doi:http://doi.org/10.15578/jra.19.3.2024.177-190 *Jurnal Riset Akuakultur, 19 (3), 2024, 177-190*

THE POTENTIAL USE OF SIAM WEED (*Chromolaena odorata*) LEAF EXTRACT AS AN ALTERNATIVE ANTIBACTERIAL COMPOUND TO TREAT *Vibrio parahaemolyticus* INFECTION IN PACIFIC WHITE SHRIMP (*Litopenaeus vannamei*)

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(Submission: 07 May 2024; Final revision: 03 July 2024; Accepted: 03 July 2024)

ABSTRACT

Siam weed plant or Siam weed (*Chromolaena odorata*) is an herb commonly used as a medicinal plant in Asian countries, including Indonesia, particularly in Southeast Sulawesi. This study explores the effectiveness of different Siam weed leaf extract concentrations in treating *Vibrio parahaemolyticus* infection in Pacific white shrimp (*Litopenaus vannamei*). In this study, the infected shrimps were soaked in *C. odorata* leaf extract solution at 1, 2, and 3 ppt concentrations and no soaking of the extract (control). The parameters measured were recovery rate, survival rate, percentage of total haemocyte count (THC) and differential haemocyte count (DHC). The results showed that the *V. parahaemolyticus*-infected Pacific white shrimps soaked in 3 ppt *C. odorata* leaf extract had the highest recovery and survival rates compared to shrimp treated with *C. odorata* leaf extract at 1 and 2 ppt. Similarly, the shrimp group treated with 3 ppt of *C. odorata* leaf extract had better haemolymph profiles than those treated with the other concentrations of *C. odorata* leaf extract. This study concludes that *C. odorata* leaf extract enhances the immune response of *L. vannamei* by increasing the activity of semi-granular cells in eliminating the pathogenic cells of *V. parahaemolyticus*.

KEYWORDS: granular cell; herb medicine; immunity; shrimp; vibriosis

ABSTRAK:* *Potensi Pemanfaatan Ekstrak Daun Krinyuh Sebagai Senyawa Antibakteri Alternatif untuk Mengobati Infeksi Vibrio parahaemolyticus pada Udang Vaname *(Litopenaus vannamei)*

*Tanaman krinyuh **(Chromolaena odorata)** merupakan tanaman herbal yang umum digunakan sebagai tanaman obat di negara-negara Asia, termasuk Indonesia, khususnya di Sulawesi Tenggara. Penelitian ini bertujuan untuk mengetahui efektivitas berbagai konsentrasi ekstrak daun tanaman krinyuh dalam mengobati infeksi **Vibrio parahaemolyticus** pada udang vaname **(Litopenaus vannamei)**. Pada penelitian ini, udang yang terinfeksi direndam ke dalam larutan ekstrak daun **C. odorata** pada konsentrasi 1, 2, dan 3 ppt dan tanpa perendaman ekstrak (kontrol). Parameter yang diukur adalah tingkat kesembuhan, tingkat kelangsungan hidup, persentase jumlah hemosit total (JHT), dan jumlah hemosit diferensial (JHD). Hasil penelitian menunjukkan bahwa udang vaname yang terinfeksi **V. parahaemolyticus** yang direndam dalam ekstrak daun **C. odorata** 3 ppt memiliki tingkat kesembuhan dan kelangsungan hidup*

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*tertinggi disbanding udang yang diobati dengan ekstrak daun **C. odorata** pada konsentrasi 1 dan 2 ppt. Demikian pula, kelompok udang yang diberi 3 ppt ekstrak daun **C. odorata** memiliki profil hemolim yang lebih baik daripada yang diberi konsentrasi ekstrak daun **C. odorata** lainnya. Penelitian ini menyimpulkan bahwa ekstrak daun **C. odorata** meningkatkan respons imun **L. vannamei** dengan meningkatkan aktivitas sel semi-granular dalam menghilangkan sel patogen **V. parahaemolyticus**.*

KATA KUNCI: kekebalan tubuh; sel granular; tanaman obat; udang; vibriosis

INTRODUCTION

Pacific white shrimp has become a national flagship aquaculture product in Indonesia. This shrimp species is considered one of the strategic commodities supported by the Indonesian government to increase the contribution of the fisheries sector on revenue and foreign exchange. In 2020, the volume of shrimp exports reached 239,282 tonnes and continued to increase in 2021, reaching 250,715 tonnes. Due to the high selling price and popularity of the shrimp as an aquaculture species, The Pacific white shrimp farms have been developed and operated at various technological levels in almost all parts of Indonesia (Ministry of Marine Affairs and Fisheries, 2022). Such intense development has triggered frequent disease outbreaks, which could backfire on the future sustainable development of Pacific white shrimp farming. These disease outbreaks are caused mainly by the interaction between the shrimp as the host, the presence of bacterial or viral agents and the poor environmental condition. In this interaction, environmental factors trigger both positive and negative effects on the host-pathogen relationship (Mahulauw *et al.*, 2022). Intensive shrimp farming technology frequently leads to declining water quality as high stocking densities and feed inputs produce a large amount of suspended and deposited waste in shrimp ponds, thereby triggering diseases (Pratiwi *et al.*, 2020). *Vibrio* sp. bacteria often infect shrimp in hatchery and grow-out ponds causing mass mortality of shrimp. One type of *Vibrio* sp. that has been identified to infect shrimp is *Vibrio parahaemolyticus* (Siregar *et al.*, 2021). *Vibrio parahaemolyticus* is well-known as the causative pathogen of early mortality syndrome (EMS) or acute hepotopancreatic necrosis disease (AHPND) of the Pacific white shrimp culture in Mexico (Nunan *et al.*, 2014) and a number of Asian countries such as China, Vietnam, Malaysia, Thailand, India, and Indonesia. The AHPND outbreaks in shrimp farms in Indonesia are reported in several provinces, including Serang and Banten (Saputra *et al.*, 2023). The signs of an infected shrimp include the pale color of the carapace and empty midgut, as well as clinical and pathological symptoms such as swimming into the corner of the container. The shrimp showed melanization due to substantial hemocyte infiltration, necrosis, atrophy, granulomas, and cell sloughing (Mulyadi *et al.*, 2020; Saputra *et al.*, 2023).

Shrimp mortality due to vibriosis has become a widespread problem, not only in Indonesia but around the world. Farmers have used various methods to treat the disease, one of which is the use of antibiotics and synthetic chemical compounds. In recent years, the use of synthetic chemicals in pathogen control has been restricted because antibiotics and chemical-type medicinal compounds can accelerate resistance to pathogenic microorganisms, pollute the environment and affect human health when continuously exposed to these antibiotics and chemical residues (Hossain *et al.*, 2021). Unsurprisingly, there are cases of the export rejection of Indonesian shrimp products abroad due to the detection of significant amounts of antibiotics accumulated in the exported products. Therefore, alternatives to replace antibiotics with natural ingredients that are environmentally friendly and easily biodegraded are necessary.

A natural ingredient which is often used as a herbal medicine is the Siam weed (*Chromolaena odorata*) leaf. The plant's leaves contain flavonoids, which can act as antiviral and antibacterial agents (Ernawati & Jannah, 2021). Extract of *C. odorata* leaves has also been reported to have high antioxidant activity in in vitro and is rich in tannins, phenols, flavonoids, saponins and steroids (Alisi *et al*., 2011). Due to its active ingredients, we strongly argue that *C. odorata* leaf extract has the same potential to be used in aquaculture as an antibacterial and antiviral agent, especially to control infectious diseases.

The potential of this plant described above merits further research to confirm its prospects. Therefore, this study aimed to determine the effect of different concentrations of *C. odorata* leaf extract to treat *V. parahaemolyticus* infection in Pacific white shrimp, focusing on the aspects of recovery rate, survival rate and haemolymph profile of the shrimp. We expect that the natural compounds in Siam weed leaves could be an alternative treatment to overcome vibriosis infection in Pacific white shrimp that is safer for both the cultured organisms and the environment.

MATERIALS AND METHODS

Study Design

The research design used in this study was a complete randomised design (CRD) with four treatments and three replicates. The treatments were treatment A (1 ppt *C. odorata* leaf extract), treatment B (2 ppt *C. odorata* leaf extract), treatment C (3 ppt *C. odorata* leaf extract), and control treatment (0 ppt *C. odorata* leaf extract).

Preparation of Chromolaena odorata Leaf Extract

Fresh and undamaged *C. odorata* leaves were washed using clean freshwater and drained. The leaves were then dried in the sun for nine hours. After completely drying, the *C. odorata* leaves were crushed into powder and sieved using a 0.9 mm sieve. The maceration process of *C. odorata* leaf powder was conducted using methanol (1:4) for 3 × 24 hours (Fadia *et al*., 2020). The macerate was then filtered using Whatman No. 40 filter paper. The extract solution was heated to 60°C using a rotary vacuum evaporator (Stuart). The extract was transferred to a sterile bottle. The result of the pure extract is considered as 100% initial concentration (Lingga *et al*., 2016).

Preparation of Containers and Test Shrimp

The test shrimps used were 40-45 days old and weighed 7.9±0.83 g of Pacific white shrimps collected from shrimp ponds in Lainea District, South Konawe Regency, Southeast Sulawesi, Indonesia. The shrimp were first acclimatized for seven days in a 2 × 1 × 0.3 m fibre tank filled with seawater with a salinity of 35 ppt, up to 50 % of the tank volume. The shrimp used were selected based on their physical condition (defects-free). The number of shrimp used was 120 individuals per tank. This study has received ethical consideration from the Halu Oleo University Research Code of Ethics Institute to design and conduct field research on shrimps.

Preparation of Pathogenic Bacteria

Vibrio parahaemolyticus bacteria was sourced from the isolates of *V. parahaemolyticus* bacteria rejuvenated on thiosulfate citrate bile salt sucrose (TCBS) media and then incubated at 28°C for 24 hours. The enrichment was performed on tryptic soy broth (TSB) media containing 2.5% NaCl for 24 hours at 28°C.

Figure 1. Siam weed plant (*Chromolaena odorata*) (Magfira, 2023)

Bacteria growing on the TSB media were centrifuged at 2000 rpm for 15 minutes to separate the bacteria from the media and then washed twice with phosphate-buffered saline (PBS) . The density of infected bacteria was determined using the spectrophotometric method, following the procedure developed by Rosmania & Yanti (2020). The test shrimp were infected with *V. parahaemolyticus* bacteria by intramuscular injection in the third segment of the abdomen of 0.1 mL per shrimp. The bacteria were injected at a density of 10^8 cfu.mL^{-1}.

Disease Treatment

The treatments were conducted 24 hours after bacterial infection by immersing the shrimp for 8 minutes in water mixed with *C. odorata* leaf extract at the concentration specified for each treatment. The shrimp were then placed in aquaria sized 35cm×35cm×40cm at a density of 10 shrimp per aquarium and kept for seven days to observe the number of shrimp that recovered, the number of shrimp alive at the end of the study, the percentage of total haemocyte count (THC) and the percentage of differential haemocyte count (DHC). Feeding is given in the morning and evening and water quality maintenance was regularly done by siphoning fecal and uneaten feed before the subsequent feeding.

Variables Observed

Recovery Rate

Observation of the level of shrimp recovery was carried out visually by directly observing the morphology of the test shrimp, how they swam, and their response to the feed given. The success of vibriosis treatment at the end of the study was analysed by calculating the recovery rate (RR) using the following formula (Ramani *et al.*, 2020):

$$\text{Recovery Rate} = \frac{\text{Number of shrimp cured}}{\text{Number of infected shrimp}} \times 100\,\% \quad ......(1)$$

Survival Rate

According to Effendie (2002), the survival rate can be calculated using the formula:

$$\text{Survival Rate} = \frac{\text{Number of shrimp at the beginning of rearing}}{\text{Number of shrimp at the end of rearing}} \times 100\,\% \quad .....(2)$$

Total Haemocyte Count

The THC was calculated by collecting 0.1 ml of shrimp haemolymph from the base of the first swimming leg using a 1 ml syringe filled with 0.1 ml of 3.8% sodium citrate anticoagulant and then homogenise. After homogenisation, the first drop was discarded, and the second was placed on the haemocytometer. The total number of haemocytes was then observed, and the number of cells was counted under the microscope with 100x magnification based on the modified equation of Blaxhall & Daishley (1973) as follows:

$$\text{Total haemocyte count} = \frac{\text{Number of cells}}{\text{Volume of the box}} \times \text{Dilution factor} \times 10^3 \quad \text{.........(3)}$$

Differential Haemocyte Count

The procedure for calculating the DHC was that after homogenisation, the first drop was discarded, and the second drop was dropped onto the object glass, then immersed in Giemsa solution for 10-15 minutes, collected and dried. The differential haemocytes were then observed and counted under a light microscope at 100x magnification. The number of differential haemocytes was counted until it reached 100 cells, and then the percentage of each cell type (hyaline and granular) was determined. According to Jones (1962), the percentage of each haemocyte type was calculated using the formula as follows:

$$\text{Haemocyte cell counts} = \frac{\text{Number of haemocyte cell types}}{\text{Total haemocyte}} \text{x100 \%} \quad \text{.......(4)}$$

Data Analysis

Survival rate (SR), recovery rate, total haemocyte count (THC), and differential haemocyte count (DHC) were subjected to analysis of variance (ANOVA) at a 95% confidence level using SPSS (IBM SPSS Statistic 21, Chicago, US). When the results showed differences, the Duncan's test was performed.

RESULTS AND DISCUSSION

Herbal compounds are considered safe for use in aquaculture disease control. Many plants have been studied and shown to have antimicrobial or immunostimulant properties to treat antiviral/antibacterial diseases in aquatic organisms. In this study, we propose the potential efficacy of the Siam weed plant to treat vibriosis disease in shrimp. The plant is herbal medicine which is part of the local wisdom among the people of the Southeast Sulawesi region to treat humans.

A study by Kurang & Penlaana (2022) reported that results of the phytochemical tests showed that the plant contains active ingredients such as flavonoids, alkaloids, saponins, and tannins, which can be used as antibacterial. To assess the immune response of shrimp to the Siam weed plant, we evaluated the survival rate and haemolymph profile of shrimp infected by *V. parahaemolyticus* after being treated with different plant extract concentrations via immersion.

Recovery Rate

Observations of the recovery rate of Pacific white shrimp during the study are shown in Figure 1. The analysis of variance showed that the variations in the immersion concentrations of *C. odorata* leaf extract had significantly affected the recovery rate of Pacific white shrimp.

Vibriosis disease, caused by *V. parahaemolyticus* bacteria, is an important disease that often affects shrimp at the hatchery level and grow-out stage in ponds, causing mass shrimp mortality. According to Saputra *et al.* (2023), the virulence of *V. parahaemolyticus* strains causes up to 100% mortality of Pacific white shrimp within 73 hours post-infection. *Chromolaena odorata* leaf extract is used to treat vibriosis diseases based on its content of active compounds such as flavonoids, alkaloids, terpenoids, saponins and tannins. These compounds have been known to act as antibacterial agents (Kurang & Penlaana, 2022)

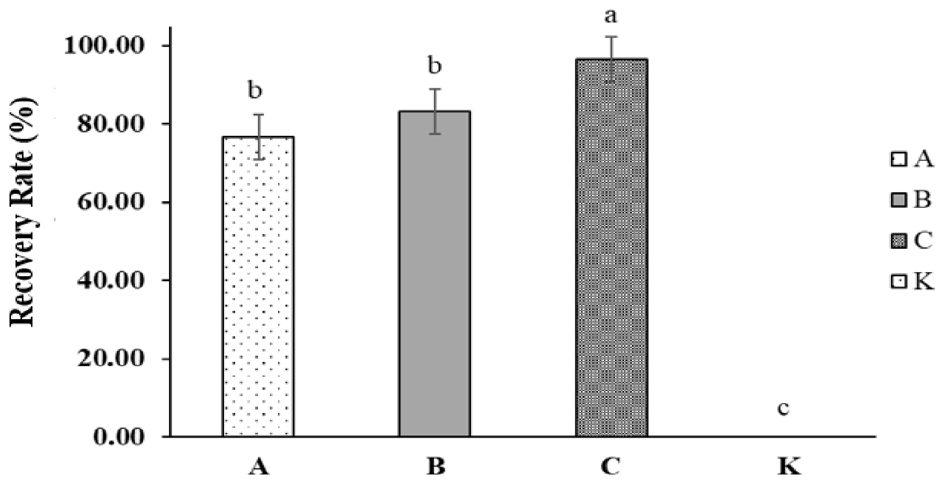

Figure 1. The recovery rate of Pacific white shrimp with immersion treatment of *Chromolaena odorata* leaf extract at different concentrations (A = 1 ppt, B = 2 ppt, C = 3 ppt, K = control). Each data is n = 3 shrimps. Different superscripts on each histogram bar indicate differences among treatments ($p<0.05$)

and do not compete with human needs and economically insignificant use (Tamrin, 2013).

The recovery rate post-infection is the number of organisms that recover after being infected with the pathogen (Roh & Kannimuthu, 2023). The recovery rate of Pacific white shrimp in this study was observed based on the clinical signs seen in shrimp after infection with vibriosis. The test organisms are considered cured when the clinical symptoms of vibriosis disease disappear. The observation of clinical signs in shrimp infected with *V. parahaemolyticus* bacteria included changes in both behavior and morphology. Behavioral changes such as reduced appetite, unstable swimming, always swimming, and staying in the corner of the tank were evidently present during the observation. The analysis of variance showed that different concentrations of *C. odorata* leaf extract had a significantly different effect on the recovery rate of Pacific white shrimp.

Survival Rate

Survival rate is the percentage of shrimp alive at the end of rearing compared to the number of organisms at the beginning of observation. The results showed significantly higher shrimp survival rates in the treatments immersed in *C. odorata* leaf extract solution (96.67-100%) compared to shrimp in the control treatment or without immersion in *C. odorata* leaf extract solution (56.67%). The high average survival rate of shrimp strongly indicates that *C. odorata* leaf extract, with its multi-beneficial characteristics (antibacterial, anti-inflammatory, antimicrobial, and antioxidant), successfully suppressed the viral infection.

The recovery rate strongly influences the survival rate. The higher the recovery rate, the higher the potential survival rate of shrimp. The highest recovery rate of Pacific white shrimp was found in the treatment of *C. odorata* leaf extract at a concentration of 3 ppt, namely 96.67%, which translated into a survival rate of 100%. The results obtained in this study also showed that the application of *C. odorata* leaf extract with a concentration of 3 ppt did not exceed the threshold where the solution will have toxic effects on Pacific white shrimp. The results of observations of survival rates in Pacific white shrimp at the end of the experiment are presented in Figure 3.

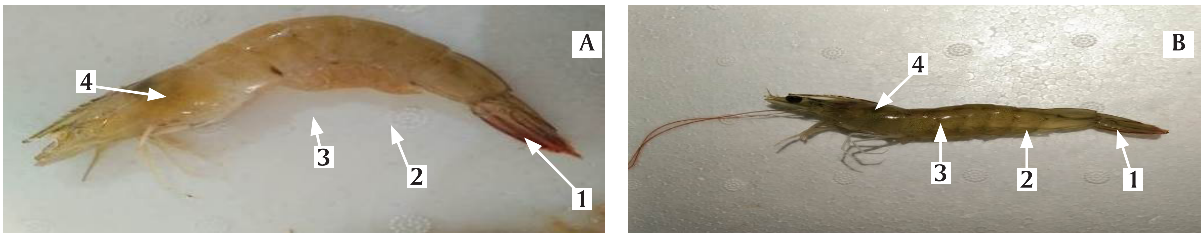

Figure 2. Morphological differences between unhealthy and recovered Pacific white shrimp. (A) Un-healthy shrimp (1. The tail is red, 2. The body looks pale, 3. The swimming legs are red, and 4. The head appears to have a red spot) and (B) Recovered shrimp (1. The tail is no longer red, 2. The body color returns to normal (not pale), 3. The swimming legs are no longer red, and 4. There are no red spots on the head)

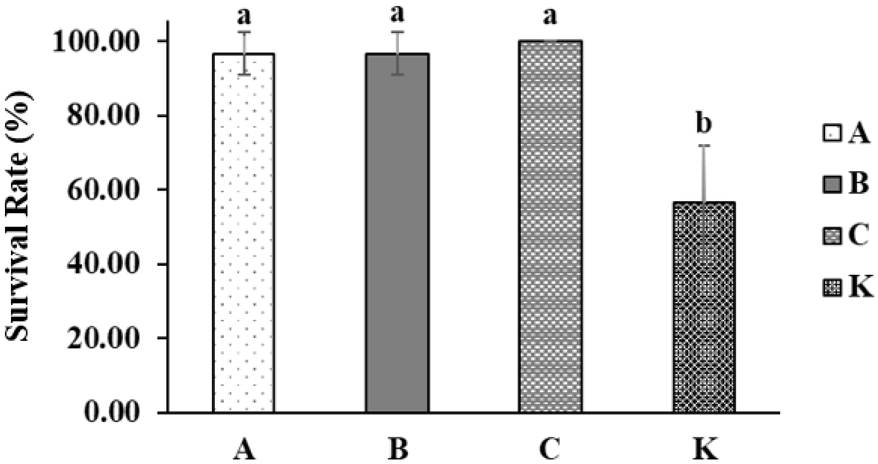

Figure 3. The survival rate of Pacific white shrimp treated with different concentrations of *Chromolaena odorata* leaf extract solution (A = 1 ppt, B = 2 ppt, C = 3 ppt, K = control). Each data is n = 3 shrimps. Different superscripts on histogram bars indicate significant differences among treatments ($p<0.05$)

Total Haemocyte Count

The observation of total haemocyte count (THC) in Pacific white shrimp during the study is presented in Figure 4. The THC level on the 3rd day after treatment showed that the THC level had decreased compared to the previous observation. This change is suspected due to the disruption of the shrimp's immune system by *V. parahaemolyticus* bacteria causing the shrimp to require time to recover. According to Suleman *et al.* (2020), the low amount of THC was due to the process where haemocyte cells undergo degranulation, cytotoxicity and lysis processes resulting in the decrease of haemocyte count. On day 6 after treatment, the THC level increased again in the three treatments of *C. odorata* leaf extract. In contrast, the THC level in the control treatment had a lower THC level. These results suggest that the increase in THC was strongly affected by *C. odorata* leaf extract.

An increase in THC indicates an increase in inter-cellular and humoral immune responses. Mulyadi *et al.* (2020) argued that an increase in the number of haemocytes indicates haemocyte proliferation. This response implies that the treated shrimp's immune system is ready to deal with pathogenic infections. Similarly, Mahenda (2021) also asserted that the increase in the number of haemocytes in the Pacific white shrimp showed that the

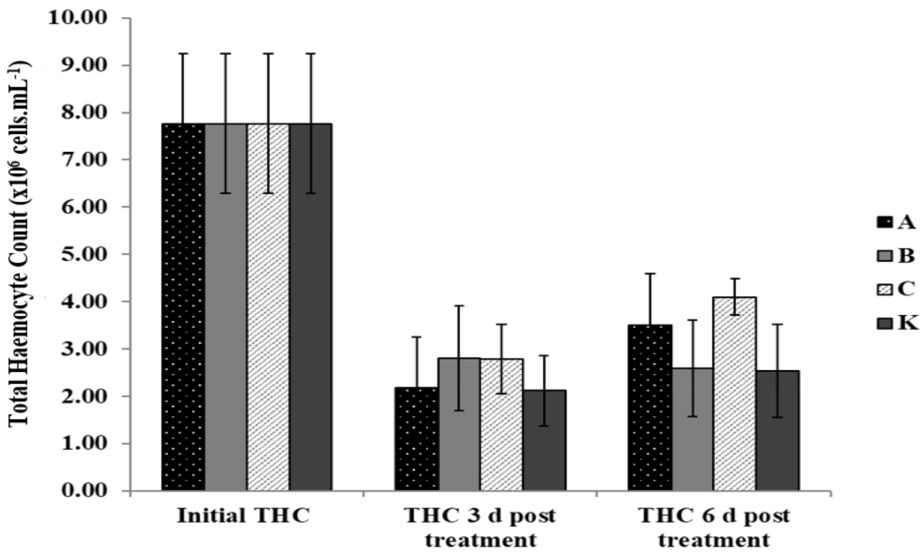

Figure 4. Total haemocyte count of Pacific white shrimp treated with *Chromolaena odorata* leaf extract solution at different concentrations (A = 1 ppt, B = 2 ppt, C = 3 ppt, K = control). Each data is n = 3 shrimps

shrimp had entered the recovery phase after being attacked by a disease. These findings confirm that the *C. odorata* leaf extract plays a role in stimulating the formation of haemocyte cells in the Pacific white shrimp. In addition, considering the ability of *C. odorata* leaf extract as an antimicrobial, the treatments may have affected the reduction in the number/density of pathogenic bacteria, giving the Pacific white shrimp a better opportunity to increase the formation of haemocyte cells. The increase in total haemocytes indicates that the body's defense response to foreign particles, including pathogenic microorganisms, entering the shrimp's body has increased. Foreign particles that enter the shrimp body are recognised by haemocyte cell receptors, which generate a cellular response such as intercellular signaling cascades, phagocytosis, encapsulation and nodular aggregation (Rodriguez & Moullac 2000).

Differential Haemocyte Count

Differential haemocyte count (DHC) generally acts as a cellular response for the crustacean immune system, including phagocytosis, cytosis, encapsulation, cytotoxicity, haemolysis, cell adhesion and degranulation. Both Johansson *et al.* (2000) & Mulyadi *et al.* (2020) concurred that crustaceans have three types of haemocytes that are morphologically distinct but interrelated, consisting of granular cells that function to recognise and bind to the cell wall, semi-granular cells that function to process βGBP complexed with glucan through degranulation and function in the release of the proPO activating system including cell adhesion proteins and peroxynectin proteins. The released peroxynectin further stimulates the phagocytosis process of pathogens by hyaline cells or the encapsulation process by semi-granular cells. The three cell types are shown in Figure 5.

The results of variance analysis on the percentage of granular cells data on day 3 and day 6 post-treatment showed that the different concentrations of *C. odorata* leaf extract solution had no significant effect on the percentage of granular cells of the tested Pacific white shrimp (Figure 6). The percentages of granular cells generally increased on day 3, which was thought to be a granular cell response to pathogen invasion. The increase in granular cells on day 3 could be the response of the shrimp's immune system to increase the recognition capability of haemocytes in detecting the pathogenic cells. Therefore, It is

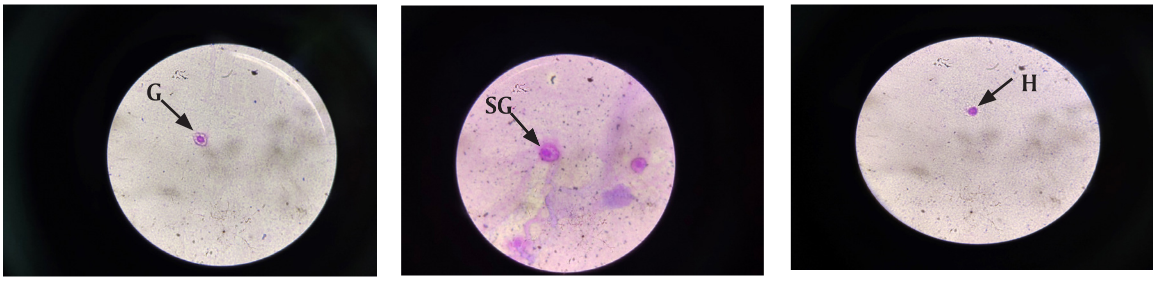

Figure 5. Haemocyte cells in crustaceans. H= Hyaline, SG= Semi Granular, G=Granular

suggested here that the bioactive compounds in *C. odorata* leaf extract may have enhanced the immune response in the shrimp body, thereby inducing the shrimp body's defense mechanism. Van de Braak (2002) suggested that an increase in the immune response due to the presence of bioactive compounds in the shrimp body will stimulate haemocytes to release proPO and protein binding enzyme Fenilpropanilain (PPA). Such a response can trigger the haemocytes to increase their activity as defense cells.

The percentages of granular cells in the shrimp treated with the extract concentrations of 1, 2 and 3 ppt on day 6 were higher than that of the control treatment. It is highly likely that the increase in the granular cell count is a post-infection response. Although the test shrimp have entered the recovery phase where some shrimp have completely recovered (Figure 8), the percentage of granular cells in shrimp treated with different concentrations of *C. odorata* leaf extract remains in a high state, supposedly aimed at preparing haemocyte cells in the event of re-infection. This is implied by Hauton (2012), who stated that when the concentration of granular cells in the haemolymph is high, proPO activity also increases, increasing the shrimp's resistance to disease. This is also supported by Smith *et al.* (2003), who stated that granular cells have antibacterial peptide compounds capable of blocking the entry of pathogenic bacteria into the shrimp's body. According to Johansson *et al.* (2000), when a pathogen infection occurs, the process that occurs at the beginning of infection is the introduction and binding of pathogen cells by semi-granular and granular haemocytes.

The analysis of variance on the percentage of semi-granular cells on the 3rd day after treatment showed that different concentrations of *C. odorata* leaf extract did not produce a significantly different effect on the percentage of semi-granular cells produced in Pacific white shrimp. Observation on the 6th day after the treatments showed that different concentrations of *C. odorata* leaf extract solution produced a significantly different effect on the percentage of semi-granular cells released by the test shrimp (Figure 7).

The percentages of semi-granular cells on day 3 were higher in shrimp treated with different concentrations of *C. odorata* leaf extract compared to that of the control treatment. It is suggested that by day 3, encapsulation of pathogenic cells had occurred in the test shrimp treated with the extract solutions. At the same time, the control treatment was still late in responding to the disease, indicated by the low percentage of semi-granular cells. Ardiansyah *et al.* (2023) stated that the low percentage of semi-granular cells after bacterial infection is one of the consequences of an increase in granular or hyaline cells in areas infected with bacteria. According to Van de Braak (2002), semi-granular cells are the maturation process of hyaline cells, where when pathogens enter the body of shrimp, hyaline cells will play the most important role, resulting in a decrease in the number of semi-granular cells.

The percentages of semi-granular cells on day 6 in shrimp treated with different concentrations of *C. odorata* leaf extract

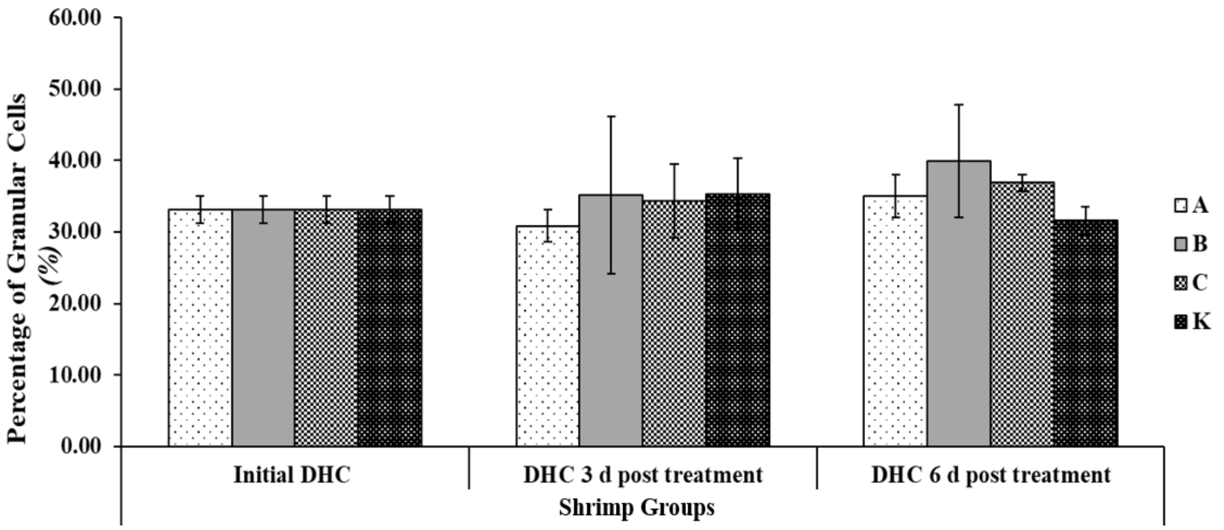

Figure 6. Percentage of granular cells in Pacific white shrimp treated with different concentrations of *Chromolaena odorata* leaf extract (A = 1 ppt, B = 2 ppt, C = 3 ppt, K = control) at different observation times. Each data n = 3 shrimps

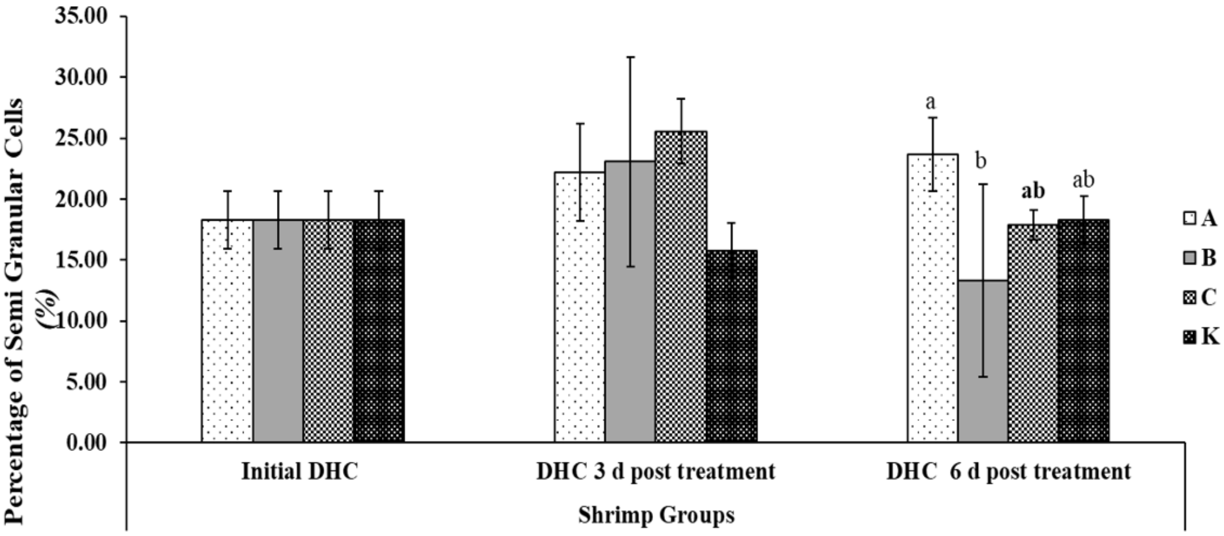

Figure 7. Percentage of semi-granular cells in Pacific white shrimp treated with *Chromolaena odorata* leaf extract solution at different concentrations (A = 1 ppt, B = 2 ppt, C = 3 ppt, K = control) at different observation times. Each data n = 3 shrimps. Different superscripts on the histogram bars indicate significant differences among treatments ($p<0.05$)

solution at 1, 2 and 3 ppt has decreased, supposedly because the pathogenic cells have been successfully eliminated by encapsulating pathogenic cells in the previous days. Encapsulation of pathogenic cells will affect the decrease of semi-granular cells by rendering the semi-granular cells involved inactive. According to Suleman *et al.* (2019), during a pathogen attack, granular and semi-granular cells will carry out the process of degranulation, cytotoxicity and lysis of foreign material, thus reducing the number of granular and semi-granular cells circulating in the shrimp body. Encapsulation is a defensive response to large numbers of foreign particles (Danwattananusorn, 2009). The increase in semi-granular cells in the control treatment was thought to be due to the late production of semi-granular cells in the control treatment. This suggests that the pathogen encapsulation process also occurs late in the control treatment so that when observing the shrimp recovery rate parameters, the shrimp in the control treatment are all still in a diseased state.

The percentages of hyaline cells in Pacific white shrimp during the study are shown in Figure 8. The analysis of variance showed that at the beginning and 6 days after the treatments, different concentrations of *C. odorata* leaf extract did not significantly affect the percentages of hyaline cells produced by Pacific white shrimp. However, on the 3rd day after the treatment, the *C. odorata* leaf extract solution significantly affected the percentage of hyaline cells produced (Figure 8).

Semi-granular cells (by encapsulation) and hyaline cells (by phagocytosis) play a role in eliminating pathogenic cells. Thus, the increase in semi-granular cells influences the percentage of hyaline cells. The elimination of pathogenic cells had likely occurred on day 3, but the role of semi-granular cells was more significant than that of hyaline cells. This was indicated by an increase in the percentage of semi-granular cells and a decrease in hyaline cells. This is in line with the findings of Ekawati *et al.* (2012), who found that granular cells play a greater role than hyaline and semi-granular cells.

The percentages of hyaline cells on day 6 increased in the shrimp body treated with and without different concentrations of *C. odorata* extract compared to that of day 3 beyond the initial hyaline percentages. This occurred because at concentrations of 1, 2 and 3 ppt, the test shrimp have entered the recovery phase where some organisms have fully recovered. Observed recovery signs were indicated by changes in the test shrimp's morphological appearance, which have returned to normal. In addition, the percentages of hyaline cells in shrimp treated with the extract solution at 1, 2 and 3 ppt, previously decreased, had increased again. In the control treatment, the percentages of hyaline cells increased. However, considering the decreased percentages of granular cells while the semi-granular cells

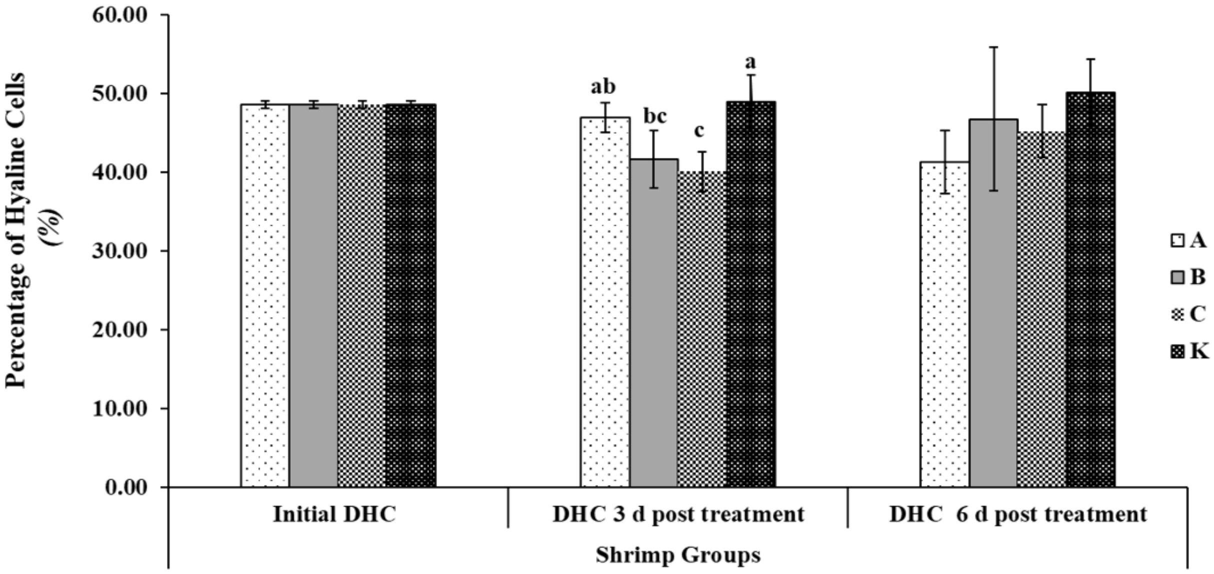

Figure 8. Percentages of hyalin cells in Pacific white shrimp treated with *Chromolaena odorata* leaf extract solution with different concentrations (A = 1 ppt, B = 2 ppt, C = 3 ppt, K = control) at different times. Each data n = 3 shrimps. Different superscripts on the histogram bars indicate differences among the treatments ($p<0.05$)

increased suggests that the recovery process occurred late in the control treatment.

Based on the observations of the percentage of semi-granular cells and hyaline cells, it appears that the *C. odorata* leaf extract treatments contributed to eliminating pathogenic cells by increasing the activity of semi-granular cells. In the control treatment, pathogenic cell elimination occurred through increased hyaline cell activity. However, the activity of hyaline cells is argued to occur slower from which it had a negative effect on the recovery rate of Pacific white shrimp.

CONCLUSIONS

Immersion of *V. parahaemolyticus*-infected Pacific white shrimp in *C. odorata* leaf extract has an effect on improving the hemolymph profile, recovery rate and survival of Pacific white shrimp. Immersion treatment of Pacific white shrimp in *C. odorata* leaf extract with a concentration of 3 ppt resulted in the highest recovery rate and survival rate of Pacific white shrimp compared to treatments with concentrations of 1 and 2 ppt. Based on the observations of the percentage of semi-granular cells and hyaline cells, it appears that the *C. odorata* leaf extract treatments contributed to the elimination of pathogenic cells by increasing the activity of semi-granular cells. In the control treatment, pathogenic cell elimination occurred through increased hyaline cell activity, which it had a negative effect on the recovery rate of Pacific white shrimp.

ACKNOWLEDGEMENTS

The authors would like to thank the managers for the use of the facilities at the UHO FPIK Aquaculture. The authors also would like to thank the staff in Hatchery and Fisheries Production Laboratory and the UHO FPIK Aquatic Productivity and Environment Laboratory for the assistance during the research.

FUNDING

This work was entirely self-funded.

AUTHOR CONTRIBUTION

All authors are contributed. M: Conducting research, maintaining research data, and writing the initial draft of the article; LOBA: Methodology and design development; and IN: Leading and supervising the planning and implementation of research activities, including reviewing manuscripts.

DECLARATION OF COMPETING INTEREST

The authors declare that there is no conflict of interest between the authors and the commercial antibiotics and immunostimulants mentioned in this paper.

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