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Research Article
Efficacy of Guava (*Psidium guajava*) Leaves Extract to Prevent Vibriosis in White Shrimp (*Litopenaeus vannamei*)

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Abstract

Background and Objective: One of the problems which concern the cultivation of vannamei shrimps is the attack of vibriosis diseases caused by *Vibrio harveyi* bacteria. This study was aimed to examine the antibacterial potential of guava leaf extract (against *V. harveyi* bacteria to determine the effectiveness of guava leaf extract for the prevention of diseases. **Materials and Methods:** Treatments included administration of guava leaf extract mixed into feed at different dosages level and without administration of guava leaf extract (control). The control treatment consists of positive control (K+) and negative control (K-). After 14 days of trial period, all shrimp were challenged with *V. harveyi* RfR concentration of 10^6 CFU mL^{-1} except negative controls. **Results:** The results showed that administration of guava leaf extract at a dose of 5250 ppm (treatment C) was more able to suppress the growth of *V. harveyi* RfR bacteria on the 18th day which was 3.72 log CFU g^{-1} and decreased on the 21st day that is equal to 2.90 log CFU g^{-1}. The highest total hemocyte count (THC) was found in the treatment of 5250 ppm (treatment C) i.e., on the 14th day, the 18th day and on 21st day while the percentage of hyaline cells after giving guava leaf extract for 14 days has increased, then decreased on the 18th day then increased again on the 21st day. While the percentage of granular/semigranular cells has decreased on the 14th day then increased again on the 18th and 21st days. **Conclusion:** Administration of guava leaf extract in feed could improve physiological status and to increase the relative survival of vaname shrimp when facing an infection of vibriosis.

Key words: Guava leaf extract, *Vibrio harveyi*, vaname shrimp, physiological status, relative survival

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Disease infection is one of the main causes of failure of vaname shrimp production. Disease control in vaname shrimp has often been done using various antibiotics, but in reality the use of antibiotics to control the disease is considered harmful to the environment and surrounding organisms. To avoid the negative effects of antibiotic use, immunostimulant is an alternative to antibiotics and vaccines in protecting and controlling disease attacks. Immunostimulants are chemical compounds, drugs or other materials that can increase the nonspecific defense of animals and develop antigenic responses that were not previously stimulated[1].

Several antibacterial active ingredients from guava leaf plants have been tested, which contain tannin, flavonoid, saponim and alkaloid compounds[2]. According to Akiyama *et al.*[3], tannins are antibacterial by precipitating proteins. Antimicrobial effects of tannins through reaction with cell membranes, enzyme inactivation, destruction or inactivation of genetic material. Tannin, alkaloids and flavonoids can inhibit the growth of *Staphylococcus aereus* bacteria[4]. Saponins, including triterpenoid compounds, can be used as antimicrobials[5].

According to several studies, guava leaves have been shown to have various pharmacological effects of anti-diarrhea[6], anticough[7], antibacterial[8-10], dental anti-plaque[11], antidiabetic[12-14], antiinflammatory and antitumor generation[15] and antioxidants[16].

In addition to being useful for increasing the non-specific defense of shrimp, the use of natural ingredients mixed in the feed is intended to meet energy needs. The research was conducted on guava leaves natural ingredients, mixed in feed aimed at preventing disease in vaname shrimp infected with *Vibrio harveyi* RfR bacteria.

MATERIALS AND METHODS

Study area: This research was conducted from March to August, 2016, located in the Pharmacy Laboratory, Fish Health Laboratory and Wet Laboratory at the Faculty of Fisheries and Marine Science, Halu Oleo University, Kendari, South east Sulawesi, Indonesia.

Preparation of guava leaf extract: The process of making guava leaf extract (Fig. 1) begun by taking guava leaves from nature and then cleaned and dried (wind dried) for 1 week. Leaves that have been dried may be marked with leaves that will soon be broken into flakes when crushed. The dried leaves were then ground using a grinding tool until smooth and filtered to obtain a really fine powder.

The process of extraction steps carried out by the fine powder was macerated using 96% ethanol solvent for 3×48 h and then filtered with a Buchner funnel to obtain the filtrate. The filtrate obtained was then concentrated using a rotary evaporator at a maximum temperature of 60°C until the ethanol solvent evaporated and a paste-shaped extract was formed which was left on the pumpkin wall. The paste extract was then taken using a spatula then weighed and stored in a dark vial bottle.

Phytochemical test: Phytochemical examination is a qualitative screening test used to determine the presence (+) or absence (-) of the content of secondary metabolites in pure extracts of guava leaves. The examination of secondary metabolite content consists of alkaloid test, terpenoid test, flavonoid test, saponin test and tannin test[17].

Lethal dose 50% (LD_{50}) test: LD_{50} test was conducted to determine the concentration of bacteria that can make the vaname shrimp population die by as much as 50% which will be used for in vivo tests (challenge test). Test shrimp used in this study were healthy vaname shrimp (*L. vannamei*) with a size of 7 g of 10 shrimps/aquarium. The bacteria used was *V. harveyi* RfR derived from the isolation of vaname shrimp from the Fish Health Laboratory, Bogor Agricultural University which was then re-cultured and enhanced its pathogenicity in the Fish Health Laboratory, Faculty of Fisheries and Marine Science, Halu Oleo University, Kendari.

Fig. 1: Guava leaves (*Psidium guajava*)

LD_{50} test was done by injecting *V. harveyi* RfR bacteria. In the test shrimp with different concentrations, namely 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU mL^{-1} per shrimp. Each treatment consisted of 10 shrimps. The concentration of each bacterium to be used with serial dilution techniques. Injections were carried out intramuscularly as much as 0.1 mL per shrimp. Observation was carried out for 3 days by counting the number of dead shrimps.

Shrimp test maintenance: Containers used for raising shrimp were aquariums with a size of 60×50×40 cm as many as 15 aquariums. Before the research was conducted, the aquarium was cleaned and filled with sterilized sea water 50% of the aquarium volume. After the sea water was filled then aeration was given in it, then 10 juveniles of vaname shrimp were added per container. Before the treatment of test shrimps were adapted and given commercial feed for 1 week as the process of acclimation of test animals to the environmental conditions of the experiment. Provision of test shrimp feed was carried out 4 times. Feeding was done *ad libitum* with feeding rate (FR) of 10% of shrimp biomass.

Maintenance of the container was carried out by removing impurities that settle to the bottom of the aquatic by change water every day (morning) as much as 10% water volume in each aquarium, this was done before the test shrimps were given food.

***In vivo* test:** *In vivo* test in this study included the application of guava leaf extract on vaname shrimp through feed and testing the resistance of vaname shrimp to *V. harveyi* RfR infection. The treatment was carried out for 14 days, then on the 15th day the shrimp were infected with *V. harveyi* RfR with a concentration of 10^6 CFU g^{-1}. Pathogen bacteria used for infection was *V. harveyi* RfR, bacteria that was resistant to rifampicin antibiotics and their virulence was increased by the Koch Postulate test. Infection was carried out by injection method. Negative control was injected with 0.85% Sodium chloride (NaCl). Furthermore, the shrimp was re-maintained for 6 days by still being given food according to treatment then observing every day.

Research treatment and design: The study design used a completely randomized design with 5 treatments and 3 replications, namely: guava leaf extract mixed into feed at doses of (A) 1250 ppm, (B) 3250 ppm, (C) 5250 ppm and without administration of guava leaf extract (control).

Observed parameters: The experimental parameters observed were LD_{50}, immune response, relative percent survival (RPS) and population of *V. harveyi* RfR bacteria in the intestine.

LD_{50} calculations were based on Reed and Muench[18]:

$$\text{Proportional Distance (PD)} = \frac{\text{50\%-Mortality at concentration next below}}{\text{Mortality next above-Mortality next below}}$$

The immune response parameters measured at 0, 14 days of treatment period and at 3, 6 days post challenge test included total hemocyte count (THC) and differential hemocyte count (DHC). The THC was calculated to discover the number of the shrimp's hemocyte in reference to the method of Blaxhall and Daisley[19], whereas DHC calculation was based on Martin and Graves[20]. Observation of bacterial population in the intestine was carried out on the 18th day (3 days after the challenge test) and the 21st day (6 days after the challenge test).

Relative percent survival (RPS) versus control was calculated at the end of 21 days of infection using the formula in Amend[21] in which:

$$\text{RPS (\%)} = 1 - \frac{\text{Number of shrimp mortality at the treatment group}}{\text{Number of shrimp mortality at the control group}} \times 100$$

LD_{50} test was conducted to determine the density of bacteria that can cause the population of vaname shrimp (*L. vannamei*) to die by as much as 50% within 72 h.

Statistical analysis: Data from the test results on all treatments were analyzed using ANOVA, if the results were significantly different, Duncan's test then was performed.

RESULTS

Phytochemical test: Phytochemical tests conducted on guava leaf extract using the Bontrager method can be seen in Table 1.

Table 1 above shows that the results of phytochemical analysis of guava leaf extracts contain strong alkaloids, saponins, tannins and flavonoids while terpenoids was weak.

Table 1: Phytochemical analysis results of guava leaf extract

Compounds	Analysis results
Alkaloid	++
Flavonoid	++
Tannin	++
Terpenoid	+
Saponin	++

++: Strong, +: Weak

Positive results of alkaloids in the Mayer test were characterized by the formation of white deposits, it was estimated that these deposits are potassium-alkaloid complexes. In the identification of flavonoids using a Walstater test showed orange and red which means positive the presence of flavonoids. In tannin identification experiments using iron (III) chloride reagents the results obtained in positive guava leaf extract contain tannins with the formation of dark blue or blackish green. Identification of terpenoids in this experiment using the Liebermann-Burchard test gave a positive result that is marked by the formation of red or green rings. The identification of saponins using the forth test shows that the positive guava leaf extract containing saponins was evidenced by the presence of foam in the solution.

LD_{50} test: The results of the bacterial LD_{50} calculation for vaname shrimp (*L. vannamei*) can be seen in Table 2.

$$\text{Proportional Distance (PD)} = \frac{\text{50\%-Mortality at concentration next below}}{\text{Mortality next above-Mortality next below}}$$

$$= \frac{50\%-33.33\%}{63.16\%-33.33\%} = \frac{16.67}{29.83} = 0.559$$

LD_{50} = Log concentration at mortality next below 50%+PD

$= \text{Log } 10^5 + PD = 5+0.559 = 5.559$

$LD_{50} = 10^{5.56} = 10^6$ CFU mL^{-1} (approximately)

Based on Table 2, the LD_{50} test result for bacteria that can kill 50% vaname shrimp populations (*L. vannamei*) within 72 h was bacteria with a density of 10^6 CFU mL^{-1}.

Population of *V. harveyi* in vaname shrimp intestine: Below is an observation of the abundance of *V. harveyi* bacteria in vaname shrimp intestines (Fig. 2).

On the 18th day (3 days after the challenge test) the population of *V. harveyi* RfR bacteria in the intestines of vaname shrimp for all treatments was significantly different. Population of *V. harveyi* RfR bacteria for treatment with a dose of 1250 ppm (treatment A) of 4.45 log CFU g^{-1}, dose of 3250 ppm (treatment B) of 4.05 log CFU g^{-1} and treatment with a dose of 5250 ppm (treatment C) of 3.72 log CFU g^{-1}. On the 21st day (6 days after the challenge test) the population of *V. harveyi* RfR bacteria in vaname shrimp intestines for all treatments was not significantly different. Population of *V. harveyi* RfR bacteria for treatment with a dose of 1250 ppm (treatment A) in the amount of 3.90 log CFU g^{-1}, dose 3250 ppm (treatment B) in the amount of 3.48 log CFU g^{-1} and treatment with a dose of 5250 ppm (treatment C) that is equal to 2.90 log CFU g^{-1}.

Figure 2 explains that on the 18th day the highest total of *V. harveyi* RfR bacteria was found in the treatment with a dose of 1250 ppm (treatment A) and the lowest was in the treatment with a dose of 5250 ppm (treatment C). On 21st day, all treatments experienced a decrease in the population of *V. harveyi* RfR bacteria in the intestine. The treatment with

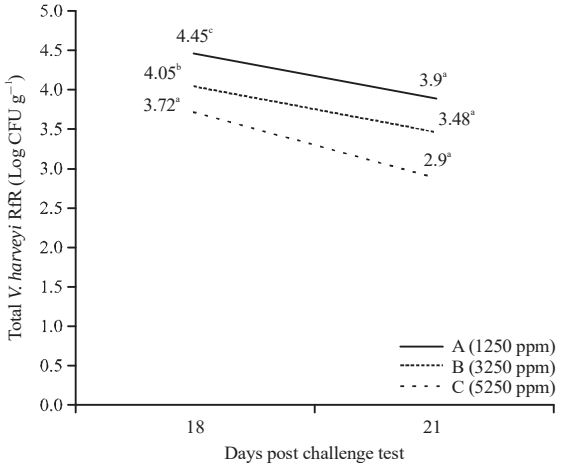

Fig. 2: Population of *V. harveyi* RfR count in the intestines of vaname shrimp (*L. vannamei*) post challenge test
Data (Mean±SD) with different superscript letters indicate statistical significant difference ($p<0.05$) compared with the other concentration extract as assessed by ANOVA followed Duncan's test as a multiple range test

Table 2: LD_{50} bacteria *V. harveyi* RfR in vaname shrimp

			Accumulation			
Density bacterium	Σ shrimps die	Σ shrimps alive	Death	Alive	Ratio	Death (%)
10^8	10	0	30	0	30/30	100.0
10^7	8	2	20	2	20/22	90.91
10^6	5	5	12	7	12/19	63.16*
10^5	3	7	7	14	7/21	33.33*
10^4	2	8	4	22	4/26	15.38
10^3	2	8	2	30	2/32	6.25

*Range of bacterial concentration caused 50% of shrimp population death

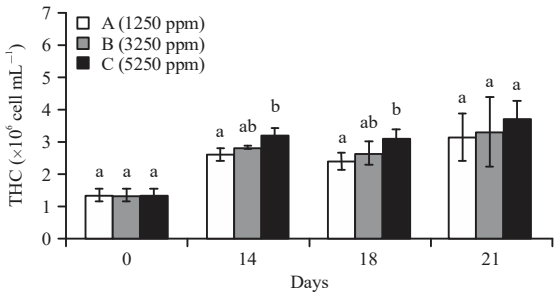

Fig. 3: Total hemocyte count (THC) of vaname shrimp (*L. vannamei*) pre and post challenge test with *V. harveyi*

Data (mean±SD) with different superscript letters indicate statistical significant difference (p<0.05) compared with the other concentration extract as assessed by ANOVA followed Duncan's test as a multiple range test

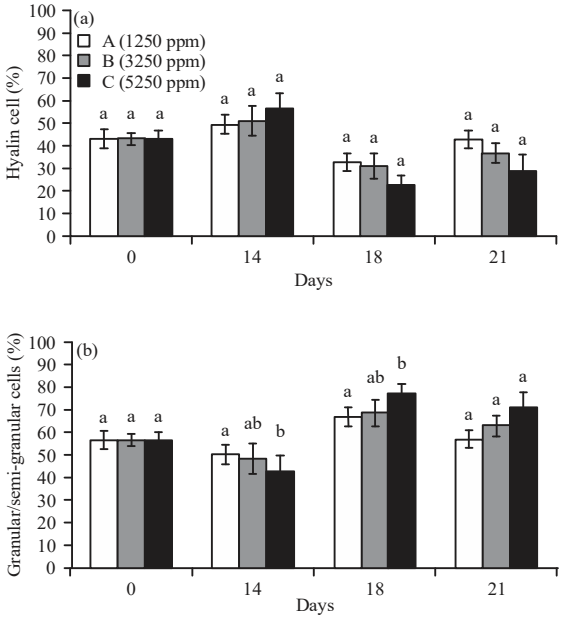

Fig. 4(a-b): (a) Percentage of hyalin and (b) Granular/ semi-granular vaname shrimp (*L. vannamei*) cells at pre and post challenge test with *V. harveyi*

Data (Mean±SD) with different superscript letters indicate statistical significant difference (p<0.05) compared with the other concentration extract as assessed by ANOVA followed Duncan's test as a multiple range test

a dose of 5250 ppm (treatment C) experienced the greatest decrease compared to the treatment with a dose of 1250 ppm (treatment A) and a dose of 3250 ppm (treatment B).

Immune response

Total hemocyte count (THC): The THC observation was done on a day before entering the treatment period (day 0), day 14 (treatment period), day 18 (3 days after bacterial infection *V. harveyi* RfR) and day 21 (6 days after bacterial infection) *V. harveyi* RfR). The THC measurement results are presented in Fig. 3.

The total hemocyte on the 0th day (before entering the treatment period) for all treatments was not significantly different from the total hemocyte value for all treatments, which was relatively the same, namely 1.34×10^6 cells mL^{-1}. On the 14th day (treatment period) the treatment with a dose of 1250 ppm (treatment A) was not significantly different from the dose of 3250 ppm (treatment B) but it was significantly different from the treatment with a dose of 5250 ppm (treatment C) with a total hemocyte value of each 2.61×10^6, 2.84×10^6 and 3.21×10^6 cells mL^{-1}. On the 18th day (3 days after the challenge test) the treatment with a dose of 1250 ppm (treatment A) was not significantly different from the treatment with a dose of 3250 ppm (treatment B) but it was significantly different from the treatment with a dose of 5250 ppm (treatment C) with a total value hemocytes, respectively were 2.40×10^6, 2.65×10^6 and 3.11×10^6 cells mL^{-1}. On the 21st day (6 days after the challenge test) the total hemocytes for all treatments did not differ significantly with their respective values of ie for treatment with a dose of 1250 ppm (treatment A) of 3.15×10^6 cells mL^{-1}, treatment with a dose of 3250 ppm (treatment B) in the amount of 3.15×10^6 cells mL^{-1} and treatment with a dose of 5250 ppm (treatment C) in the amount of 3.70×10^6 cells mL^{-1}.

Differential hemocyte count (DHC): Observation of DHC in vaname shrimp was done at the beginning before entering the treatment period, the 14th day (treatment period), the 18th day (3 days after the challenge test) and the 21st day (6 days after the challenge test). DHC observations can be divided into two, namely observation of hyalin cells and granular cells. Semi-granular cells are categorized as granular cells. The percentage of hyalin cells is inversely proportional to the percentage of granular/semi-granular cells. Figure 4 is a graph of DHC observations on vaname shrimp treated with the addition of guava leaf extract into the feed.

Hyalin cells are the smallest cell types with high cytoplasmic nucleus ratio and without granular. Granular cells are the largest cell type with smaller nuclei and are encased in granules. The comparison between hyaline cells, granular cells and semi-granular cells is known as hemocyte differentiation or DHC. The three types of hemocyte cell types each play a role in the shrimp immune system.

Table 3: Relative survival values (RPS)

Treatments	Mortality (%)	RPS (%)
A (1250 ppm)	43.33	43.5
B (3250 ppm)	36.66	52.4
C (5250 ppm)	20.00	73.8
K (Control)	76.66	-

Hyalin cells on the 14th day (treatment period) before the challenge test for all treatments experienced an increase and decreased on the 18th day (3 days after the challenge test) then increased again on the 21st day (6 days after the challenge test). The highest hyalin cell value on day 14 (before the challenge test) is found in the treatment with a dose of 5250 ppm (treatment C) then decreased on the 18th day (3 days after the challenge test) and on the 21st day (6 days after the challenge test).

Relative survival (RPS): Observations on relative survival were carried out to determine the effectiveness of guava leaf extract in protecting the test shrimp after the challenge test. The data in Table 3 shows the varied values of each treatment, namely the dose of 1250 ppm (treatment A) which is 43.5%, the dose of 3250 ppm (treatment B) that is equal to 52.4% and treatment with a dose of 5250 ppm (treatment C) which is 73.8%.

Based on the results of statistical analysis shows that the highest relative survival of the test shrimp is found in the treatment with a dose of 5250 ppm (treatment C) which is 73.8% and significantly different from the treatment with a dose of 1250 ppm (treatment A) and treatment with a dose of 3250 ppm (treatment B).

DISCUSSION

This pathogenicity test covers the pattern of death and LD_{50}. Death occurs on the first day after infection. Most deaths were experienced by all treatments on the 2nd and 3rd day. It is suspected that the peak of *V. harveyi* RfR bacterial infection occurs after 24 h after infection. This is consistent with the statement of Rey *et al.*[22], *V. harveyi* RfR infection causes clinical symptoms after a few hours after infection and death begins after 7 h after infection, which in turn will cause more deaths after 72 h after infection. Vaname shrimp infected by *V. harveyi* RfR bacteria showed symptoms of stress by swimming without direction, low appetite and parts of the body that are pale to reddish.

According to Rahayu *et al.*[23], a high level of bacterial pathogenicity can cause death to reach 100%. It can be concluded that vaname shrimp infected with different bacterial concentrations experience mortality rates that are in line with increasing bacterial density, the higher the concentration of infected bacteria the higher the mortality rate in vaname shrimp.

LD_{50} test results in this study (Table 2) showed that the concentration of bacteria that can kill 50% of the population of vaname shrimp (*L. vannamei*) within 72 h is 10^6 CFU mL^{-1}. Pathogen *V. harveyi* RfR bacteria used in this study are included in the virulent category. Santos *et al.*[24] and Rico *et al.*[25], classifies the level of virulence of bacteria based on the LD_{50} value of the bacteria, namely bacteria that have LD_{50} values between 10^4-10^7 CFU mL^{-1} belonging to the group of virulent bacteria while bacteria that have $LD_{50}>10^7$ CFU mL^{-1} are the avirulent.

The decline in *V. harveyi* RfR population in the intestines of vaname shrimp shows that the administration of guava leaf extract mixed into the feed can suppress the growth of pathogenic bacteria. This is because guava leaves contain active compounds that can inhibit bacterial growth. Guava leaves contain compounds of saponins, tannins, flavonoids and alkaloids[26]. This is consistent with the results of phytochemical analysis that have been carried out that guava leaf extract contains tannins, flavonoids, alkaloids and saponins (Table 1).

The mechanism of tannin inhibition against bacteria according to Akiyama *et al.*[3] is by reacting with cell membranes, inactivation of essential enzymes and destruction or inactivation of genetic material functions. The antibacterial power of tannin is caused by the presence of a pyrogallol group and a galois group which is a phenol group that can inhibit bacterial growth or kill it by reacting with protein cells from bacteria so that protein denaturation occurs. Denaturation of proteins in bacterial cell walls causes disruption of bacterial metabolism resulting in damage to the cell wall which ultimately causes lysis[5]. According to Panche *et al.*[27], flavonoids have an antibacterial effect through their ability to form complex bonds with bacterial cell wall extracellular proteins, this will damage the integrity of the cell wall and eventually the cell wall is damaged and cause lysis. Akter *et al.*[28] states that saponins can suppress bacterial growth, because these compounds can reduce the surface tension of cell walls and when interacting with bacterial walls, the walls will break or lysis. Saponins will interfere with the surface tension of the cell wall, so when the surface tension is disturbed the antibacterial substance will enter easily into the cell and will disrupt the metabolism until finally there is bacterial death.

The mechanism of tannin inhibition is by lysis of bacterial walls due to saponin and flavonoid compounds, causing tannin compounds to easily enter the bacterial cell and coagulate the protoplasm of bacterial cells *V. harveyi* RfR as a result cells cannot carry out live activities and their growth is inhibited or even dead.

Total hemocyte can affect the ability of the host to react against foreign material and various responses to infection[29]. Low total hemocytes greatly affect the susceptibility of shrimp to pathogens, so that increased total hemocytes can improve the health status of these organisms because increasing hemocytes means increasing the chances of phagocytic cells forming which are very instrumental in controlling the attack of microorganisms. An increase in total hemocytes after being fed with guava leaf extract. in this study showed that guava leaf extract was able to play a role in stimulating shrimp immune responses compared to controls.

Total hemocyte after 14 days of guava leaf extract mixed into feed. The highest total hemocyte was found in the treatment with a dose of 5250 ppm (treatment C) in the amount of 3.21×106 mL^{-1} cells compared with treatment with a dose of 1250 ppm (treatment A) and a dose of 3250 ppm (treatment B). According to Huang *et al.*[30] that the total hemocyte value in the treatment with a dose of 5250 ppm (treatment C) is higher than the THC value of vaname shrimp that has been resistant of *V. harveyi* that is 2.4×10^6 cells mL^{-1}.

An increase in THC values indicates a rapid reaction of vaname shrimp immunity to a given infection. But on the 18th day the total hemocyte in all treatments decreased. The decrease in the number of hemocyte cells is an effect of the operation of the body's defense mechanisms such as infiltration of hemocytes in infected tissue, hemocyte cell death due to apoptosis[31]. Phagocytic activity, encapsulation, nodule formation and the occurrence of degranulation processes for the activity of the prophenoloxidase system (PO) and other body defense mechanisms[32]. Furthermore, according to Van de Braak[33] states that the decrease in hemocytes after the challenge test is associated with different defense activities. Hemocyte will migrate to the injection site causing a reduction in cell concentration in hemolymes. On the 21st day the total hemocyte has increased again because the body of the vaname shrimp has returned to normal so that the shrimp is able to reproduce hemocyte cells (recovery).

The high relative survival in treatment with a dose of 5250 ppm (treatment C) compared with treatment with a dose of 1250 ppm (treatment A) and treatment with a dose of 3250 ppm (treatment B) (Table 3), this indicates that administration of guava leaf extract mixed into the feed provides positive effect for increased shrimp resistance to *V. harveyi* RfR infection.

Based on these results it is known that the administration of guava leaf extract mixed into the feed gives a good influence on the relative survival of vaname shrimp infected by *V. harveyi* RfR bacteria. This is thought to be because the active substances contained in guava leaves can increase the shrimp's immune response. This is reinforced by the results of research conducted by Arima and Danno[8] which states that the active substances contained in guava leaves such as flavonoids and tannins function as anti-infective to fight bacterial attacks and can increase the body's resistance. Furthermore, Giri *et al.*[34] states that the active substance from guava leaves can be used as an antimicrobial agent and can also increase the immunity of fish that is able to overcome and eliminate pathogens.

The increase in the immune response was seen in the total number of hemocyte cells produced by the test shrimp (Fig. 3). The number of hemocytes in the treatment with a dose of 5250 ppm (treatment C) has a high value so that the shrimp are better prepared in the face of pathogens. Rodriguez and Le Moullac[35] state that hemocytes in crustacean play an important role in the body's defense system against pathogens such as viruses, bacteria, fungi, protozoa and metazoan.

CONCLUSION

Application of guava leaf extract at a dose of 5250 ppm as feed additives reduce *V. harveyi* in the intestines of vaname shrimp. Provision of guava leaf extract at a dose of 5250 ppm through feed can increase the immune response of vaname shrimp by observing total hemocytes, hyalin cells and granular cells. Administration of guava leaf extract at a dose of 5250 ppm through feed can increase the relative survival of vaname shrimp.

SIGNIFICANCE STATEMENT

The research discovered the use of guava leaf extract at a right dose mixed in feed that can be beneficial for aquaculture to increase the relative survival of vaname shrimp to combat vibriosis. This study will help the researchers to uncover the critical areas of shrimp diseases that many researchers were not able to explore. Thus a new theory on the use of guava leaves extract for shrimp immunostimulation may be arrived at.

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