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Effect of Ethanol and Methanol Extract of *Caulerpa lentilifera* on Hematological Parameters and Phagocytosis of *Cyprinus carpio*

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ABSTRACT

The effect of the immersion or soaking of different extracts of *Caulerpa lentillifera* in profile hematology and phagocytosis as immune responses is still unknown. In this research, the effect of three extractions of *Caulerpa lentillifera* (extracts of ethanol, methanol and water) was determined on the profile hematology and phagocytosis activity. Briefly, 8- 10cm carp (*Cyprinus carpio*) were exposed to each extract with different concentrations (0; 0.24, 0.32, 0.42, 0.65 and 0.87ppm) by immersion or soaking for 96 hours. The result showed that the ethanol extract of *Caulerpa lentillifera* increased immune response. The ethanol extract showed significant increases in erythrocyte, leukocyte and phagocytosis activity at a concentration of 0,42 ppm around $1.43.10^6$ cells/ mm^3, 30.10^3 cells/ mm^3 and 35%, respectively. However, methanol and water extracts showed increasing immune responses with no significance compared to ethanol extract. Furthermore, ethanol extract showed different responses in fish morphology. It was induced by enhancing the appearance of the fish's color, as well as swimming more actively than a sample treated with methanol and water extracts.

INTRODUCTION

Green algae seaweed contains bioactive compounds with a potential of serving as functional ingredients for human health **(Wynants *et al.*, 2020)**. Currently, the application of seaweed has led to rapid research to find and develop new therapeutic agents. *Caulerpa lentillifera* is one of the green algae that is widely grown and cultivated in the Indonesian waters **(Mao *et al.*, 2020)**. It has been reported that *Caulerpa lentillifera* exhibits several pharmacological properties **(Ji *et al.*, 2020)**. Several bioactive compounds have also been isolated from this seaweed (green algae), showing potential to

treat several diseases and are recognized as strong immunological stimulators in humans. **Putra *et al*. (2021)** postulated that, the addition of up to 20g/ kg of sea grape powder (*C. lentillifera*) to a milkfish feed mixture can provide optimal growth. Other research on black tiger shrimp (*Penaeus monodon*) gave the same result **(Putra *et al*., 2019)**.

Common carp is one of the freshwater fish that has significant economic value so that the community widely cultivates carp. Besides being kept in ponds, common carp are also often kept in the fields together with rice plants **(Rudiyanti & Ekasari, 2009)**. One of the problems facing common carp farming is the disease. Disease attacks can occur due to poor environmental conditions or due to bacterial, fungal or parasitic infections. The examination of hematology parameters can be used to record the health level of fish **(Maftuch *et al.*, 2012)**. Hematology examination includes an examination of hematocrit values, hemoglobin levels, red blood cell counts, white blood cell counts and deferential observation of blood **(Ramesh & Saravanan, 2008)**.

Improving the immune system in fish is important to prevent the occurrence of disease infections in fish. Basically, the potential for bacterial, viral or fungal diseases exists in the aquatic environment, but if the immune condition of the fish's body is strong, disease attacks can be prevented. The immune system is involved in the etiology and pathology of many diseases. The modulation of immune responses improves controlling diseases. The immune system (immune) deals with leukocytes. Leukocytes are part of the immune system that has an important role in every disease-causing agent. The main function of leukocytes is to destroy infectious and toxic materials through the phagocytosis process forming antibodies **(Lubis *et al.*, 2016)**. In the classical literature, erythrocytes have long been typecast as mere oxygen transporters. However, mounting evidence suggests that these cells also play an important role in the innate immune system **(Darbonne *et al.*, 1991; Neote *et al.*, 1993)**, thereby regulating and modulating immune responses. Internal components of erythrocytes such as hemoglobin and heme are also formidable facets of innate immunity, capable of generating antimicrobial reactive oxygen species (ROS) to defend against invading hemolytic microbes, as well as promoting pathologic inflammatory and auto-immune responses **(Anderson & Siwicki, 1994)**. One important immunomodulatory property of erythrocytes is their propensity to bind a wide variety of chemokines.

Caulerpa Lentilifera is common in Indonesian coastal waters. This type is widely known as sea grapes. The taste of this sea grape is similar to salmon roe but fresher and more fragrant. *Caulerpa lentillifera* is a source of nutrition since it has good nutritional value, and thus it is good for human consumption **(Antara *et al.*, 2022)**. Other benefits of sea grapes play a role in supporting health because they can improve the immune system so that they are not susceptible to disease. There have been many studies on various immune responses in organisms from *Caulerpa* sp. **Yuniarti *et al.* (2015)** found an increase in total leukocytes, monocytes, lymphocytes, neutrophils, macrophages and phagocytosis activity after being induced by *Caulerpa racemosa* in gourami. Furthermore, *Caulerpa lentillifera* was able to increase growth in shrimp **(Putra *et al.*, 2019)** and milkfish **(Putra *et al.*, 2021)** through the addition of extracts to feed. The effect of *Caulerpa lentillifera* on the immune system has been widely carried out in animal studies on rats. Meanwhile, the information about the effect of the immune response from immersing *Caulerpa lentillifera* on fish was very small. In this study, we will examine and explore the immune response of three extracts (ethanol extract,

methanol extract and water extract) of *Caulerpa lentillifera in vivo* by the expression of hematology parameter and phagocytosis activity. Treatment is only through immersion in media with different concentration and maintainance for 96 hours with aeration and feeding.

MATERIALS AND METHODS

1. Fish preparation

Ten carp fish (*Cyprinus carpio*) with size ranging from 8- 10cm (At this size, it is easy to take fish blood samples without causing the fish to die) were maintained in well-aerated aquarium conditions in freshwater at 29- 31°C. Carp fish were maintained for one week for acclimatization before experiment. The fish were fed twice per day with commercial feed.

2. *Caulerpa lentillifera* extraction

All research activities were carried out in the Biosciences Institute laboratory, Brawijaya University, Malang. Seaweed (*C. lentillifera*) was obtained from Gresik Regency, East Java, Indonesia (seaweed cultivation). Next, it was dried in a hot air oven at 50°C, ground into a powder and filtered with a filter size > 80 mesh. Furthermore, extract of *Caulerpa lentillifera* was obtained by soaking the seaweed powder for 4 days with ethanol, methanol and water was then filtered, and the solvent was removed using a rotary evaporator.

3. *Caulerpa lentillifera* treatment

Ten carp fish were placed in 2 liters of water in the experimental fiberglass tank clean and pathogen-free facilities in the laboratory of freshwater aquaculture, Faculty of Fisheries and Marine Science, the University of Brawijaya. Further, the fishes were induced with extracts of ethanol and methanol, following concentration control, 0.24, 0.32, 0.42, 0.65 and 0.87ppm for 96h. Test medium was not renewed during the experiment, and no food was provided to the animals. During the test, the fish were fasted so that the effect of the *Colerpha lentilifera* extract treatment was not disturbed by other ingested food ingredients. After 96 days, blood samples were collected for profile hematology analysis and phagocytosis activity analysis. Each experiment was repeated three times respectively.

4. Hematology analysis

Blood was drawn using a syringe needle containing 3.8% Na citrate as an anti-coagulant. Blood was drawn from the veins in the lateral line above the ventral fins in the 45˚ position and slowly pulled until the blood entered the syringe. Then, the blood was transferred into a tube for examination. The measurement of the erythrocyte and leukocyte were performed as described by **Nemzek *et al*. (2001)**. Briefly, 0.5ml sample was collected by taking the blood sample from the caudal vein using a capillary thoma pipette. Furthermore, the hayem solution (MERCK, USA) was added to the sample for erythrocyte analysis. To the other sample, turk solution (MERCK, USA) was added for leukocyte analysis. Immediately, each sample was mixed well by shaking gently. The erythrocyte and leukocyte were measured under microscope using the counting chamber and calculated with total cell erythrocytes, multiplied by 10^4 cells in per mm^3 and total cell leukocyte multiplied by 50 cell in per mm^3.

5. Phagocytosis

Phagocytic activity was performed following the method of **Wulansari (2009)**, first 50µl of blood sample was put into a sterile Eppendorf tube, and 50µl of baker's yeast cell suspension was added. Then, the mixture was homogenized and incubated at room temperature for 20 minutes. Furthermore, 5µl of the mixture of blood and baker's yeast was observed using a glass slide with a size of 1- 1.2mm, with Giemsa staining. The staining process for the smear preparation with Giemsa was carried out to the following procedure: phagocytosis activity was expressed by the number of phagocytic cells/100 and the observed phagocytic cells were multiplied by 100%.

6. Data analysis

Data were 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. Morphology fish during treatment

Each extract of *Caulerpa lentillifera* showed a different response of carp at each concentration (Table 1). This study recorded the behavior of swimming, operculum pattern and the color of carp fish.

Table 1. Morphology and behavior response to each extract *Caulerpa lentillifera*

Concentration (ppm)	Response	Ethanol extract	Methanol extract	Water extract
control	Color	Normal	normal	normal
	Swim	normal	normal	normal
	Operculum	Stable	stable	stable
0.24	Color	Normal	normal	Normal
	Swim	normal	normal	normal
	Operculum	Stable	stable	stable
0.32	Color	Normal	normal	normal
	Swim	normal	normal	normal
	Operculum	Stable	stable	stable
0.42	Color	Lighter	normal	normal
	Swim	Active	slower	To bottom
	Operculum	Stable	faster	slower
0.65	Color	Lighter	normal	normal
	Swim	Active	slower	To bottom
	Operculum	Stable	faster	slower
0.87	Color	Normal	darker	normal
	Swim	Slower	slower	To bottom
	Operculum	Faster	faster	slower

It was noticed that, *Caulerpa lentillifera* caused no mortality rate in fish. The carp induced by *Caulerpa lentillifera* showed no effect at concentrations of the control, 0.24 and 0.32 ppm. Furthermore, the effect this seaweed showed at concentrations of 0.42; 0.65; 0.87ppm were determined in detail in the following data. At concentrations from 0.42- 0.65ppm, it was found that the color of fish became lighter and they swam actively in ethanol extract of *Caulerpa lentillifera*. At the concentration of 0.87ppm of ethanol extract, the swimming behavior slowed down, with a faster operculum opening pattern. Furthermore, in the methanol extract of *Caulerpa lentillifera* at a concentration of 0.42ppm, fish swam slowly with an unstable movement pattern of the operculum as well as at the concentration of 0.65ppm. While, at the concentration of 0.87ppm, the fish in the methanol extract had a darker color. At the concentration of 0.42- 0.87ppm of the aqueous extract of *Caulerpa lentillifera*, the fish swam at the bottom of the test container, with a slower movement for the operculum.

2. Profile hematology

In this study, further analysis of erythrocyte total and leukocyte total were addressed to observe the effect of each extract (ethanol, methanol, water) of *Caulerpa lentillifera*. These results are shown in Fig. (1). The ethanol extract of *Caulerpa lentillifera* induced an elevation in the total erythtocyte for carp significantly at concentration 0,42 ppm around $1.43.10^6$ cells/mm^3. In contrast, at the concentration of 0.87ppm of the ethanol extract of *Caulerpa lentillifera*, a decrease was detected in the total erythrocytes as well as in terms of using methanol and water extractions of *Caulerpa lentillifera.* Moreover, the erythrocyte was obtained in methanol and water extractions of *Caulerpa lentillifera* with no significant increase.

In the current study, the effect of *Caulerpa lentillifera* extract was observed (Fig. 2). *Caulerpa lentillifera* enhanced the immune response by increasing the total leukocyte in carp. At the concentration of 0.42ppm of ethanol extract of *Caulerpa lentillifera*, an induced increase was recorded in the total leukocyte significantly around 30.10^3 cells/mm^3. In this case, increasing total leukocyte was found in methanol and water extractions of *Caulerpa lentillifera* at the concentration of 0.42ppm, without significance; around $20.\ 10^3$- $21.\ 10^3$ cells/mm^3. Furthermore, suppressed immune response was observed in all type of extract *Caulerpa lentillifera* at concentration 0,87ppm; around 18.10^3-19.10^3 cells/mm^3

3. Phagocytosis activity

To find out whether *Caulerpa lentillifera* is also able to increase the immune system, in this study a phagocytosis test was carried out. Interestingly, all extracts of *Caulerpa lentillifera* were capable of increasing the phagocytic activity at the concentration of 0,42ppm. In this case, ethanol extract of *Caulerpa lentillifera* significantly increased the phagocytosis activity for about 35%. In contrast, the methanol extract and water extract did not significantly increased the phagocytosis activity, recording values of 8-9%. Interestingly, the suppression in immune system by reducing phagocytosis activity was observed for the concentrations of 0.87ppm from all extracts of *Caulerpa lentillifera* (around 5- 9%).

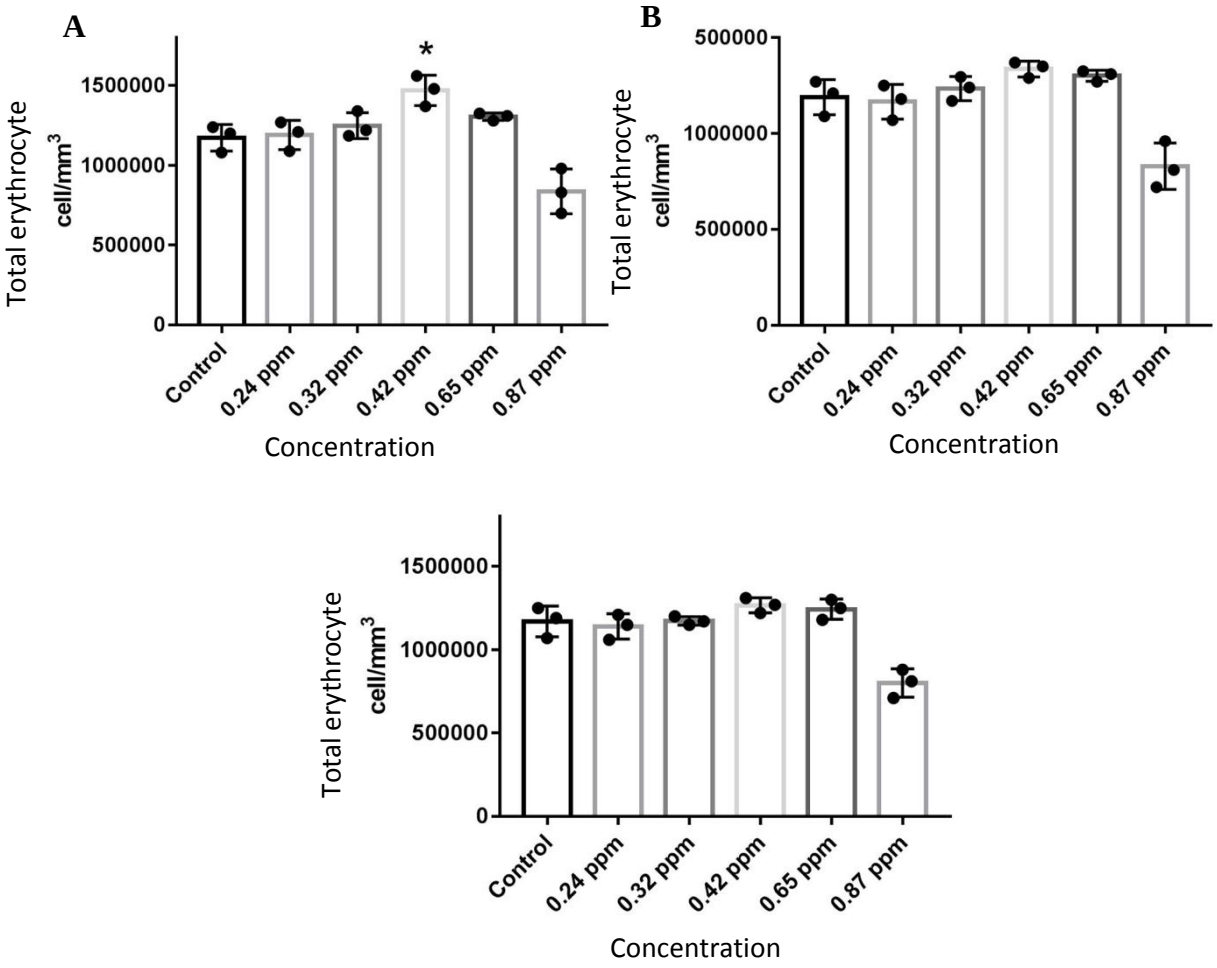

Fig. 1. Analysis of the effect of *Caulerpa lentillifera* on the total erythrocyte of carp. **A**. Ethanol extract of *Caulerpa lentillifera*, **B**. Methanol extract of *Caulerpa lentillifera*, **C.** Water extract of *Caulerpa lentillifera*. Results were shown as the mean ± SD; **P*<0.05 was significant.

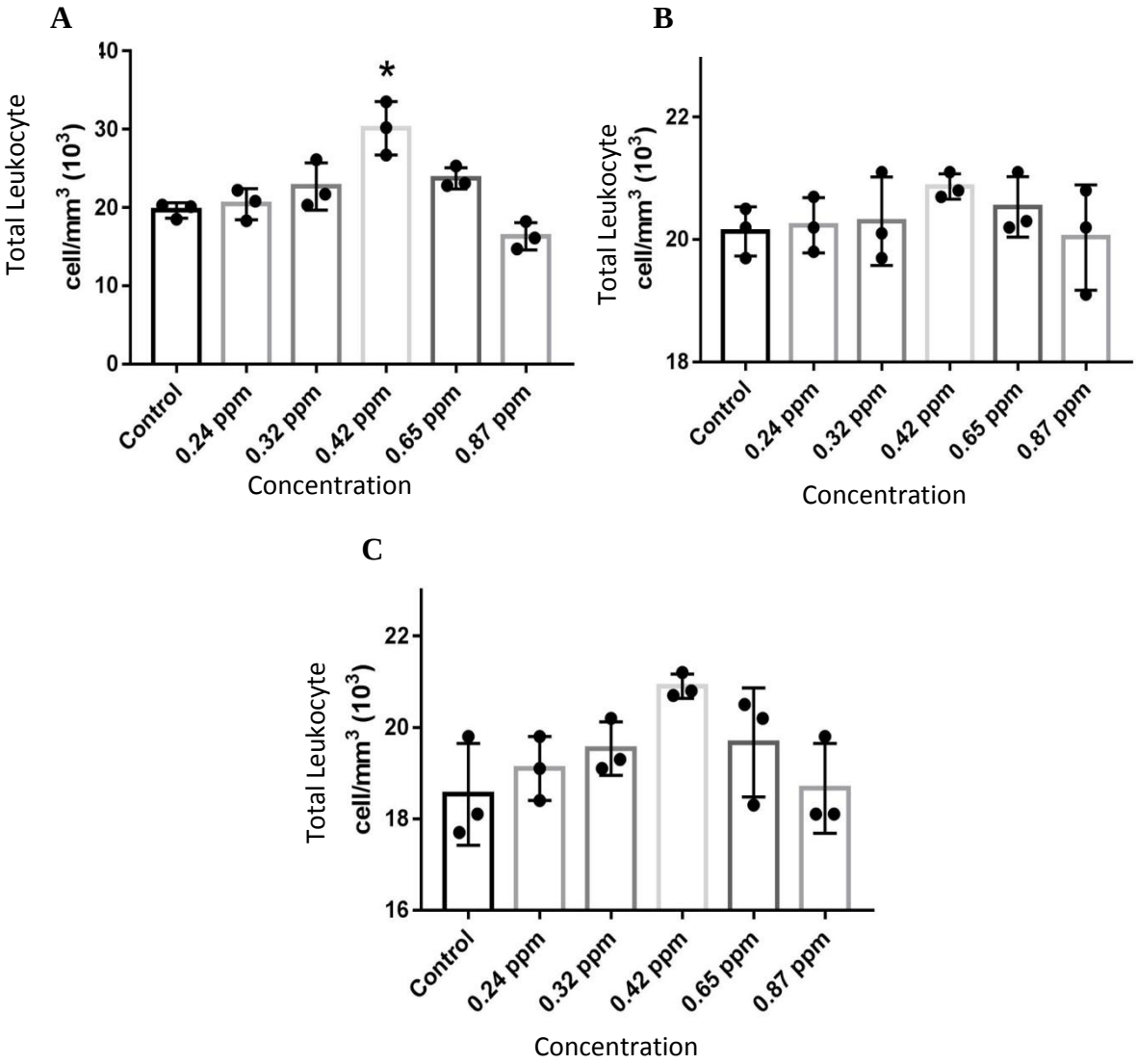

Fig. 2. Analysis of the effect of *Caulerpa lentillifera* on the total leukocyte of carp. **A**. Ethanol extract of *Caulerpa lentillifera*, **B**. Methanol extract of *Caulerpa lentillifera*, **C**. Water extract of *Caulerpa lentillifera*. Results were the mean ± SD *p<0.05 was significant

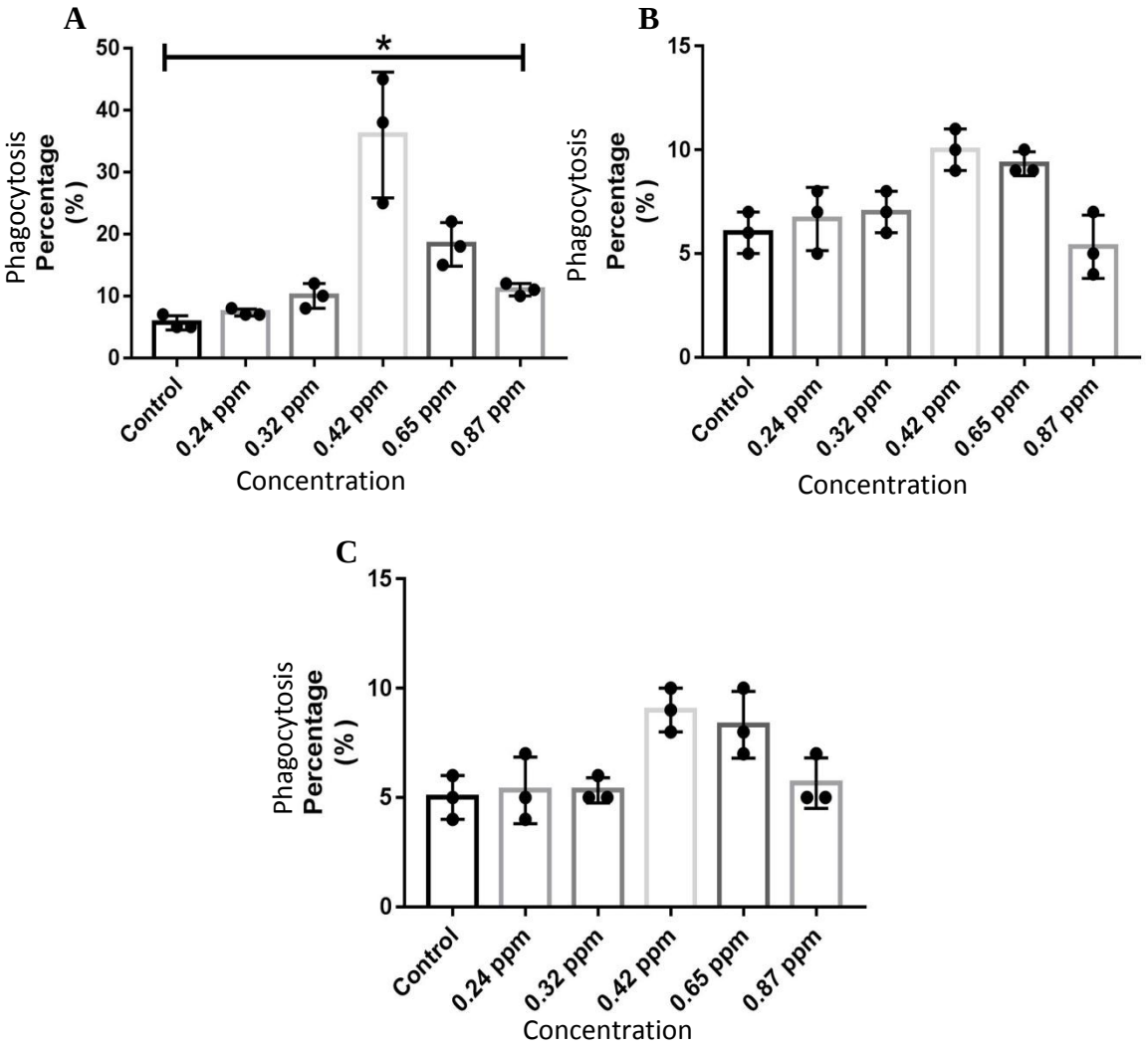

Fig. 3. Analysis of the effect of *Caulerpa lentillifera* on the phagocytosis of carp. **A**. Ethanol extract of *Caulerpa lentillifera*, **B**. Methanol extract of *Caulerpa lentillifera*, **C**. Water extract of *Caulerpa lentillifera*. Results were assessed with the mean ± SD ; **P*<0.05 was significant.

DISCUSSION

In this study, the ethanol extract recorded the highest result in increasing the hematology profile and phagocytosis activity in a significant immune response, compared to methanol extract and water extract. This finding is similar to that of **Yuniarti *et al*. (2015)** upon applying immunostimulants by soaking *Caulerpa racemosa* extract and obviously detected an improvement in a non-specific immune response by 5% through parameters, including total leukocytes, lymphocytes, monocytes, neutrophils,

macrophages and phagocytosis activity of the giant gourami fish. According to **Yengkhom *et al.* (2018)**, the results clearly showed that the intraperitoneal administration of the extract of *Caulerpa scalpelliformis* had a stimulating effect on the nonspecific immune responses, immune gene expression and disease resistance. It enhanced all the tested non-specific serum immune responses, including lysozyme, myeloperoxidase, antiprotease and bactericidal activities. There was an upregulation of the genes encoding IL-1β, lysozyme and TNF-α in the spleen of polysaccaharides injected fish, compared to the control group. Polysachcaarides have played an important role in enhancing the immune response by macroalgae. Plant polysaccharides from seaweed has been studied for their immunomodulatory activity in terms of the activation of the macrophage immune response, viz. the enhanced respiratory burst activity and generation of proinflammatory cytokines and in the expression of immune genes **(Schepetkin *et al.*, 2005; Schepetkin & Quinn, 2006)**.

In this study, it was found that, all extracts of *Caulerpa lentillifera* enhanced the immune response by increasing the total erythrocyte, leukocyte, and phagocytosis in carp. However, ethanol extract increased significantly at the concentration of 0.42ppm, compared to methanol and water extracts. Interestingly, at the concentration of 0,87ppm of all extracts of *Caulerpa lentillifera*, the suppressing immune response was detected by reducing total erythrocyte, leukocyte and phagocytosis in carp. Innate immunological parameters are important indicator of fish health and its physiological status. It is one of the most important tool for fish disease diagnosis, and its change depends on the health condition of fish **(Hrubec *et al.*, 2000)**. Increase or decrease in neutrophil, monocyte and lymphocytes number may occur during microbial infections, bioactive compound, etc… **(Haney *et al.*, 1992)**. **Maftuch *et al.* (2012)** reported a decrease in erythrocytes and attributed it to the condition of the gills getting worse, thus causing the disruption of the gills' function, which has an impact on the difficulty of Hb binding oxygen. The reduced number of erythrocytes is also thought to be caused by anemia in fish. Phagocytic activity is a cellular mediated nonspecific defense mechanism in fish and may also vary according to its health status. Phagocytic activity of fish blood can be used as a significant nonspecific immunological indicator of immune-suppression in fish **(Anderson, 1990)**. According to **Brown (2000)**, the improvement of immunity was noticed from the increase in the phagocyte cells activity of hemocytes. Phagocyte cells function to perform phagocytosis to foreign particles invading the host body. Polysaccharides from marine alga involve in enhancing immune response. Polysaccharides induce an inflammatory response as fish perceive the polysaccharide as a foreign agent due to their conformity with the polysaccharide of fungal or gram-negative bacteria, and thereby imparting protection **(Anderson & Siwicki, 1994)**. It will promote increasing erythrocyte, leukocyte and phagocytosis.

CONCLUSION

In conclusion, ethanol extract, methanol extract, and water extract of *Caulerpa lentillifera* enhance immune response by increasing erythrocyte, leukocyte and phagocytic activity. However, the ethanol extract at 0.42ppm significant increase to induce an immune response in carp by immersion or soaking treatment. Furthermore, this

study showed that concentrations higher than 0.87ppm were not recommended for immunomodulation due to suppressed profile hematology and phagocytosis activity.

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