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The Growth and Hemocyte Analysis of the Shrimp (*Litopenaeus vannamei*) Induced by Different Concentration of Artificial Seawater and Water Extract Of Sea Grapes (*Caulerpa lentillifera*)

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ABSTRACT

This study aimed to investigate the growth rate and hematocyte (THC and DHC) profiles in white shrimp (*Litopenaeus* vannamei) induced by different concentration of artificial seawater and (*Caulerpa lentillifera*) extract. In this study, white shrimp observing approximately 6-7 cm in length were subjected to various concentrations of different concentration artificial seawater and sea grapes extract. The findings of the study indicated that the treatment of different salinities and water extract of sea grapes shows obvious effect on the growth rate and hemocyte (THC and DHC) in white shrimp. The growth value (SGR and average daily growth) and hemocyte number was significantly increased at the concentration of 10 ppt with 0.65 gram sea grapes extract with around 3.8%, 0.07%, respectively. THC was around 50×10^4, semi-granul, granul, and hyaline with value 35×10^4, 30×10^4 and 70×10^4, respectively. The concentrations of 10 ppt with 0.65 gram sea grapes extract was also showing the lowest value of ROS below 5 ng/L.

INTRODUCTION

White shrimp (*Litopenaues vannamei*) aquaculture has grown rapidly to become one of the most important commodities in international seafood trade **(Elshopakey *et al.*, 2018)**. White shrimp is known as the representation of over 90% shrimp in the Western hemisphere **(Wurmann *et al.*, 2004)**. This species is also a type of shrimp that widely cultivated in Indonesia due to the promising prospects and profits **(Supono *et al.*, 2019)**. White shrimp is generally cultivated in semi-intensive systems located near coasts. Successful culture of white shrimp depends on the quality of seawater used in the system, as well as use of a wastewater treatment plant to prevent pollution of adjacent areas

(Araneda *et al.*, 2008). The increasement of the shrimp production can be maintain by monitoring the water quality, including the high organic matter content, providing the proper feed, and appropriate administrations of probiotics or antibiotics doses **(Taslihan *et al.*, 2015)**. The usage of antibiotics is not recommended in shrimp farming nowadays because it leaves residue that could be harmful to human body if it consumed. Safe ingredients that have antibacterial content can be obtain from some plants. Herbal plants are now widely being used to replace the usage of antibiotics because they are safe to consume due to zero residue produced and do not harm the environtment so it is completely safe **(Aminzare *et al.*, 2015)**. One of the plant that contain antibacterial content is sea grapes (*Caulerpa lentillifera*).

Caulerpa lentillifera, also known as sea grapes, are green seaweeds eaten raw as salad and cultivated in different parts of the world, particularly in the Indo–Pacific region **(de Gaillande *et al.*, 2017; Nagappan and Vairappan, 2014)**. *Caulerpa lentillifera* is mainly cultivated in the Phillipines, Vietnam, and Japan and instantly become popular because it contains high amount of polysachharides as well as dietary fiber, protein, and several essential unsaturated fatty acids **(Zhang *et al.*, 2020)**. Marine organisms such as seaweeds are known to be rich in source of natural bioactive constituents, such as proteins, polysaccharides, and secondary metabolites **(Yap *et al.*, 2019)**. These bioactive compounds has been found to contribute to anti-inflammatory, antioxidant, and antimicrobial properties **(Alshalmani *et al.*, 2014).** Studies have shown that seaweeds possess bioactive compounds with strong antioxidant capacity to protect seaweeds against reactive oxygen species (ROS). The antioxidant compounds from seaweeds may belong to the main classes of phytochemicals known as phenolics and flavonoids due to the presence of hydroxyl groups, which act as hydrogen-donors to stabilize the free radicals and to terminate the generation of new free radicals in fish body **(Pereira *et al.*, 2009)**. *Caulerpa lentillifera* can be used as the herbal plant to replace the usage of antibiotics to prevent shrimp from diseases.

Besides the usage of herbal plant, the increasement of white shrimp productivity can be done by serving a proper environment for it to develop and grow. A number of factors have limited expansion of white shrimp culture, including the high cost of coastal real estate and the constant appearance of viral disease such as white spot syndrome (WSSV) which could lead to the failure of shrimp aquaculture. One proposed solution to white shrimp production problem is the use of water with salinities lower than seawater **(Arneda *et al.*, 2008)**. Artificial seawater can be used for about three production cycles using the same water before elevated nitrate levels begin to degrade shrimp growth and survival **(Furtado *et al.*, 2014; Kuhn *et al.*, 2010)**. The potential benefits of applying different concentrations of artificial seawater in shrimp cultivation added with water extract of *Caulerpa lentillifera* have not been clarified yet. Therefore, the objective of this study was aimed to determine the best concentrations of artificial seawater and water extract of *Caulerpa lentillifera* to support the growth and hemocytes of white shrimp (*Litopenaues vannamei*).

MATERIALS AND METHODS

1. Shrimp preparation

This experiment was conducted in an indoor laboratory located at University of Brawijaya, Malang. A group of white shrimps (*Litopenaeus vannamei*) with size 6-7 cm

in length were kept and maintained in a couple of containers and provided with a daily ration of commercial feed which was administered twice per day. The samples were subjected to a one-week acclimatization prior to the commencement of the experiment.

2. Growth Shrimp analysis

Growth rate of a shrimp is basically influenced by feed factors, environtment, and water quality, which play an important role in aquaculture operational cycle **(Addo *et al.*, 2021)**. The shrimp productivity will increase if throughout the cultivation the water quality history is good. Water quality is driven by biochemical processes in the aquatic ecosystems **(Madusari *et al.*, 2022)**.

a) Average Daily Growth

White shrimp (*Litopenaeus vannamei*) is one of the crustaceans that has faster growth rate compared to all types of crustaceans **(Anand *et al.*, 2019).** The measurements of white shrimp average body weight and average daily gain were weighed using an analytical digital scale and the average daily growth rate value was obtained by subtracting the current shrimp sampling weight from the previous shrimp sampling weight and then by dividing it with the time period (days) of rearing.

b) Specific Growth Rate (SGR)

Unlike mammals, fish and crustaceans exhibit indeterminate growth wherein growth continues afer sexual maturity is reached **(Powell *et al.*, 2019)**. Growth curves of species that exhibit indeterminate growth are more hyperbolic. Given the hyperbolic nature of growth exhibited by fish and crustaceans, exponential type growth functions are commonly applied with the specific growth rate (SGR) model seeing widespread application in both fish and shrimp culture. Specific growth rate (SGR) was calculated at the end of the experiment period using the formula by **(Muralisankar *et al.*, 2021).**

$$\text{SGR} = \frac{\text{Ln (Final wet body weight)} - \text{Ln (Initial wet body weight)}}{\text{Time (days)}} \text{ x } 100$$

c) Food Conversion Ratio (FCR)

Food conversion ratio (FCR) was defined as the rate of feed intake to body weight gain. The value of feed conversion ratio (FCR) was also calculated at the end of the experiment periode using formula by **(Jaffer *et al.*, 2020).**

$$\text{FCR} = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

3. Hemocyte analysis

In relation to immune system, hemocytes play a central role in the defense mechanism of crustaceans and total hemocytes and the proportions of different cell types can respond to environmental fluctuation stresses **(Prates *et al.*, 2023)**. Therefore, hemocyte count has been considerate an adequate indicator of shrimp immune condition. Shrimp immune system and its activities is actively related to proteins and in case hemocytes, there is a production of antimicrobial peptides that are active against a large range of pathogens, essentially gram-positive bacteria **(Destoumieux *et al.*, 2001)**.

a) Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC)

The hemocytes were counted using hemocytometer (Boeco, Hamburg, Germany) and light microscope at 400× magnification **(Liu *et al.*, 2004)**. One hundred microliters of anticoagulant-hemolymph mixture were incubated with 100 µl fixative (10% formalin in 0.45 M NaCl) for 20 min at room temperature. Fixed hemocyte suspension (20 µl) were mixed with the same volume of rose bengal solution (1.2% rose bengal in 50% ethanol) and incubated for another 20 min at 25 °C. Smears were prepared and completely air-dried before counterstaining with hematoxylin solution for 7–10 min. The cells were then counted in random areas, and the numbers and relative proportions of hemocyte types were calculated by counting at least 200 cells on each slide. The differential hemocyte count (DHC) was determined using the following equation **(Sritunyalucksana *et al.*, 2005)**.

$$\text{DHC} = \frac{\text{Number of hemocyte cells types}}{200} \text{ x THC}$$

Shrimp hemocytes are mainly classified into three types; hyaline (a granular), semi-granular (small granular), and granular (large granular) hemocytes, based on the existence or relative size of granules **(Martin *et al.*, 2013)**.

4. Reactive Oxygen Species (ROS) Analysis

The ROS analysis was conducted in accordance with the procedure outlined in the MedikBio ROS ELISA Kit (E0347Fi). In a concise manner, the 40 microliter sample and 10 microliter shrimp ROS antibody were introduced into the designated sample wells. Next, a volume of 50 microliters of streptavidin-HRP was added to the sample wells and thoroughly mixed. The well sample was subjected to incubation at a temperature of 37°C for a duration of 60 minutes. Subsequently, it was immersed in a wash buffer of 300 µl for a period ranging from 30 seconds to 1 minute for each washing step. In addition, 50 microliters of substrate solution A and substrate solution B were introduced into each respective sample well. Subsequently, the specimen well was subjected to an incubation period of 10 minutes at a temperature of 37°C, while being kept in a light-restricted environment. The Stop Solution was added to each well with a volume of 50 µL. The determination of reactive oxygen species (ROS) was conducted by measuring the optical density (OD value) of each well using a microplate reader set to a wavelength of 450 nm. This measurement was performed within 10 minutes after the addition of the stop solution.

5. Data analysis

Data are expressed as mean ± SD. Statistical significance of pairwise differences among three or more groups were determined using one-way analysis of variance (ANOVA) followed by LSD test. $P<0.05$ was considered statistically significant. Analysis was performed using SPSS for Windows (SPSS Inc., Version 20.0, Chicago, USA). Graph was performed using GraphPad Prism 7 (GraphPad Software, Inc. USA).

RESULTS

1. Growth analysis

To observed the effect of different concentrations of artificial seawater and sea grapes extract, growth analysis of white shrimp was performed. In this study, further analysis of average daily growth, specific growth rate (SGR), food conversion ratio (FCR), and growth rate were assessed. These results are shown in fig. 1. Artificial seawater and extract sea grapes given at certain concentration shows an increased in average daily growth, SGR, FCR, and growth rate of white shrimp (*Litopenaues vannamei*).

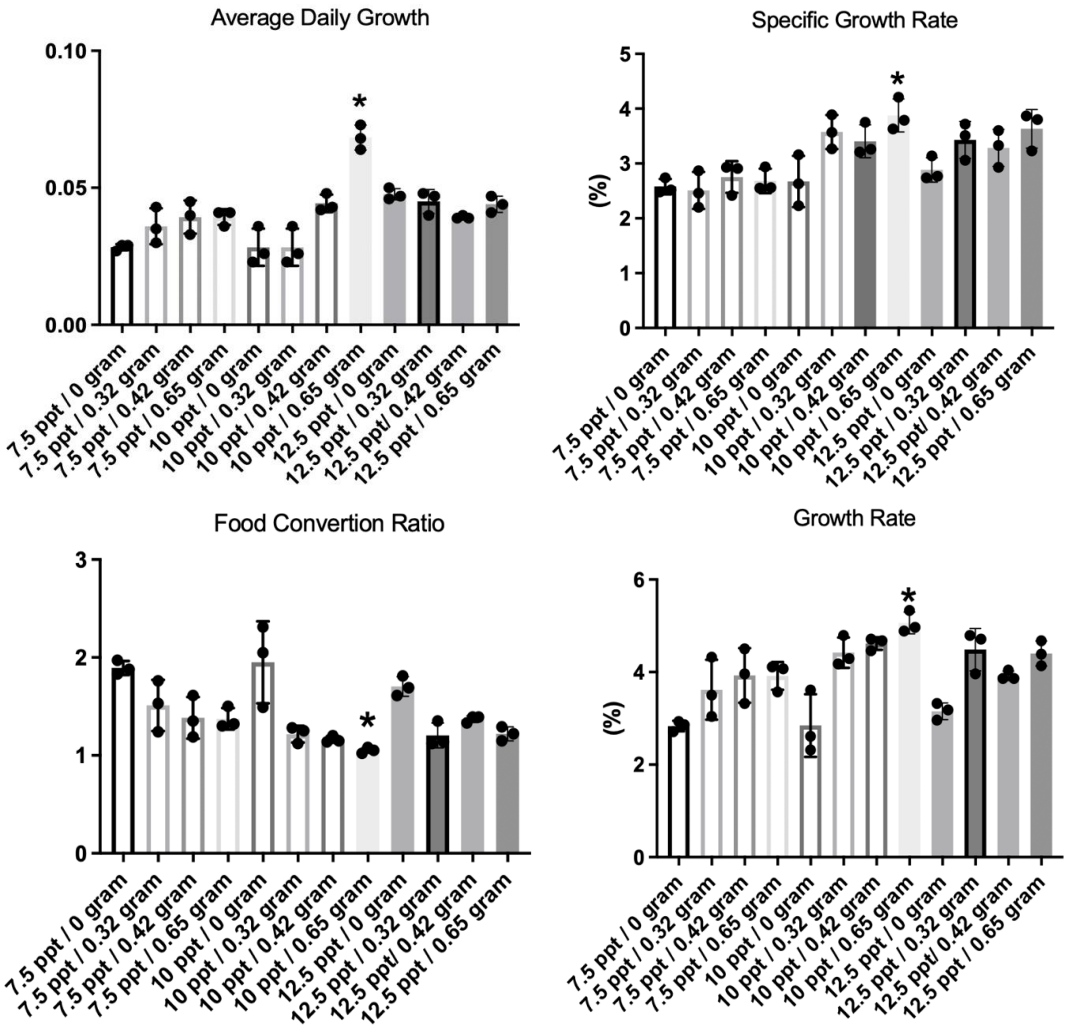

Fig. 1. Growth analysis of shrimp induced by different concentration of artificial seawater and water extract of *Caulerpa lentillifera.* Results were the mean ± SD *P<0,05 was significant

At the concentration of 10 ppt with 0.65 gram sea grapes extract shows a significant increased with a value around 0.07% for average daily growth followed by the concentration of 12.5 ppt with no sea grapes extract added with value around 0.05% per

day. In contrast, the treatment with no extract of sea grapes shows the lowest number of average daily growth in white shrimp. The value of specific growth rate (SGR) was significantly increased at the same concentration, which is 10 ppt and 0.65 gram with around 3.8% value followed by concentrations of 12 ppt with 0.65 gram sea grapes extract with value around 3.4%. At the concentration of 7.5 ppt with no sea grapes extract, the value of SGR was at the lowest number among all the treatments. Feed conversion ratio (FCR) shows the smallest number around 1 at the concentration of 10 ppt with 0.65 gram sea grapes extract. The smaller FCR value indicate the better food intake by the shrimps. The growth rate of white shrimp (*Litopenaues vannamei*) shows a significant increased with around 5% at concentration of 10 ppt with 0.65 gram sea grapes extract. This indicates that at the certain concentration, the growth performance of white shrimp induced by different concentration of artificial seawater and sea grapes extract are affected.

2. Hemocyte analysis

The hemocyte analysis was divided into 2 types, total hemocyte count (THC) and differential hemocyte count (DHC), including hyaline, granul, and semi-granul. The results of these differential hemocyte is shown in fig. 2.

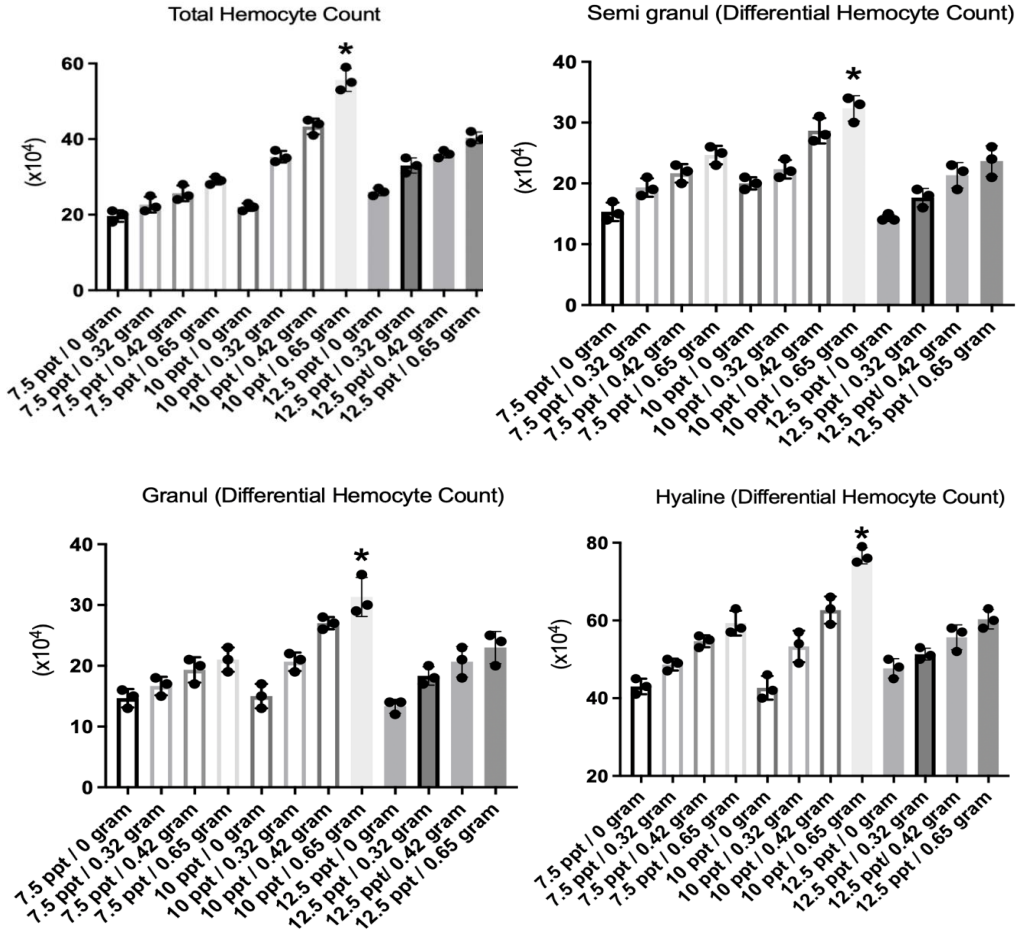

Fig. 2. Hemocyte analysis of shrimp induced by different concentration of artificial seawater and water extract of *Caulerpa lentillifera*. Results were the mean ± SD *P<0,05 was significant.

Total hemocyte count (THC) shows a significant increased at the concentration of 10 ppt with 0.65 gram sea grapes extract with value around 50×10^4 followed by the treatment with 10 ppt and 0.42 gram sea grapes extract with value around 40×10^4. The lowest THC shown in the treatment with 7.5 ppt and 0 gram sea grapes extract around 20×10^4. Differential hemocyte count for semi granul shows a significant increased at the concentration of 10 ppt with 0.65 gram sea grapes extract around 35×10^4 followed by the lowest number at the concentration of 12.5 ppt with no sea grapes exctract added with value around 10×10^4. Granul and hyaline count at the concentration of 10 ppt and 0.65 gram sea grapes extract shows a significant increased with value around 30×10^4 and 70×10^4, respectively.

3. ROS analysis

Induction of different concentration of artificial seawater and sea grapes extract and its effect on ROS was shown in fig 3.

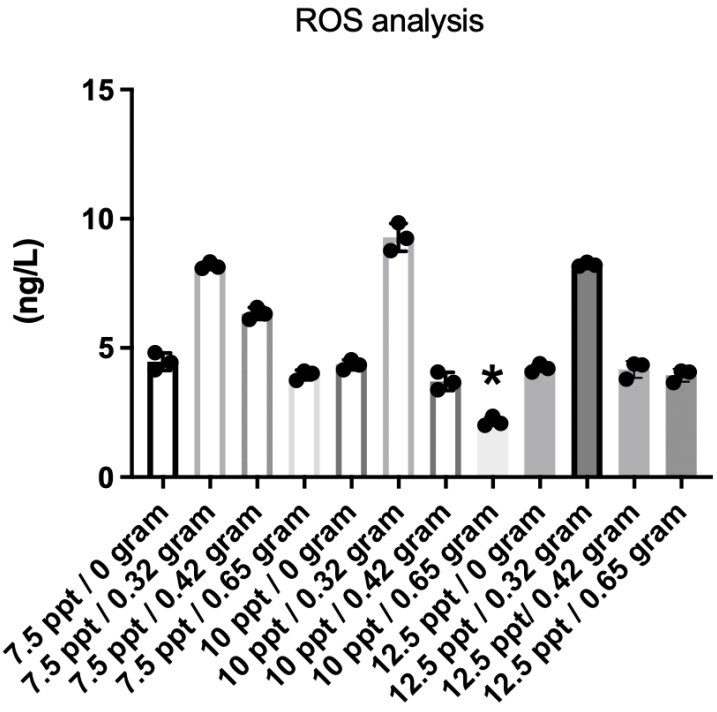

Fig. 3. ROS analysis of shrimp induced by different concentration of artificial seawater and water extract of *Caulerpa lentillifera*. Results were the mean ± SD *P<0,05 was significant.

The result showed that at the concentration of 10 ppt with 0.65 gram sea grapes extract significantly affecting the ROS number with value below 5 ng/L. The highest number of reactive oxygen species (ROS) was shown in concentration of 10 ppt with 0.32 gram sea grapes extract with value around 10 ng/L. This indicates that at certain number of sea grapes extract the reactive oxygen species (ROS) value will be affected either decreasing or increasing. The combination between water salinity and herbal plant extract needs to be balance in order to avoid the increasing number of ROS in shrimp.

DISCUSSION

Growth rate analysis of a shrimp is basically influenced by feed factors, environtment, and water quality, which play an important role in aquaculture operational cycle **(Addo *et al.*, 2021)**. The induction of different concentration of artificial seawater and sea grapes extract is an environment condition that affects the growth rate analysis of white shrimp. Like in fish, the use of the natural logarithm of body weight in the SGR model results in significant underestimation of predicted body weight when interpolating between initial and final body weight in shrimp. Given that growth models are used as tools to predict feeding requirements, significant underestimation of predicted weights can result in underfeeding and subsequently an inability for the growth potential of the shrimp to be realized. In contrast, extrapolating past the final body weight used to calculate SGR generates greatly overestimated predicted body weights resulting in feed wastage, with feeding costs representing 50%–60% of total production costs in intensive shrimp culture **(Powell *et al.*, 2019)**.

The different concentration of salinity in water and herbal plant extract had a considerable effect on the overall activiy, food consumption and growth, and also hemocyte profile in white shrimp **(Palafox *et al.*, 1997)**. The effect of each herbal plant is different depends on the species used and bioactive compounds contained within. The THC and DHC analysis were showing an interesting result, which at certain concentration of salinity and sea water extract could affect the total hemocyte count (THC) and different hemocyte count (hyaline, granul, and semi-granul). The composition of these combination has to be balance in order to avoid the increasing number of ROS in shrimp. The increasing of ROS will not only cause lipid oxidation but also protein oxidation in shrimp **(Hematyar *et al.*, 2019)**. Free radicals are highly ROS that can cause damage to biological materials.

CONCLUSION

In conclusion, the induction of different concentration of artificial seawater and sea grapes extract are effecting the growth and hemocyte in white shrimp (*Litopenaues vannamei*) certainly at concentration of 10 ppt and 0.65 gram sea grapes (*Caulerpa lentillifera*) with value of average daily growth, SGR, and growth rate were 0.07%, 3.8%, and 5%, respectively. The best feed conversion ratio (FCR) was showed at the same concentration with value around 1. Total hemocyte count (THC) and differential hemocyte count (DHC) were also at its best value with the same concentration of 10 ppt and 0.65 gram sea grapes extract.

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