Journal of Aquaculture and Fish Health Vol. 10(3) - September 2021
DOI : 10.20473/jafh.v10i3.25973

The Addition of Red Betel Leaf Extract (*Piper crocatum*) in the Feed of Vannamei Shrimps (*Litopenaeus vannamei*) for Vibriosis Prevention

Fariq Azhar[1*], Muhammad Junaidi[1], Bagus Dwi Hari Setyono[1] and Andre Rachmat[1]
[1] Study Program of Aquaculture, Faculty of Agriculture, Universitas Mataram, Jl. Pendidikan 37, Mataram 83125, West Nusa Tenggara, Indonesia

*Correspondence :
fariqazhar@unram.ac.id

Received : 2020-03-17
Accepted : 2021-07-30

Keywords :
Red betel leaf, Vibriosis, Vannamei shrimp, Feed

Abstract

Vibriosis is a type of shrimp disease caused by *Vibrio* sp. In the disease control of consumption biota, it is highly recommended to use natural ingredients that are not carcinogenic, one of which is red betel leaves (*Piper crocatum)*. This study aims to determine the best dose of red betel leaf extract (*P. crocatum*) mixed in Vannamei shrimp feed to prevent vibriosis. In this study, shrimps were reared for 40 days at a density of 20 fish/container. The treatments included positive control, P1 (without extract + bacterial infection), negative control P2 (without extract and without bacterial infection), P3 (0.5% extract + bacterial infection), P4 (1% extract + infection), and P5 (2% extract + bacterial infection). The application of red betel leaf extract at a dose of 0.5% resulted in 75% survival after infection with *V. parahaemolyticus*, Vannamei shrimps of THC 7.70×106 cells/mL, and DHC (hyaline 82.94% granular 20.10%). The number of bacteria and the number of vibrio in the intestine were 52×10^8 CFU/mL and 12×10^8 CFU/mL accordingly. The best dose was obtained at P3 (Feed +0.5% red betel leaf extract), seen from the increase in survival rate, the number of hemocytes, differential haemocyte counts, and a decrease in total bacteria, so the application of red betel leaf extract can be used in the cultivation of Vannamei shrimps as an immunostimulant.

INTRODUCTION

Vannamei shrimp species are increasingly being cultivated because they have high economic value and high market demand. In addition, physiologically Vannamei shrimps are in great demand by cultivators because it has a relatively fast growth rate so that the production period is shorter (Purnamasari *et al*., 2017). However, disease attacks are a major threat in shrimp culture (Chang *et al*., 2012), one of which is vibriosis caused by *Vibrio* sp. bacteria (Chandrakala and Menaka, 2017). The mortality rate of Vannamei shrimps that are attacked by vibriosis is recorded at 80-100% and is pathogenic (Annisa *et al*., 2015). Treatment of bacterial-infected Vannamei shrimps with synthetic antibiotics can increase bacterial resistance (Putri *et al*., 2015), so antibiotics from natural ingredients that are more environmentally friendly and not carcinogenic are highly needed. One of the natural ingredients

that can be used is red betel leaves (*Piper crocatum*).

Red betel leaves (*P. crocatum*) are plants that are widely used in bacterial control. Annisa *et al*. (2015) stated that red betel leaves can be utilized as a natural antibacterial and antifungal material so that it can inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* sp., *Klebsiella,* and *Pasteurella* as well as kill *Candida albicans*. Vibriosis attacks can be prevented through the active ingredients contained in red betel leaf extracts such as flavonoids, alkaloids, tannins, polyphenolic compounds, and essential oils (Puspita *et al*., 2018). These flavonoid compounds play a role in preventing cell oxidation and increase the immune system for the body's defense of Vannamei shrimps, while alkaloids play a role in inhibiting the process of the formation of the peptidoglycan component of bacterial cells so that these cells are not formed (Syahida *et al*., 2013).

Through this research, red betel leaf extract was added to the Vannamei shrimp feed with different doses. Therefore, the quite virulent attack of *V. parahaemolyticus* bacteria can be prevented through the administration of betel leaf extract with the right dose because it will affect the health of Vannamei shrimps. This study aims to determine the effect of the dose of red betel leaves (*P. crocatum*) on Vannamei shrimp feed for the prevention of vibriosis.

METHODOLOGY

Place and Time

This research was conducted for 60 days in January-April 2020 at the Aquaculture Laboratory, Department of Fisheries and Marine Sciences, Faculty of Agriculture, University of Mataram.

Research Materials

The tools used in this study were containers, blowers, Petri dishes, ose needles, autoclaves, Erlenmeyer, analytical balances, micropipettes, syringes, haemocytometers, microscopes, driglaski, glass slides, hot plates, test tubes, and serological pipettes. The materials used in this study were PL-20 shrimp, commercial feed, seawater, red betel leaf extract, TCBS media, liquid SWC media, TSA media, 75% alcohol, pure isolate of *Vibrio parahaemolyticus* bacteria, Giemsa dye, 10% EDTA, 0.9% NaCl solution, trisodium citrate, and methanol.

Research Design

The treatment consisted of positive control (P1) in which the feed was not given extract and infected with bacteria, and negative control (P2) in which the feed was not given extract and infected with NaCl 0.9%, (P3) in which the feed was given 0.5% red betel extract and infected with bacteria, (P4) in which the feed was given 1% red betel extract and infected with bacteria, and (P5) in which the feed was given 2% red betel extract and infected with bacteria at doses of 0.5%, 1%, and 2%. Bacterial isolates obtained from BKIPM Mataram City were re-cultured and purified, and then recharacterization and total plate count (TPC) were performed. The isolates of *V. parahaemolyticus* used were cultured in 25 ml of liquid SWC medium for 18 hours in a water shaker at 29 °C.

Work Procedure

The initial stage of this study was to make extracts using the maceration method of powdered red betel with 96% ethanol, which was then filtered and evaporated with a Rotary Vacuum Evaporator at a temperature of 50 °C until a concentrated extract with a concentration of 100% was obtained (Putri *et al.*, 2015). In the next stage, the maintenance of PL 20 seeds was conducted for 40 days to be later injected with a pure isolate of *V. parahaemolyticus* bacteria obtained from semisolid media with a density of 10^6 CFU/mL as much as 1 mL/head on day 45 (Sarjito *et al*., 2015). Furthermore, observations were made after 7 days post-infection.

Container and Fish Testing

The seeds used were 20 days old or PL-20 and weighed 0.02 g/individual with a stocking density of 20 fish/container. The seeds came from the Center of Superior Shrimp and Oyster Production (BPIUUK) Karangasem Bali which was selected based on the weight of the seeds. Before being put into the research container, the seeds were adapted for 1 week in a reservoir. The container used in this study was a plastic container with a size of 40x30x28 cm, with a volume of seawater of 20 L and a salinity of 30 ppt per container, equipped with aeration devices, Pvc shelters, and water changes with siphoning done every day in the morning.

Test Feed

During the study, the shrimps were fed with crumble 681 feed which had a protein content of 30% mixed with red betel leaf extract. The making of red betel leaf extract included the process of the washing process, drying, and pollinating the red betel leaves. Afterward, the maceration process was carried out using 96% methanol. Then, the material was filtered 3 times to obtain a thick extract which was concentrated using a Rotary Vacuum Evaporator at a temperature of 50°C (Setiawan *et al.*, 2016). Following this, the extract was mixed with the feed until evenly distributed. Shrimps were fed as much as 5% of the weight of the biomass per aquarium. Feeding was done 3 times a day, specifically at 09.00; 15.00, and 21.00 WITA with doses of 0.5%, 1%, and 2% accordingly.

Data Analysis

Several parameters which were tested in this study were survival rate (SR) by taking into account the number of live shrimps at the beginning and end of the study, total hemocyte count (THC) which was carried out by taking 0.1 ml of shrimp hemolymph from the base of the first swimming leg using a syringe which had already contained 0.3 ml of 3.8% Sodium citrate anticoagulant. Afterward, the obtained mixture was homogenized, and the mixture was dripped into the hemocytometer after the first drop had been removed. Then, the number of cells per ml was observed and counted under a microscope with 400 times magnification.

Differential hemocyte count (DHC) was determined by some processes in which the hemolymph of shrimps was taken, and then it was dripped on an object glass and made into smear preparation before air-dried and then given fixation with 100% methanol for 5 minutes. After that, it was air-dried again and then stained with 10% Giemsa solution for 10 minutes. The preparations were observed using a microscope and distinguished by the number of hyaline and granular cells. Calculation of total bacteria and total vibrio were carried out at the end of the study, in which intestines were taken and homogenized in phosphate-buffered saline (PBS) solution.

Bacterial counts used the cup count method, using SWC (Sea Water Complete) media for the calculation of TBC (Total Bacterial Count), and TCBS (Thiosulfate Citrate Bile Salts Sucrose) specific media for VBC (Vibrio Bacterial Count) calculations. The data were later processed statistically in one way - ANOVA using the SPSS application (Version 16.0) at a 95% confidence level ($P<0.05$). Significantly different results were further tested using Tukey HSD.

RESULTS AND DISCUSSION

The survival of Vannamei shrimps after infection can be seen in P2 or negative control, which showed a figure of 76.67%, P3 of 75.00%, P4 of 61.67%, and P5 of 60.00%, while the smallest figure was in P1 of 26.67% (Figure 1). However, after the One-Way Annova test and Tukey's follow-up test, the administration of red betel leaf extract had a significant effect ($P>0.05$) on P1 or positive control compared to other treatments. This means that the positive control (feed not given extract and infected with bacteria) was significantly different from all treatments.

The total hemocyte count of Vannamei shrimps after infection was noted in P3 of 7.70 × 10^6 cells/ml, P4 of 6.25 × 10^6 cells/ml, P5 of 5.55 × 10^6 cells/ml, negative control (P2) of 3.89 × 106 cells/ml, and positive control (P1) as the smallest figure of 3.15 × 10^6 cells/ml (Figure 2). However, after the One-Way Annova test and Tukey further, test, the administration of red betel leaf extract showed a significant effect (P>0.05) between the control and other treatments, where P3, P4, and P5 were not significantly different but significantly different from P1 and P2, meaning that the positive and negative controls were significantly different from the treatment given red betel leaf extract.

Based on the results of DHC observations based on the numbers in Figure 3, the largest amount of hyaline was obtained at 82.94% in P3 and the smallest was obtained at 35.75% in P1. Meanwhile, the highest number of granulocytes was in P1 of 64.25% and the lowest was found in P4 of 17.06% (Figure 3). However, based on the One-Way Annova test, significant results were obtained (P<0.05) between positive controls P1 and P3, P4 and P5 with the addition of red betel leaf extract.

Based on the results of the total count of bacteria obtained after bacterial infection, the highest number was mostly found in P1, accounting for 136 × 10^8 CFU/mL and the lowest was found in P2, accounting for 116 × 10^6 CFU/mL. The number of *V. parahaemolyticus* in P1 of 66 × 10^8 CFU/mL was the highest total vibrio, while the lowest was P2 of 64 × 10^6 CFU/mL (Table 1).

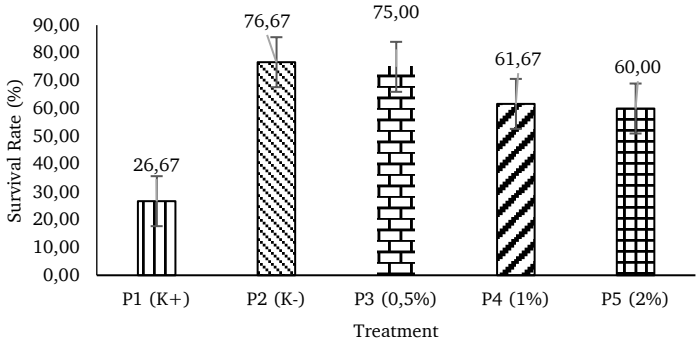


Figure 1. Vannamei shrimp survival after infection with *V. parahaemolyticus*.

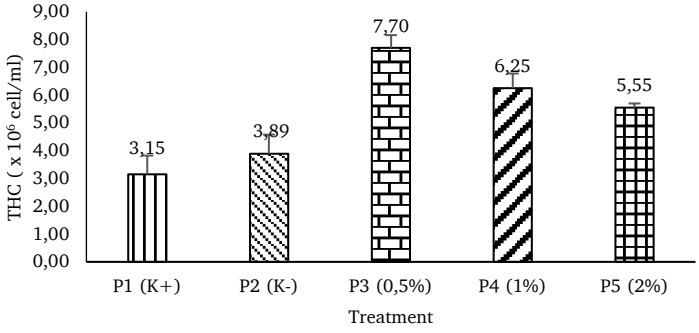
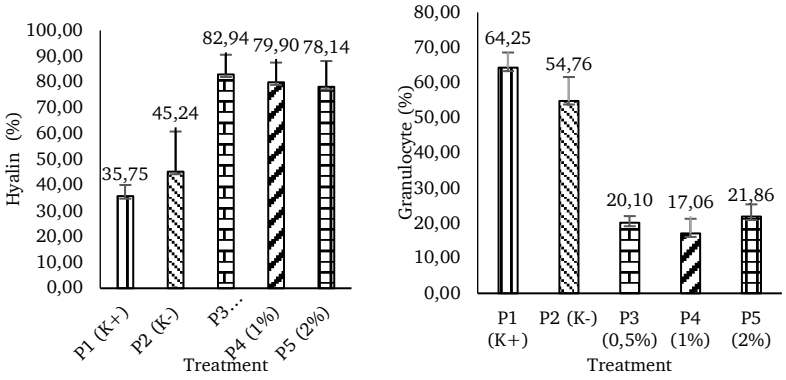


Figure 2. Total Haemocyte Count of Vannamei shrimps after *V. parahaemolyticus* infection.



(A) (B)

Figure 3. Vannamei shrimp survival after infection with *V. parahaemolyticus*; A (hyaline); B (granulocytes).

Table 1. Total bacteria and total *Vibrio*.

Treatment	Total Bacteria (cfu/ml)	Total Vibrio (cfu/ml)
P1 (K +)	136×10^8	66×10^8
P2 (K -)	116×10^6	54×10^6
P3 (0,5%)	52×10^8	12×10^8
P4 (1%)	66×10^8	23×10^8
P5 (2%)	75×10^8	34×10^8

Based on the results of this study (Figure 1), there was a high survival rate in P2 (76.67%), which acted as a negative control because the treatment was not infected with *V. parahaemolyticus* bacteria compared to other treatments that were infected with bacteria. However, P1 (26.67%) which was a positive control without extract and infection with bacteria showed the lowest survival rate. In general, the survival of shrimp post-larvae was closely related to the quality of feed, which can optimize digestive function to provide good body resistance (Nimrat *et al.*, 2011).

The concentration of *V. parahaemolyticus* bacteria as much as 104 CFU/mL injected intramuscularly was able to cause 50% mortality in the test shrimp population within one week (Rahmanto *et al*., 2014). *Vibrio* sp. is the causative agent of vibriosis in shrimps, resulting in mortality reaching 80-100% (Annisa *et al*., 2015). *Vibrio* sp. is a normal flora that can increase in conditions that are not favorable for shrimps (Chandrakala and Menaka, 2017). In Oktaviana's (2014) study, the highest survival rate was found in negative controls not being infected with *V. harveyi*. Based on Azhar's (2018) research, to increase the survival value, it is necessary to increase the immune response in shrimps as a preventive measure against pathogen attack and resistance.

The use of red betel leaf extract affected the number of hemocytes, thereby increasing the resistance of shrimps against *V. parahemolyticus* attack. Based on the results of this study, the highest number of hemocytes as described in Figure 2 was obtained in P3 which was treated with *V. parahemolyticus* infection and the addition of red betel leaf extract as much as 2%, accounting for 7.70×10^6 cells/mL, while P1 (positive control) obtained the lowest number of hemocytes overall treatments, accounting for 3.15×10^6 cells/mL. Increased THC can increase shrimp immunity, which leads to disease resistance (Hsieh *et al*., 2008). Based on research by Rohmin *et al.* (2017), an increase in the number of hemocytes indicates an immune response of Vannamei shrimps as seen from the number of parasites and the decreased impact of infection. These hemocytes themselves play a role in the crustacean body's defense process by reducing foreign

particles in the shrimp body (Hauton, 2012).

Based on the results of the study, the highest number of hyaline cells was noted in P3, accounting for 82.94% with the number of granulocytes of 20.10%. The indicator of the occurrence of pathogenic infection, which can cause inflammation as a form of non-specific defense that can be influenced by bacteria, fungi, and viruses, can be seen from the number of shrimps hemocytes (Rohmin *et al.*, 2017). The increase in hemocytes is related to the uptake of the extract as an immunostimulant in the Vannamei shrimp's body, thereby encouraging hemocyte activities to degranulate and phagocytose (Darwantin *et al*., 2016). The increased number of hyaline cells is also associated with phagocytic activities, while the granular cells themselves play a role in the production of melanin in the cytotoxic process (Hauton, 2012).

At the end of the study, the number of bacteria and the number of vibrios showed a significant increase between the control and the treatment groups were given red betel leaf extract, as shown in Table 1. In the treatment group given red betel leaf extract, after looking at the whole body of the infected shrimps, the number of bacteria and the number of vibrios were much lower than that in the treatment groups not given red betel leaf extract as an immunostimulant. This is thought to be due to the active ingredient as an antibacterial in red betel leaves.

In Kartika *et al.*'s research (2018), red betel leaves in the form of particles with a micrometer size are effective in inhibiting bacterial growth by penetrating bacterial cells. Prevention of vibriosis can be done through the provision of natural ingredients of red betel leaf extract which contains active ingredients such as flavonoids, alkaloids, tannins, polyphenolic compounds, and essential oils (Puspita *et al.,* 2018). Flavonoid compounds play a role in preventing cell oxidation and in increasing the immune system for the body's defense of Vannamei shrimps, while alkaloids play a role in inhibiting the process of the formation of the peptidoglycan component of bacterial cells so that these cells are not formed (Syahida *et al*., 2013).

CONCLUSION

The best application of red betel leaf extract which was able to prevent the vibriosis attack was noted in P3 (Feed +0.5% red betel leaf extract), which was seen from the increase in the survival rate, the number of hemocytes, differential hemocyte counts and a decrease in total bacteria.

ACKNOWLEDGMENT

The authors would like to thank the University of Mataram for facilitating and fully supporting the process of this research.

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