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Potential Analysis of *Lemna* sp. Extract as Immunostimulant to Increase Non-Specific Immune Response of Tilapia (*Oreochromis niloticus*) against *Aeromonas hydrophila*

Eric Armando [1*)], Ayu Lestiyani [2)] and R. Adharyan Islamy [3)]

[1]Study Program of Akuakultur, Faculty of Agriculture, Tidar University
[2]Study Program of Agroecotechnology, Faculty of Agriculture, Tidar University
[3]Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya

Email Address : ericarmando@untidar.ac.id

KEYWORDS

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Lemna sp.;
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Abstract *Lemna* sp. is known to have several bioactive compounds and polysaccharide macromolecules that can function as immunomodulators to affect non-specific immune responses to increase the body's resistance to pathogens. This study aims to determine the potential of catfish eye extract as an immunostimulant by observing non-specific tilapia immune parameters. The extraction method used was 96% ethanol maceration for 2 days with a ratio of 1: 4. The experimental design used a Completely Randomized Design with 5 treatments (doses 0.2, 0.4, 0.6 mg/kg, control + and control -) and 3 replications. The non-specific parameters of immunity observed included total plasma protein (Bradford method), superoxide dismutase and lysozyme activity. The data obtained will be analyzed using ANOVA, if there is a significant difference, it will be further tested with Duncan Multiple. Range Test (DMRT). The results showed that the highest total plasma protein was found in treatment C (giving an extract of 0.3 mg/kg body weight) with an average total plasma protein after 12 days of maintenance of 4.99 g / dL. The extract dose of 0.3 mg/body weight showed a rapid decrease in SOD and increase Lysozyme activity.

Introduction

Tilapia (*Oreochromis niloticus*) is fish from the Cichlidae family scattered and found in public waters (Insani *et al*., 2020). They are one of the leading aquaculture commodities. The national production of aquaculture experienced an average growth of 3.36% in the 2015-2018 period with an increase in tilapia production by 14% (Ministry of Maritime Affairs and Fisheries of the Republic of Indonesia, 2018), and on a global scale, tilapia production reached 4.1 million tons in 2016 (FAO, 2019). The high level of intensive cultivation activity with high stocking density raises the potential for diseases that can cause economic losses.

One of the problems in aquaculture is a decrease in the immune system of cultivated fish. This can result in fish susceptible to disease (Isnansetyo *et al.,* 2015). Efforts to increase fish immunity can be made by providing immunostimulants for aquaculture as an alternative disease prevention system to suppress the use of antibiotics and are known to be effective in increasing the immune response in fish and shrimp (Deivasigamani and Subramanian, 2016). The use of plant extracts is known to increase immune response activity (Hai, 2015; Vallejos-Vidal *et al*., 2016; Awad & Awaad, 2017; Kilawati and Islamy, 2019; Kilawati and Islamy, 2021).

Lemna sp. is the smallest flowering monocot plant that lives floating in the water and is known to have high protein content, bioactive compounds (Aguilera-morales *et al*., 2018) and the potential for polysaccharide content reaching 20-50% consisting of several

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Monosaccharides, i.e. Rhamnogalacturonan, Pectins, Galactose, Arabinose, Apiosa, Xylogalacturonan and Silose (Gyunter *et al.,* 2008; Zhao *et al.,* 2014). This plant is available in abundance and can live in various water conditions, but its utilization is still low both as a food source and a source of bioactive compounds. This study intends to analyze the potential of *Lemna* sp. as an alternative raw material for making immunostimulants to increase the non-specific defence system of Tilapia. This plant is categorized as a fast-growing plant, so the potential for the development of *Lemna* sp. As a source of bioactive compounds is very prospective.

Materials and methods

Sample preparation and extraction

The research was conducted in January to May 2021 at the Laboratory of the Faculty of Agriculture, Tidar University. The extraction method carried out according to Islamy *et al.* (2017). Lemna plant samples were washed under running water and rinsed with distilled water to remove any remaining residue, dried and mashed. Extract of *Lemna* sp. using the ethanol 96% (EtOH) maceration method with a 1: 4 (w / v) ratio for 2 days, stirring occasionally. After that, the filtration is carried out, and the filtration results are evaporated to the final volume of crude ethanol extract of *Lemna* sp. (CEEL) is reduced by 95% (Haggag *et al*., 2017). Then proceed with testing the CEEL moisture content by heating in an oven at 105°C for 24 hours to obtain the yield content of the extract from the callus of *Lemna* sp.

Animal test preparation

According to a published method, acclimation of test fish for 7 days (Islamy *et al*., 2017). Previously, the place for maintenance was sterilized by immersing PK (Potassium Permanganate) solution for 24 hours. The fish were acclimated for 7 days in a bucket with a diameter of 46.5 cm and a height of 23 cm. The number of fish used was 96 fish because there were 24 experimental units, each filled with 4 fish. The body length and bodyweight of the fish were also measured.

Bactrial preparation

The isolate of *A. hydrophila* was originated from Jepara Brackish Water Aquaculture Center. These bacteria were kept in Trypticase Soy Agar (TSA) media at 4°C and sub-cultured in Trypticase Soy Broth (TSB) overnight before use.

Experimental design

The research design used a completely randomized design (CRD) with 5 treatments and 3 replications. *Lemna* sp. The extract was given by intraperitoneal injection at doses of 0.1, 0.2, and 0.3 mg/kg Fish Weight (FW). The negative control was injected using sterile PBS, while the positive control used a commercially available immunostimulant. After 24h acclimatization, the fish then infected by *A. Hydrophilla*.

Observation of Immunological Parameters

Observation of non-specific immune parameters was carried out on days 0, 4, 8, and 12 after injection. The fish blood was collected from the caudal artery and placed on a microtube given 10% Na-EDTA anticoagulant. The parameters observed were humoral non-specific immune parameters, including total plasma protein, superoxide dismutase (SOD) and lysozyme activity (LA).

Total plasma protein and SOD were measured using spectroscopic methods and observed at a wavelength of 600 nm and 505 nm, respectively, according to published protocols (Siwicki *et al*., 1994; Chang *et al*., 2013). The total absorbance measurement of plasma protein was converted to protein mg/ml using standard protein curves using bovine serum albumin (BSA), while the absorbance value of SOD was converted to U / ml.

Observation of and lysozyme activity (LA) carried out using the turbid metric assay system (Parry *et al*. 1965). Lysozyme activity assay at

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the end of the feeding trial was sampled from the sacrificed fish. Mucus was taken with soaking paper and dissected; the fish took the desired tissue (kidney and liver). After taking the weight of the tissue, acetate buffer (0.5 M Acetic acid + 0.5 M Sodium acetate; pH = 5) was added five times to the tissue and homogenated. After homogenization, the tissue suspension was centrifuged at 4000 rpm for 15 min, and the supernatant was collected. One hundred forty µl *Aeromonas hydrophila* solution [1 mg/ ml lyophilized *Aeromonas hydrophila* in 0.05 M sodium phosphate buffer (pH = 6.2)] was mixed with 10 µl tissue homogenates. Then, the reduction in absorbance at 450 nm (delta A) was recorded at 0 and 30 min. after incubation at room temperature (20°C). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min, and the formula used to calculate the activity: ⊿ A X 1000/min/ml (Lie *et al*., 1989).

Data Analysis

The data that has been obtained were analyzed by using the ANOVA test. This data is used to determine how exposure to batik waste on levels of SOD (Superoxide Dismutase) in Tilapia. This ANOVA analysis used a confidence level of 95% and an error rate of 5%. The follow-up test used was the Duncan test.

Results and Discussion

Total plasma protein

The calculation of Total Plasma Protein (TPP) of Tilapia (*O. Niloticus*) after being challenged with *A. Hydrophilla* can be seen as in Figure 1.

Total plasma protein is the total amount of protein contained in blood plasma, including albumin, fibrinogen, and globulin. Plasma protein consists of 60% albumin, 35% globulin, and 4% fibrinogen (Hastuti, 2012). The concentration of protein in plasma can be used as a reference to measure the extent to which the level of immunity in a living thing, including fish. The mean total protein blood plasma of Tilapia is shown in Figure 1. The results showed that the highest total plasma protein was found in treatment C (giving an extract of 0.3 mg/kg body weight) with an average total plasma protein after 12 days of maintenance of 4.99 g / dL.

In comparison, the lowest total plasma protein occurred in treatment A (control, without giving the extract dose. Based on the results of the analysis of variance, it was found that the administration of *Lemna* sp. extract had a very significant effect on the total plasma protein of Tilapia after being challenged with *A. hydrophilla* bacteria.

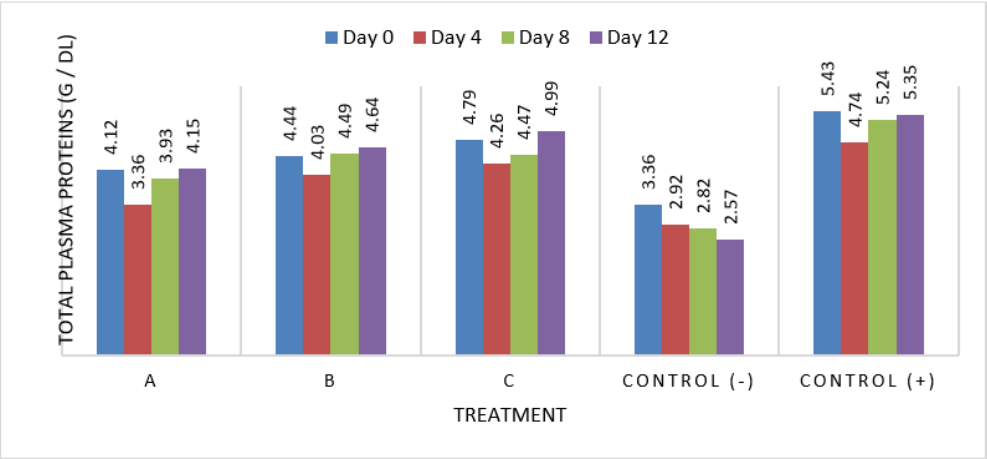

Figure 1. Total Plasma Protein (TPP) of Tilapia (O. Niloticus) after Being Challenged with A. Hydrophilla

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Showed that the compounds in the extract of *Lemna* sp. can act as immunostimulants that can repair and enhance fish's immune system. Smith *et al*. (2003) stated that immunostimulant is a material used to increase immune system activity and resistance to pathogens, but it is also increasing show the survival of organisms when exposed to harmful pathogens. Matsuyama *et al*. (1992) reported that injection of immunostimulants could increase fish resistance to bacterial infections. In this study, the best extract dose to increase total plasma protein was at an extract dose of 0.3 mg/kg body weight. We assume that this dose is the right dose to provide immunity to Tilapia against A. Hydrophilla bacteria optimally. However, giving too high a dose can also be toxic to fish (Islamy, 2019) and causes immunosuppression in fish to reduce the non-specific immune system of fish (Sahan and Duman, 2010).

Superoxide Dismutase (SOD)

Based on the sublethal toxicity test results in Figure 2, it can be seen that the activity of Superoxide Dismutase or SOD for each treatment increases on day 4 and begins to decline on day 8 and 12.

An increase in SOD is an indicator that the fish's body is activating its immune system to fight bacterial infections. This increase in the SOD enzyme aims to reduce cellular superoxide explosion during the defence against viral infection and to protect shrimp cells from damage (Anduro *et al*., 2012 in Ramadhani *et al*., 2017). Decreasing SOD on day 8 and 12, it can be assumed that the fish's immune system activity has finished fighting bacterial infection and returned to normal. In this study, the extract dose of 0.3 mg/body weight showed a rapid decrease in SOD. We assume that this dosage is the best dose to fight bacterial infection and improve the fish's immune system because it can accelerate the decrease in SOD and accelerate the fish to be healthy and in a normal condition.

Lysozyme activity

Lysozyme is one of the critical bactericidal enzymes in the natural immune. That non-specific immune response during infection, such as stress conditions, acts as an acute-phase protein that plays a role in defense against fish disease infections (Swain and Nayak, 2009). Lysozyme activity during the research shown in Figure 3.

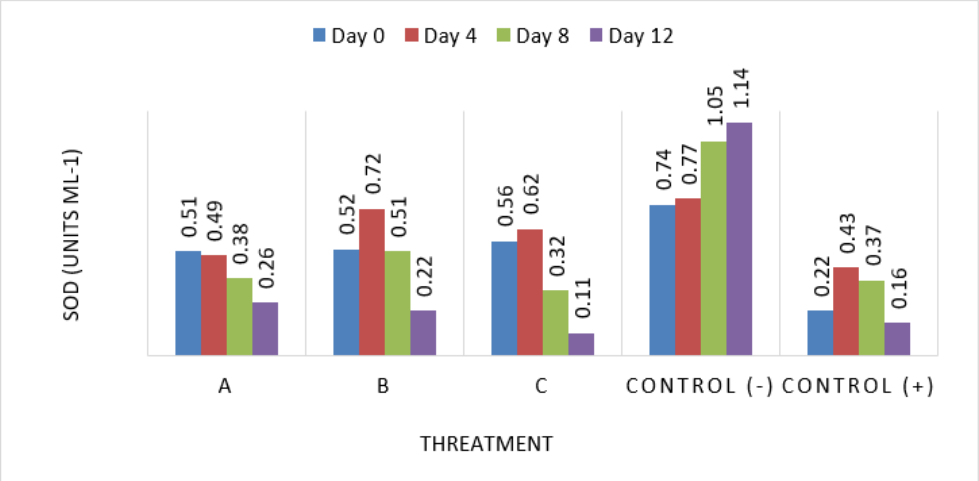

Figure 2. Activity of Superoxide Dismutase or SOD for Each Treatment.

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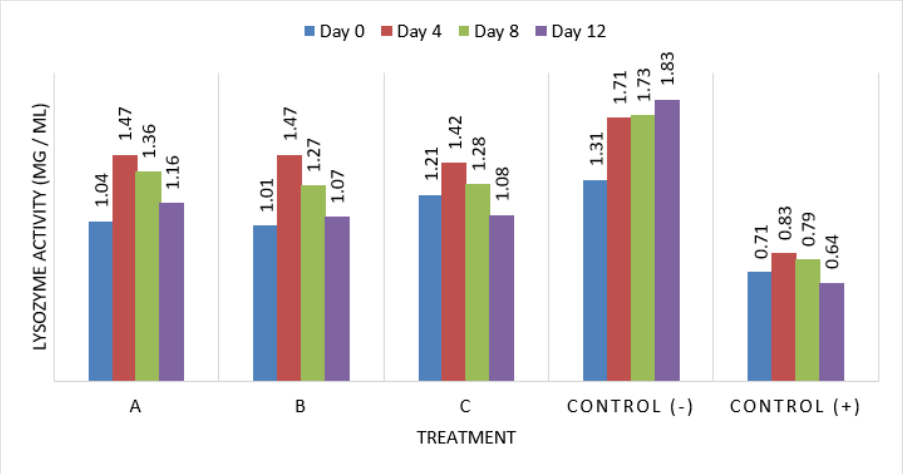

Figure 3. Lysozyme Activity During the Research

Data in Figure 3 shows that giving *Lemna* sp. at each dose reduces lysozyme activity. We assume that this enzyme facilitates the immune system of Tilapia in fighting infection with A. hydrophilla bacteria. Lysozymes are hydrolytic enzymes present in the mucus, serum and phagocytic cells of various fish species. Several studies have suggested that this enzyme is capable of providing important immunity against microbic pathogens. Neutrophils and monocytes from fish contain lysozyme in their cytoplasm and serum lysozyme derived from leukocytes (Uribe *et al*., 2011).

Lysozymes are enzymes that have an anti-bacterial activity that acts as hydrolases by destroying the β (1-4) bonds in the peptidoglycan layer on the bacterial cell wall (Ellis, 1990). Bacteria are destroyed either directly or opsonized by phagocytosis. At that time, lysozyme in fish blood will be anti-bacterial, which means that the immune element has been activated. This shows that the anti-microbial compounds in the mangosteen peel xanton can increase the macrophage mechanism so that the phagocytosis mechanism increases, which in turn the body's defence system will rise through an increase in lysozyme enzymes. Apart from having a direct effect as anti-bacterial, lysozyme is also reported to increase phagocytosis (Engstad *et al.,* 1992).

Conclusions and Suggestion

Lemna sp. extract had a very significant effect on the immune system of Tilapia (*O. niloticus*) after being challenged with *A. hydrophilla* bacteria.

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