	71 × 10^8 c	Significantly different from P4
P4 (1%)	52 × 10^8 b	Significantly different from P5
P5 (2%)	77× 10^8 c	Not significantly different from P3

The addition of meniran leaf extract to the feed showed a significant effect (P< 0.05) on the total shrimp gut bacteria. P1 showed significantly different results with all treatments, the results between P3 and P5 were not different but significantly different from P2 and P4. The positive control (P1) showed the highest number of bacteria compared to other treatments (168×10^8 cfu/ml), because P1 (positive control) was infected with bacteria without the addition of meniran leaf extract which can function as an antibacterial. At P2 without the addition of meniran leaf extract and bacterial infection, the value was lower than P1 which was 61 × 10^6 cfu/ml. In P2 (negative control) a physiological solution

of NaCl was injected with a dose of 0.1 mL as a comparison with other treatments that were infected with *V. harveyi,* so that it could be seen that shrimp mortality was indeed affected by *V. harveyi* infection. While the total bacteria in P3 is 71×10^8 cfu/ml, followed by P4 with 52×10^8 cfu/ml, and P5 with 77×10^8 cfu/ml. Mangunwardoyo *et al*. (2009) stated that the tannin compounds in meniran plants are effective as antimicrobials and antibacterials. Sapara *et al.* (2016) revealed that the working system of tannins as an antibacterial is by attacking the polypeptide walls of bacterial cells so that the formation of bacterial cell walls is less than perfect and then the bacterial cells will die.

Total Vibrio Count

The total Vibrio count (TVC) in the vannamei shrimp gut after being reared for 60 days with commercial feed given to various treatments and added meniran leaf extract, namely 24×10^4 - 58×10^6 cfu/ml (Table 2).

Table 2. Total vibrio count.

Treatment	Total Vibrio (cfu/ml)	Information
P1 (K+)	$58 \times 10^{6\,c}$	Significantly different from P2
P2 (K -)	$24 \times 10^{4\,a}$	Significantly different from P3
P3 (0.5 %)	$30 \times 10^{6\,b}$	Not significantly different from P4
P4 (1%)	$27 \times 10^{6\,b}$	Not significantly different from P5
P5 (2%)	$32 \times 10^{6\,b}$	Significantly different from P1

The TVC contained in the intestines of vannamei shrimp after infection showed that the addition of meniran leaf extract to the feed had a significant effect ($P< 0.05$). P1 showed the highest value and significantly different with all treatments. P2 without the addition of meniran leaf extract and without *V. harveyi* infection showed the smallest value of vibrio growth in shrimp intestines contrast to other treatments (Table 2).

Chandrakala and Menaka (2017), revealed that the presence of *Vibrio* sp. is a normal flora in the brackish water environment which has a maximum limit. The high value of TVC in this study was due to the challenge test which was carried out on the 50th day of rearing and the shrimp needed time to adapt and cope with the vibrio infection. This has no effect on the survival rate of vannamei shrimp, because the survival rate of shrimp in this study is still categorized as good (Figure 1). From the results of phytochemical tests that have been carried out in this study, it shows that the meniran plant content in the form of steroids functions as an antibacterial. Sapara *et al.* (2016) revealed that the mechanism of steroid compounds as antibacterial is by the interaction between steroids and bacterial cell phospholipid membranes which are permeable to lipophilic compounds, causing cell membrane morphology to change, causing bacterial cells to become brittle and lyse.

CONCLUSION

Based on the results, the addition of meniran leaf extract *(Phyllanthus niruri L.)* with a dose of 1% gave a significant effect on increasing the immune system of white vane shrimp infected with *Vibrio harveyi*, with the results of 78% SR, 56.7% RPS, 29.04×10^6 cells/ml THC, DHC is hyaline 57%, semi-granulocytes are 18.7%, and granulocytes are 24.3%, phagocytic activity is 78.4%, TBC is 52×10^8 (cfu / ml) and TVC is 27×10^6 (cfu / ml).

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